AN EVALUATION OF THE USE OF SPECTRAL PROPERTIES IN MONITORING STRESS IN MARINE MACROALGAE.

by

SARAH JANE MAY

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

October 2000

ABSTRACT

An Evaluation of the Use of Spectral Properties in Monitoring Stress in Marine Macroalgae

By: Sarah Jane May

The overall aim of this thesis was to assess the potential of developing specific quantifiable assays of pollutant damage based on changes on the *in vivo* optical properties of macroalgae, applicable to laboratory and remote systems.

The green macroalgae, *E.intestinalis*, was exposed to selected trace metals (copper and zinc), triazine herbicides (Irgarol 1051 and atrazine) and a series of alcohols (n = 1 - 9). The algal *in vivo* spectral properties, measured using a spectrophotometer fitted with an integrating sphere, determined changes due to exposure, and results were compared with established methods, including growth and fluorescence, to assess algal health status.

Each pollutant, except zinc, had a significant ($P < 0.05$) effect on *in vivo* spectral properties at the range of concentrations used. The results indicated that the technique has potential to identify the algal systems affected by the pollutant, as signatures obtained appeared to indicate whether change was due to structure or pigments. The technique was sensitive, repeatable, and could detect individual pollutants in a copper/Irgarol 1051 mixture.

The results were mathematically interpreted to provide ratios, individual wavelengths, sensitivity figures, web diagrams and QSARs to highlight differences between pollutant effect. The QSAR, obtained from *E.intestinalis* exposed to a series of alcohols, had a R^2 value of 0.9682 using *in vivo* absorptance at 680 nm and Log K_{OW}, which corresponds with published values of 0.97 using ion leakage with the same species. However, the technique of *in vivo* spectral properties has the advantage of being non-invasive.

Samples of *E.intestinalis* were collected from different field sites and their *in vivo* spectral responses could be grouped according to potential pollutants to which they had been exposed. In addition, the potential of extending the technique for use in remote sensing is discussed. It was concluded that the technique of monitoring *in vivo* spectral properties is an appropriate biomonitor to add to the expanding range of current biomonitors.

CONTENTS

LIST OF FIGURES, PLATES AND TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ACKNOWLEDGEMENTS

Firstly, I would particularly like to thank my supervisors Dr Dave Price, Dr Maria Donkin and Dr Stuart Lane for their endless patience, time and encouragement, both academic and personal, throughout this study.

I would like to acknowledge the University of Plymouth for providing research facilities and the technical staff, especially Angela Watson, for assistance with equipment. Plymouth Marine Laboratories and the NERC unit must be thanked for help with producing the images required for this thesis. The remote sensing studies would not have been possible without Dr Richard Murphy who provided the opportunity to incorporate some of the data obtained during the LOIS project. Dr Chris Ricketts must also be thanked for his statistical advice. Special thanks go to Stella Lewis for jointly collecting some of the fluorescence and growth measurements, and Alan Scarlett for the Irgarol 1051 analysis of the algal samples.

Whilst undertaking this research I made some good friends at the University. Janice Bateson, Angela Watson and Rachael Myler all deserve a special mention for their humour, friendship, calmness and consistent moral support. Chris Coleman and Susie Bloomfield have also been invaluable in sorting out equipment and for their friendship.

I would particularly like to thank Chris and Bob Bryan for helping me write this thesis by proof-reading sections, looking after my horse Sequel and providing constant encouragement. Incidentally, Sequel must be acknowledged for keeping me sane throughout.

Finally, my parents deserve a very special mention for their continuous support in every way possible throughout this study, including putting up with me whilst writing this thesis. The decision to undertake a PhD was based on interest, concern for the environment and a personal challenge. The challenge has been significant, and I sincerely thank all my family and friends as I would not have been able to face it without their love and support.

AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was self-financed.

Short courses on statistics and remote sensing were undertaken.

Relevant scientific seminars and conferences were attended at which work was presented.

Publications

Lewis, S.A., May, S.J., Donkin, M.E. and Depledge, M.H. 1997. The influence of copper and heatshock on the physiology and cellular stress response of *Enteromorpha intestinalis*. *Marine Environmental Research* **46**(1-5): 421-424

Conferences attended

Society for Experimental Biology, 1996, Lancaster
Poster presented - Monitoring seaweed type and distribution using remote sensing techniques.

British Phycological Society, January 1997, Sheffield
Poster presented – An assessment of the toxicity of the marine antifouling triazine Irgarol 1051 to *Enteromorpha intestinalis*

Society for Experimental Biology, April 1997, Canterbury
Poster presented – Ecotoxicological responses of *Enteromorpha intestinalis* to copper exposure

Society for Experimental Biology, March 1998, York
Poster presented – Modelling the effects of non-specific narcotic hydrocarbon pollutants on green macroalgae (Chlorophyta)

Society for Experimental Biology, April 2000, Exeter
Poster presented - The effects of non-specific narcotic hydrocarbon pollutants on the spectral properties of *Enteromorpha intestinalis*

Signed .....

Date Feb 2001

1 INTRODUCTION

1.1 PROBLEMS OF POLLUTION

The problems of pollution in marine systems are well documented and can have significant effects on the ecosystem. For many years, the marine environment has been the 'dumping ground' for many wastes from human activity and the first major oil spills in the 1960s (Tait and Dipper, 1998) raised the issue of marine pollution. However, oil spills are extremely visual and it is now realised that other forms of pollution which are much more subtle can, even at low concentrations, have a dramatic effect on communities and species (Nybakken, J.W. 1997). The intertidal zone is an area of great biological diversity and productivity and there is currently serious concern over its damage by marine pollution.

There is no precise definition of the term pollutant but one possible general definition is

"substances that occur in the environment at least in part as a result of man's activities, and which have a deleterious effect on living organisms"

(Moriarty 1983)

Pollution can be caused by many factors including overexploitation, climate change or spread of exotic species. However, chemicals are one major component of pollution in marine systems. These can include oil, herbicides, heavy metals and antifouling compounds and the effect can frequently be observed at the cellular, population, community and ecosystem levels of organisation. Pollution by chemicals can be either point e.g. industrial discharges or non

point e.g. agricultural/urban runoff (Scheuert 1993), sewage outfalls or atmospheric deposition.

Common marine pollutants include:

- Domestic sewage
- Pesticides - including anti-fouling paints
- Fertilisers
- Industrial effluents – complex mixtures including trace metals, PCBs, pulp mill effluent and hydrocarbons
- Oil and its derivatives
- Thermal pollution
- Trace metals
- Enhanced UV-radiation

The marine environment is a valuable resource and there is a great need for monitoring the pollution effects on the ecosystem. One branch of science that has developed and is concerned with the study of pollutant effects on populations, communities and ecosystems is ecotoxicology. This is a relatively new branch of science but it is now considered to be an independent discipline following the explosion of interest in the well being of the environment (Depledge, 1993).

Ecotoxicology encompasses many different areas and, as with pollution, there is no precise definition but it can be loosely defined as

"the field of study that integrates the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment"

(Forbes and Forbes 1994)

Most work involving ecotoxicology is designed to either

1. generate data to be used to support decisions for risk assessment and environmental management,
2. meet legal requirements regulating the development, manufacture or release of potentially dangerous substances or
3. develop empirical or theoretical principles to further understanding of the behaviour and effects of chemicals in living systems (Forbes and Forbes 1994)

1.2 POLLUTANTS STUDIED

The pollutants studied during this research were trace metals (copper and zinc), triazine herbicides (atrazine and Irgarol 1051) and a series of alcohols (n = 1 – 9). These were chosen following a review of the literature which considered that they are representative of the major chemical pollutant groups found in marine systems.

1.2.1 TRACE METALS

The term 'heavy metal' is generally used to refer to those metals having a density greater than $5g/cm^3$ or an atomic number greater than iron. However, the term heavy metal is also often used by ecotoxicologists to refer to metals that have been shown to cause environmental problems (Depledge *et al*, 1994). This can therefore cause some confusion and alternative ways of grouping the elements have been employed. One way, devised by Nieboer and Richardson, 1980, is to group the metals and metalloid ions by their atomic properties and solution chemistry into class A (oxygen-seeking, including alkali metals and alkaline earths), class B (sulphur-seeking, including Cu^{+}, Hg^{2+}, Ag^{2+} and Pb^{2+}) and borderline (intermediate between A and B). Class A ions are biologically essential and class B ions are extremely toxic and are usually non-essential. Borderline ions have biological roles but may also exhibit toxic effects and include Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} (Vymazal, 1995). If this method of classification is used then all heavy metals would belong to either the borderline or class B group (Depledge *et al*, 1994).

Some heavy metals in the borderline group are essential micronutrients and are frequently referred to as trace metals. If concentrations of these metals are too low then algal growth may be limited but if concentrations are too high then they may become toxic to the algae. Concentrations of trace metals in water often become much higher than naturally occurring levels due to anthropogenic sources including sewerage outfalls, industrial effluents and mining operations. These can have severe toxicological effects on the aquatic ecosystem and the situation is aggravated by the lack of natural elimination processes for metals. This often results in metals shifting from one compartment in the aquatic ecosystem to another, which frequently results in accumulation in the biota (Chapman and Kimstach 1996).

Copper and zinc are both trace elements for algae and were chosen for this study as they represented two metals with a varying degree of toxicity on algae. The order of metal toxicity on algae varies with various factors eg. algal type and experimental conditions, but is generally Hg > Cu > Cd > Ag > Pb > Zn (Rai *et al* 1981). Many water quality assessment programmes are assessing the effect of metal pollution. These include the Global Environment Monitoring System (GEMS) programme that includes 10 metals and the United States Environmental Protection Agency (US EPA) that considers 8 trace elements as high priority. Copper and zinc occur on both of these lists of high priority and are consequently being monitored (Chapman and Kimstach 1996). The group of heavy metals is also a group of pollutants that the UK government is required to reduce in the environment and the UK 'red list' that has consequently been compiled contains both copper and zinc (NRA 1994).

1.2.2 TRIAZINES

Pesticides are considered to be one of the greatest pollution threats in the marine environment (Forbes and Forbes 1994) and they are increasingly becoming a problem to marine flora. Although few herbicides are added directly to water, they often reach the aquatic environment by runoff, river discharge or leaching from sources including antifouling paints. The herbicide Irgarol 1051 belongs to the triazine group. Triazines are a group of herbicides which are known to inhibit photosynthetic activity at Photosystem II (PS-II) and have differences in activity caused by various lipophilic side chains (Somerville 1990).

Photosystems I and II are large pigment-protein complexes, each having a unique chlorophyll composition (P_{700} and P_{680} respectively). In green algae, these complexes span the thylakoid membrane, thylakoid appresion occurs and the pattern of stacking can resemble that of higher

plants (see Lobban and Harrison, 1994). The appressed region shows close membrane interaction and is rich in PS-II. The triazine, Irgarol 1051, inhibits PS-II activity by displacing plastoquinone from the QB site. This prevents the electron carrier quinone binding and so electron flow is prevented (Dodge 1989).

The triazine herbicides chosen for this study were Irgarol 1051 and Atrazine because there is particular concern among ecotoxicologists about possible adverse effects of these herbicides on non-target plants especially considering their widespread and increasing use.

1.2.3 ALCOHOLS

Hydrocarbons dissolved in seawater can vary between 1 and 5 % of the total organic matter in solution (Barbier *et al* 1973). The concentration of these hydrocarbons can range from a few micrograms per litre offshore with higher values nearshore (Law, 1981). The alcohols methanol – nonanol, with carbon number ranging from n=1 to n=9, were chosen for this study as they represent a wide group of hydrocarbons. The choice of a homologous series of alcohols also allowed the possibility of constructing Quantitative Structure Activity Relationships (QSAR's) with the results obtained. QSARs are mathematical models that relate the biological activity of chemicals to their structure and corresponding chemical and physiochemical properties (Lipnick, 1995).

1.3 USE OF BIOMONITORS TO MONITOR POLLUTION

In the past, many test organisms have been used as indicators rather than biomonitors. An indicator is often used to describe an organism which can detect the presence or absence of an environmental variable whereas a monitor is often used for quantitative assessment (Whitton, 1984). An example of a typical indicator is the abundance of species of the genera *Ulva* and *Enteromorpha intestinalis* in eutrophicated waters indicating the presence of excess nitrates.

It has been recognised that measurements of pollutant residues in organisms could provide valuable information in the study of pollutants resulting in an additional parameter to the analyses of water and sediments (Phillips 1980). Phillips found that the pollutant levels in the organism could be linked to its exposure and that the method had various advantages. One advantage is that in situations where the concentration of many pollutants is higher than in the surrounding environment only the fraction of the pollutant that is biologically available is measured. This allows the possibility of ascertaining a time averaged index of pollution if the rates of uptake and excretion are known for a particular pollutant. (Forbes and Forbes 1994). An organism, which has been successfully used in biomonitoring, is the mussel, *Mytilus edulis*. (Widdows and Donkin, 1991) because of its filter-feeding and sessile habit, geographical distribution, available numbers and resistance to general stress (Livingstone and Goldfarb, 1998). However, although some good correlations between metal exposure and metal content in some organisms have been found (Phillips, 1977), care must be taken with interpreting the results because differences in the partitioning of metal within the tissues of an organism may have a large influence on toxicity, but may be masked when measurements are made up of the total body metal load (Forbes and Forbes 1994). This potential problem of the handling of the metals within an organism has resulted in the interpretation of biomonitoring of metals being more complex than as thought originally. Residue biomonitoring also has a

further limitation in that it can only be used to equate body load with exposure when a pollutant bioaccumulates. Bioaccumualtion is defined as the uptake of chemicals via food and water (Geyer and Muir, 1993).

Although the use of biomonitors can provide useful information, monitoring the residue levels of pollutants in organisms may have limited use in assessing chemicals with a high toxicity as significant biological effects can often be exhibited at pollutant levels that are not detectable with current equipment (Forbes and Forbes 1994). A potentially more useful method may be the development of biomarkers which identify changes at the molecular, biochemical or physiological level of the organism.

1.3.1 USE OF ALGAE IN ECOTOXICOLOGICAL TESTING

A considerable amount of classic ecotoxicology has been carried out using invertebrates but it is now becoming increasing popular to use algae. Many studies using macroalgae have already been carried out and these most frequently involve the use of *Fucus* and *Enteromorpha species* (Say *et al*, 1986 and 1990). There are many reasons why the use of algae can provide an important addition to ecotoxicological studies. Some important reasons for using algae are discussed below:

- Ecological importance

  Algae are extremely important as an integral and abundant component of ecosystems and constitute a significant portion of the food chain base in coastal waters. If the natural amounts of algae are affected in any way this could have a considerable effect on the balance of the ecosystem because algae are the main primary producers in coastal regions. It has also been estimated that phytoplankton generate about 70 % of the world's atmospheric oxygen (Reynolds, 1984). It is coastal regions that are often at risk from

pollution by terrestrial run-off. An additional important ecological factor involving macroalgae is that some species *e.g.* kelp actually provide an important habitat structure.

- Algal sensitivity

  There is a common misconception among scientists that plants are not as sensitive to toxicants as animals. The sensitivity of a test organism to a toxicant is dependent on a variety of factors including type of toxicant, environmental conditions and test methods (Wang and Freemark, 1995). Algae are sensitive to a variety of toxicants and the degree of sensitivity of algae in comparison to other test organisms varies with different toxicants. Miller *et al* (1985) and Thomas *et al* (1986) reported that the decreasing sensitivity order of Cd, Cu and Zn to organisms was algae>daphnids>bacteria>plant seeds>earth worms whereas decreasing order of sensitivity to herbicides was plant seeds>algae/bacteria>daphnids> earthworms. It is therefore important in ecotoxicological testing to use a variety of organisms in order to be able to assess the potential impact of a pollutant on the ecosystem.

- Economic importance

  In many countries algae has an economic importance, for example, in Japan they are farmed extensively as a food source, and so it is extremely important to be able to monitor any adverse effects from potential pollutants.

- Algae are sessile

  The fact that algae are sessile in nature offers an advantage over other organisms because they can characterise one location over a period of time. This is particularly important in areas where there may be regular pollution even at low concentrations.

- Algae take nutrients and accompanying chemicals directly from the water

  This provides the possibility that levels of pollutants found in algae can be directly attributed to levels of pollutants found in the water.

- Abundance

  Many species of algae are abundant and so are particularly suitable for biomonitoring because they can be used for testing a variety of locations.

- Ethical considerations

  The use of algae also overcomes the current concerns about animal testing in scientific testing.

Some species of algae have been used to monitor pollution in certain regions. One particular application is their use in monitoring heavy metal pollution. This is well documented and includes monitoring in fresh waters (Whitton, 1984) and marine environments (Leal *et al*, 1997). An additional, well-documented use of macroalgae is the increase of particular species in certain locations, an example being the increased quantity of large sheets of Ulva lactuca, a green macroalgae, near areas with high levels of sewage pollution (Burrows 1971).

Macroalgae therefore represent static, resident monitors of pollution in marine environments, recording in their altered morphology and constituents, effects of the specific stresses to which they have been exposed.

1.3.2 CHOICE OF SPECIES

Enteromorpha intestinalis, a member of the phylum *Chlorophyta*, order *Ulvales*, family Ulvaceae, was the species of macroalgae chosen for this study. This decision was based on many factors, some of which are outlined below:

- *Enteromorpha spp.* is found along the majority of the British coastline and so is ideal as a biomonitor because it may be monitored from both 'clean' and potentially 'polluted' areas. It is commonly found on estuarine mudflats, sandy shores or in coastal rockpools (Nybakken 1997).
- Some species of macroalgae are physiologically simple and may remain alive and even regenerate if cut into pieces (Wilkinson, 1992). *Enteromorpha intestinalis* is an example of this type of macroalgae and short segments have been used to test against anti-fouling compounds in previous studies (Goodman *et al* 1976). It was therefore considered appropriate to use it in a similar manner during this study.
- *Enteromorpha intestinalis* is a green macroalgae and, in comparison to brown and red macroalgae, has a pigment complement that is most similar to higher plants. This is important when considering the use of the fluorescence measurements obtained in order to be able to evaluate the use of optical properties.
- *E.intestinalis* has been used in other studies involving biomonitoring, especially with trace metals (Say *et al*, 1990)

Plate 1: Typical form of *E.intestinalis*

1.4 POTENTIAL METHODS USING ALGAE

A wide range of ecotoxicological tests can be utilised when using algae as biomonitors. The primary aim of this study is to assess the use of *in vivo* optical properties of algae. It was, therefore, decided that fluorescence and growth measurements should be taken to provide an indication of the health status of the algae and to provide appropriate comparisons with the use of *in vivo* spectral changes.

1.4.1 FLUORESCENCE

Chlorophyll pigments absorb light in the blue and red regions of the electromagnetic spectrum and transmit and reflect light in the green region of the spectrum resulting in plants containing chlorophyll a and b appearing green. However, a proportion of the light that is absorbed is

always lost as heat or re-emitted as fluorescence. In order to understand the application of fluorescence it is crucial that the different stages of photosynthesis are considered.

Photosynthesis consists of two reactions – the light and light-independent reactions. During the light reactions the chlorophyll molecules absorb light that is necessary for driving the chemical reactions. When the chlorophyll molecule absorbs light it makes a transition from its lowest energy or ground state to a higher-energy or 'excited' state. The energy of photons is higher when their wavelength is shorter and so the absorption of blue light excites the chlorophyll to a higher energy state than the absorption of red light. (see Taiz and Zeiger 1991)

After the pigment molecules have absorbed the light, and are therefore in a higher-energy or 'excited' electronic states with electrons in antibonding orbitals, they are unstable and need to give up their excess energy in order to return to their ground state. First, the excited molecule loses excess vibrational energy to the surroundings and comes down to a lower vibrational state. There are then several pathways that can be taken, one of which is to pass the excitation energy onto another molecule and a second possibility is simply the emission of radiation as fluorescence. The energy available for fluorescence emission is inevitably lower than that which was absorbed originally due to some of the energy being initially lost as vibrational energy (Britton, 1983). It is because of this that fluorescence always occurs at a longer wavelength than the light that was absorbed.

Chlorophyll fluorescence is red because the energy difference between the ground level and first singlet level equals the energy of a photon of red light (Bolhar-Nordenkampf and Öquist,G, 1993). As mentioned above, the red fluorescence emission peak, which occurs at

685nm *in vivo,* is a longer wavelength than the red chlorophyll absorption peak. This shift is referred to as Stoke's shift (Bolhar-Nordenkampf and Őquist,G, 1993).

If a leaf is illuminated at a constant level of light intensity it will fluoresce at a steady level (Hansatech PEA Manual). However, if a leaf is kept in darkness for several minutes and then exposed to bright illumination the Kausky effect can be observed. An example of characteristic fluorescence induction kinetics or Kausky curve is shown in figure 1.

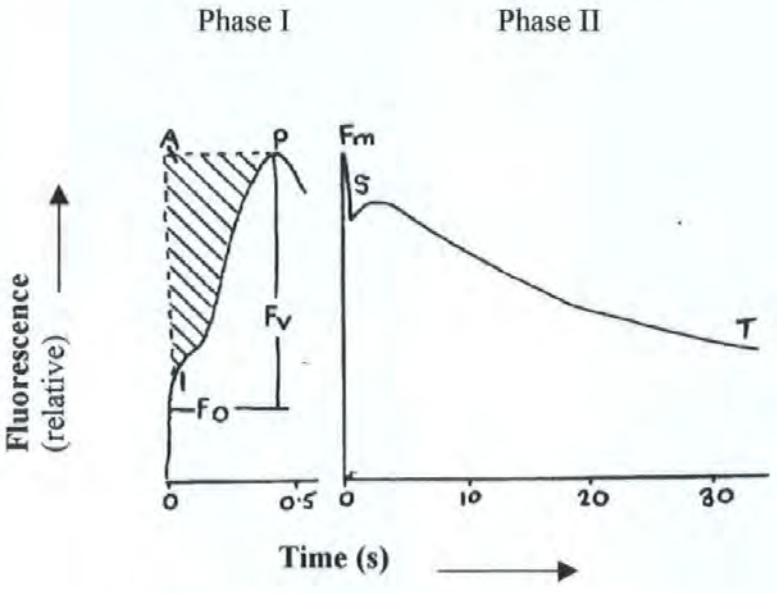

Figure 1: Typical Kausky curve (adapted from Bolhar-Nordenkampf, 1993).

When the leaf, which has previously been kept in the dark, otherwise known as dark-adapted, is exposed to light there is an initial immediate rise in fluorescence to a minimal level. This point is referred to as F_o and is attained when the chlorophyll antennae absorb the light, but before the reaction centres of PS II have trapped the excitons. It is extremely difficult to assess the real value of F_o because this state only lasts for picoseconds to nanoseconds. However, in a non-modulated measuring system it can be resolved by mathematical extrapolation or, alternatively, the use of a modulated fluorescence system ensures the exciting

light is low enough to prevent detectable photochemistry from occurring. At F_o all the reaction centres are open for primary photochemistry and so both the potential and photochemical use of excitation energy and hence photochemical quenching of fluorescence are at their maximum (Bolhar-Nordenkampf and Őquist,G, 1993).

After F_o, fluorescence rises from F_0, via an intermediate level (I), to a peak level (P) and then gradually declines to a level close to the original F_o value (PEA Manual). At I the electrons start to become transferred to the plastoquinone pool via Q_B so that a transient reoxidation of Q_A occurs. The gradual increase in fluorescence continues until P, sometimes referred to as F_{max} or F_m, when the acceptor Q_A becomes increasingly reduced as the 'traps' close. If the actinic light is strong enough then the maximum level of fluorescence, F_{max} or F_m, is obtained as all the reaction centres become closed and photochemical quenching is, therefore, zero. Therefore, if the light is of a saturating intensity for the leaf, the peak value will be the maximum achievable fluorescence level at a given light intensity. The difference between the low-level fluorescence signal (F_o) and the maximum fluorescence level (F_m) is referred to as the variable component of fluorescence (F_v).

Fluorescence measurements are non-invasive and non-destructive so they are extremely useful for monitoring the health status of a plant or leaf, especially if repetitive or repeated measurements need to be taken over a time period. Fluorescence methods have already been used extensively in phycological research (Geider and Osborne, 1992) and it is now widely accepted as an indicator of photosynthetic reactions in chloroplasts of green plants. Certain points on the curve can be used to create ratios and area measurements can also be informative.

One of the most widely used fluorescence ratios derived from the fast kinetics of the fluorescence induction curve is the variable fluorescence (F_v) divided by the maximum fluorescence (F_m). This ratio has been shown to be proportional to the quantum yield of photochemistry by calculation of rate constants for competing decay reactions at F_o and F_m (Bolhar-Nordenkampf and Öquist,G, 1993). The typical value of Fv/F_m for a healthy leaf is usually approximately 0.83 (Bjorkman and Demmig 1987). Fluorescence measurements were used in this study in order to assess the health status of the algae so that an evaluation of the use of optical properties could be obtained.

1.4.2 GROWTH

When mature macroalgae are used in pollutant studies, it is usually the sublethal effects rather than lethal toxicity that are assessed (Wilkinson, 1992). A typical sublethal effect that is often monitored is growth inhibition (Tewari *et al* 1990). When measuring growth of macroalgae in long term testing, it is not appropriate to use traditional higher plant methods of dry weight analysis, as this is a destructive method, so an alternative method is to measure change in length. The macroalgae *Ulva spp.* has diffuse growth rather than localised meristems and so the growth can be measured easily as change in diameter or surface area (Burrows, 1971, Scanlan and Wilkinson, 1987). *Enteromorpha intestinalis* has a similar growth to *Ulva spp.* and so recording the length of the excised sections can enable growth to be monitored.

1.5 OPTICAL PROPERTIES IN GENERAL

The term 'light' is commonly used to represent the narrow region of the electromagnetic spectrum whose wavelengths are visible to the human eye, plus the ultraviolet and infra-red regions.

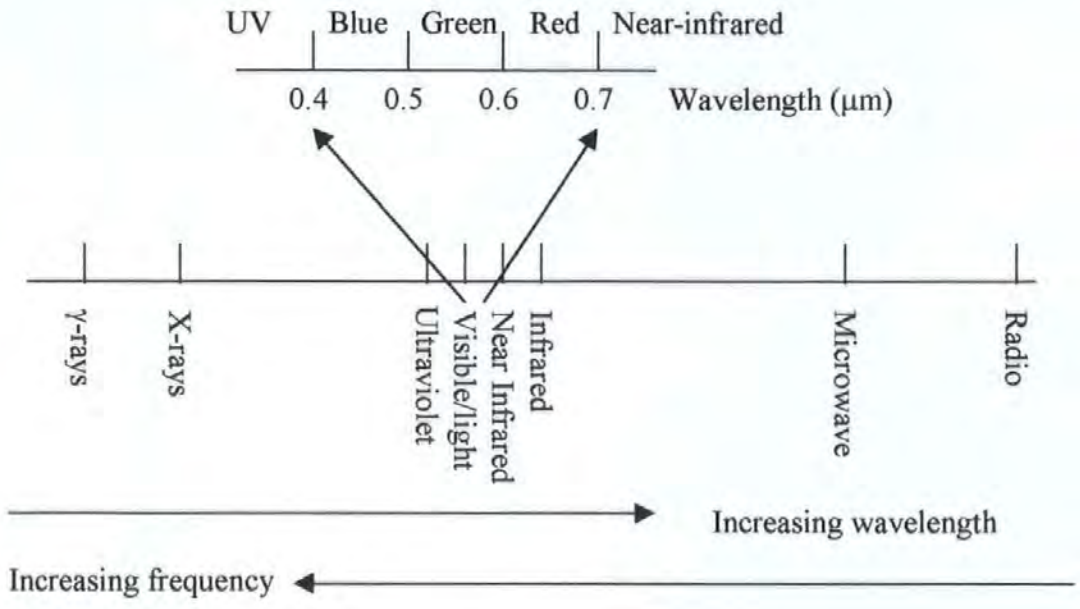

Fig. 3: **Diagram of the electromagnetic spectrum**

When light is received at an object it is reflected, transmitted or absorbed. There are primarily two types of reflectance, which are **specular**, originating at the surface, and **diffuse**, originating from light scattering within the leaf and therefore subject to differential absorption.. Light that is neither reflected nor absorbed is transmitted through the leaf. Both reflectance and transmission of a solid sample can be measured *in vivo* using an integrated reflectance accessory (Chapter 2) and *in vivo* absorptance of the sample can then be calculated by the equation:

In vivo absorptance = 100% - (%R + %T).

It must be noted, however, that only a proportion of this absorptance will be due to specific absorption by photosynthetic pigments.

Different regions of the light section of the electromagnetic spectrum are characterised by different properties of vegetation as follows (Lillesand and Kiefer, 1994):

- 400 – 750 nm: This region is characterised by the absorption by pigments consisting mainly of chlorophylls a and b, carotenes and xanthophylls. Light energy is strongly absorbed by chlorophyll at the wavelengths 450 and 680 nm. However, even green light is absorbed non-specifically by the algal thallus.

- 750 – 1350 nm: This is a region of high reflectance (usually 40 – 50 %) and low absorption (usually less than 5 %), which is greatly affected by internal leaf structure. It is often possible to discriminate between different plant species, even if they look the same in the visible part of the spectrum, due to the highly variable structure of different plant species.

- 1350 – 2500 nm: This region is partly affected by internal structure but is mainly affected by water content. The light energy is usually absorbed or reflected with very little transmittance. Beyond 1.3 μm, the reflectance of a leaf is usually inversely related to the total water present and so is a function of both the moisture content and thickness of the leaf.

During these studies, the wavelengths chosen for investigation were 250 – 800 because, as mentioned above, this represents the area most affected by changes due to pigment effects. The regions affected by internal leaf structure and water content were not studied but it must be noted that these may be slightly different in an algal thallus than in a leaf. This

would especially be a consideration when using *E.intestinalis* due to its simple tubular structure only one cell thick.

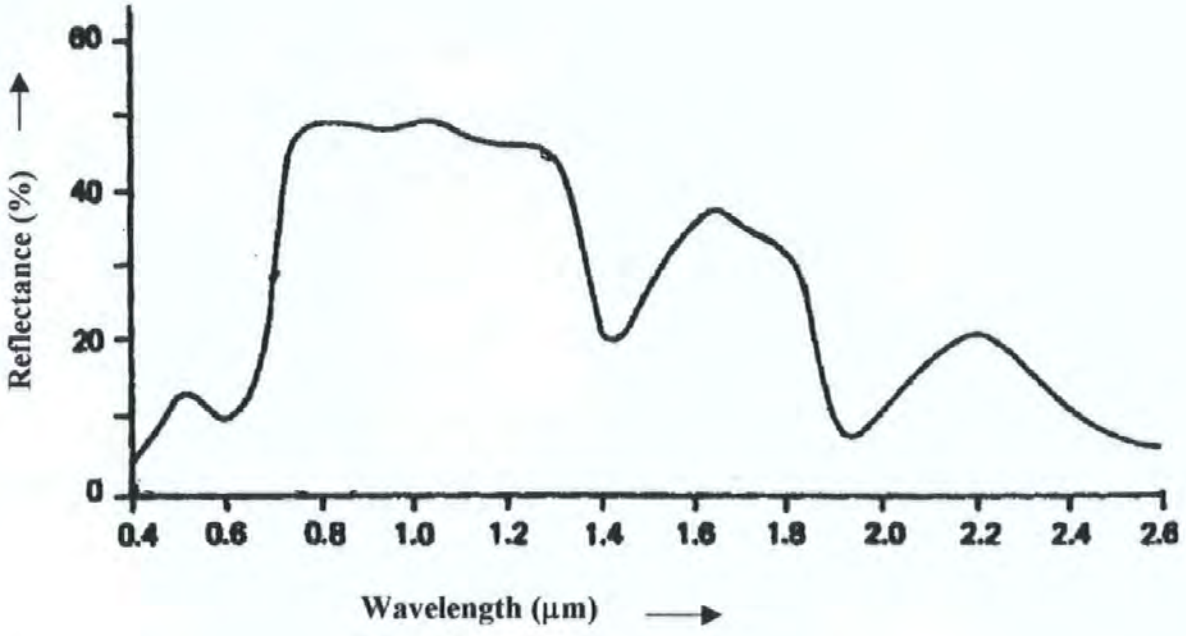

Figure 3 : A typical reflectance curve of vegetation (adapted from Lillesand and Kiefer, 1994)

It can be seen from figure 3 that dips occur in the reflectance values at 1.4, 1.9 and 2.7 μm because water in the leaf absorbs strongly at these wavelengths and these wavelengths are correspondingly known as water absorption bands (Lillesand and Kiefer, 1994).

Different algae have different pigment systems and this is apparent from observing their typical spectral signatures. The following figures have been obtained from *Ulva lactuca, Fucus serratus* and *Porphyra umbilicalis* as an example of a green, brown and red macroalgae respectively.

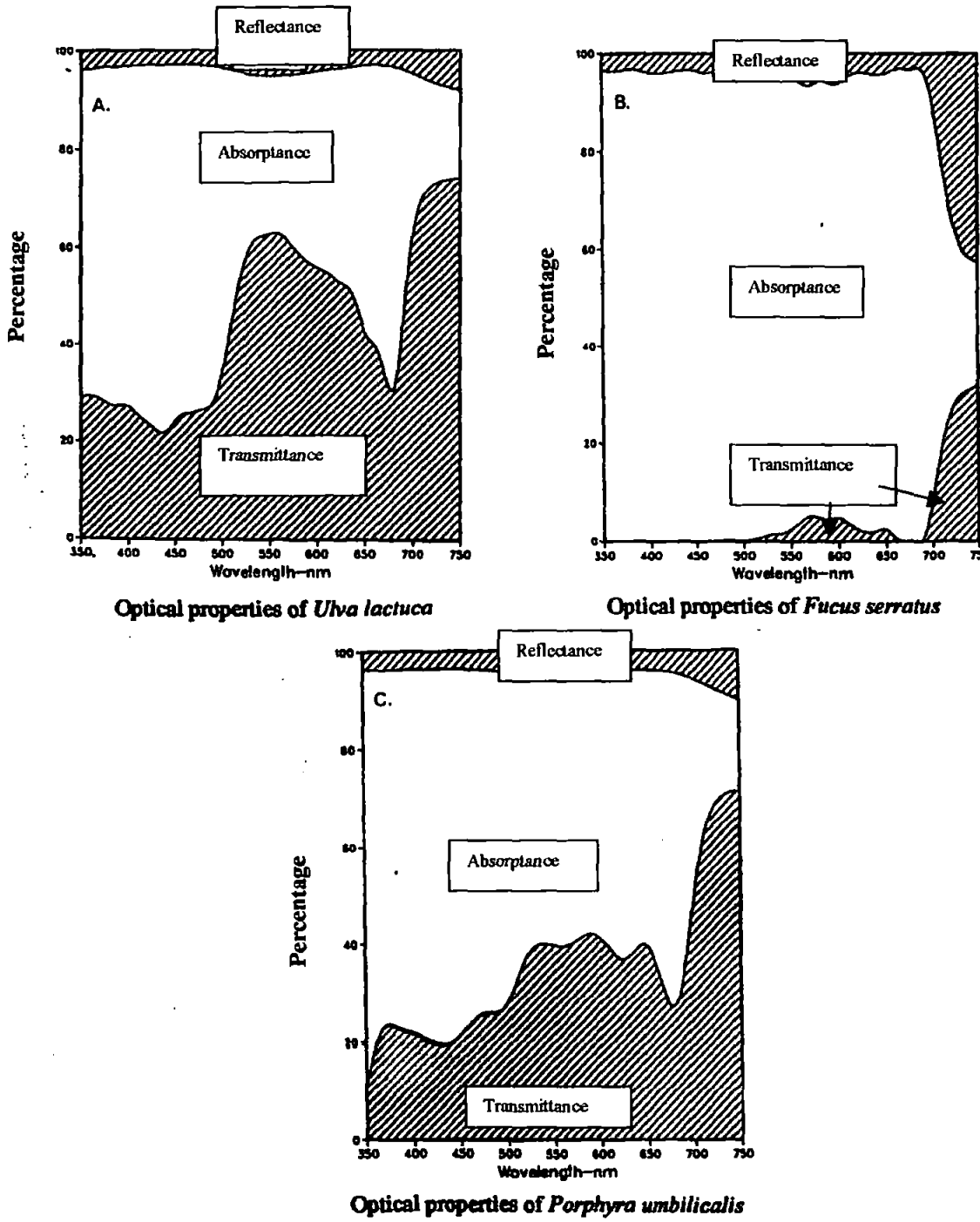

Figure 4: Typical optical properties of a green, brown and red macroalgae.

Figure 4 demonstrates that each major class of macroalgae exhibit characteristic peaks and troughs in *in vivo* transmission, reflectance and absorptance in response to their structure and

differing pigment complements. All three macroalgae types are relatively transmittant above 700 nm and the thin fronds of *Ulva lactuca* and *Porphyra umbilicas* transmit considerable energy at other wavelengths.

In vivo reflectance is low in all macroalgae except above 700 nm. The *in vivo* peak of absorption of chlorophyll at 680 nm is evident in all three. At the edge of the visible spectrum, as the absorption of red light by chlorophyll pigments begins to decline, reflectance rises sharply. This is known as the red edge effect. The combined effects of chlorophyll and carotenoids is evident in the blue and near UV in the *Ulva lactuca* and the effect of porphyrins in *Porphyra umbilicalis* and carotenoids in *Fucus serratus* produce noticeable perturbations across the green regions of the spectra.

It should also be noted that reflectance increases with an increase in the number of intercellular air spaces because diffused light passes more often from highly refractive hydrated cell walls to lowly refractive intercellular air spaces (Barrett and Curtis 1995). Therefore, an 'older' leaf has a higher reflectance than a 'younger' leaf. Previous work has included work on the effects of age on the *in vivo* optical properties of cotton leaves (Gausman *et al*, 1971) when it was found that young, compact leaves containing few air spaces within the mesophyll had a lower reflectance in the 750 – 1350 nm region than older leaves. This effect was also found during preliminary studies when it was apparent the juvenile *Fucus serratus* frond had a lower reflectance than an adult *Fucus serratus* frond (Figure 5) even though a fucus frond has less air spaces than those found in a leaf.

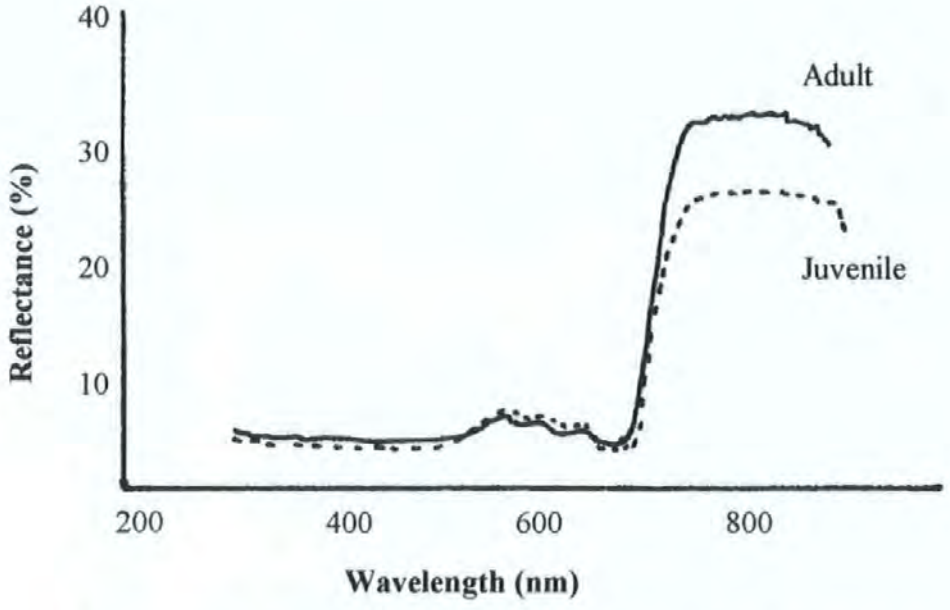

Figure 5: Percentage reflectance of adult and juvenile *Fucus vesiculosis*

1.6 USE OF *IN VIVO* OPTICAL PROPERTIES OF ALGAE TO DETECT STRESS

Potentially, some of the most attractive diagnostic features for pollution monitoring are the visual symptoms of damage, which may involve changes in overall colour or the appearance of local lesions on seaweed fronds. However, such changes are difficult to quantify and resolve into changes that are specific for particular stresses, but it may be possible that the analysis of *in vivo* optical properties of macroalgae is capable of such quantification and resolution.

If optical properties can be used to detect pollution damage then this may offer the potential of extrapolation of the method to remote sensing.

Remote sensing can be defined as

"the set of techniques used to obtain information about the Earth's surface and atmosphere at some distance from them, usually by means of the electromagnetic spectrum"

(Townshend 1981) or,

"the use of sensors to record images which can be interpreted to yield useful information"

(Curran 1987)

Remote sensing, therefore, encompasses a wide range of techniques including aerial photographs, airborne sensors and satellite systems. Aerial photographs have been used with some success to monitor plant diseases (Toler *et al*, 1981, Jackson, 1986, Deysher, 1993) but the use of multispectral scanners to record the spectral responses of plants enables more information to be determined. Satellite remote sensing has been used successfully in the past to measure various parameters including temperature, turbidity, and chlorophyll *a* determination (Khorram *et al* 1991). Airborne remote sensing offers even more potential for monitoring as the sensors are closer enabling a smaller pixel size to be used resulting in greater detail.

A valuable use of remote sensing is mapping of vegetation using their spectral response. Different vegetation types can be identified, over large areas, and changes can then be observed. This method is used frequently with terrestrial plants (Barrett and Curtis 1992, Lewis 1994 and Matheson and Ringrose 1994) and there is now an increasing use of remote sensing in mapping vegetation in aquatic environments (Armstrong 1993, Deysher 1993, Guillaumont *et al* 1993, Penuelas *et al* 1993). In addition to mapping vegetation, manipulation of the spectral data obtained can provide further information including biomass and chlorophyll estimation (Chappelle and McMurtrey 1992, Filella and Penuelas 1994) and detection of plant stress (Jackson 1986, Carter *et al* 1992, Vogelmann *et al* 1993). Remote sensing can also be used to detect stress by the use of the chlorophyll *a* fluorescence signal of vegetation (Valentini *et al* 1994). In addition, remote sensing can be used to monitor increases in chlorophyll concentration in water due to algal blooms etc. because the reflectance of the water changes as the chlorophyll concentration changes.

A method that is already used extensively in the remote sensing of vegetation is the 'red edge'. This is the slope of the reflectance spectrum over the range of 680 – 760 nm. The use of the 'red edge' has been used extensively in remote sensing studies including using the position and shape of the red edge. The position of the red edge has been related to the chlorophyll concentration of a crop (Munden *et al*, 1994). Filella and Penuelas, 1994, also found a high correlation between chlorophyll content of *Capsicum annuum* and *Phaseolus vulgaris* and the wavelength of the red edge peak. In addition, they found that the area of the red edge peak, between 680-780nm, was a good estimator of Leaf Area Index and was affected by the hydric status of the plant. If the red edge shifts to a longer or shorter wavelength then this may be linked to changes in the chemical and morphological status or vitality of plants. It has been found that trees exposed to high levels of heavy metals exhibit these shifts due to changes in the chlorophyll reflectance spectrum. (Lillesand and Kiefer, 1994).

Remote sensing methods have been used successfully in monitoring stress in vegetation. As mentioned above the spectral data obtained is usually manipulated, often including ratio analysis (Chappelle and McMurtrey 1992), to highlight certain parameters. Remote sensing, therefore, may offer potential in detecting stress in marine macroalgae as the technique has been used successfully in monitoring stress in terrestrial plants as changes that occur as a result of stress frequently affect the spectral response (Jackson 1986).

1.7 OVERALL AIMS OF THE RESEARCH

The studies for this research involve using a variety of techniques including assessing optical properties, fluorescence, growth studies of macroalgae and remote sensing. The overall study is to assess the health status of the macroalgae by using growth and fluorescence techniques in order to evaluate the use of *in vivo* spectral properties in pollution monitoring.

If changes in spectral properties can be directly attributed to pollution effects then optical changes can then be added to a growing group of biomarkers. However, in order for this to be effective, the spectral changes will have to offer appropriate quantitative parameters for monitoring pollutant stress. The basis of these spectral changes will have to be assessed and the possibility that certain wavelengths, ratios and other derivations may offer the potential of resolving damage to particular stresses needs to be investigated. An additional aim is to investigate whether remote sensing can be used to identify different macroalgae types. This work could indicate that by identifying specific reflectance changes of the macroalgae in the laboratory it might be possible, by the use of specific wavebands when using a Compact Airborne Spectrographic Imager (CASI) or an alternative similar instrument, to use remote sensing as a tool to monitor pollution along the coast. This would have a considerable advantage over current monitoring techniques as large areas could be monitored within the same time period.

This study will, therefore, address the following issues:

- Do spectral changes offer appropriate quantitative parameters for monitoring pollutant stress?
- Do certain wavelengths, ratios and other derivations offer the possibility of resolving damage to particular stresses?

- Can the use of *in vivo* spectral properties allow the detection, quantification and discrimination between pollutants in a mixture?
- Does the method exhibit a high degree of repeatability?
- Is there a concentration effect on the sensitivity signatures?
- Can the data obtained under laboratory conditions be extended to situations in the field?
- Can changes, observed in the spectra, form the basis of remote detection?
- Is the use of *in vivo* spectral changes an appropriate biomonitor to add to the expanding range of biomonitors already used?

The overall aim is of this study is, therefore, to assess the potential of developing specific, quantifiable assays of pollutant damage based on changes in the *in vivo* optical properties of macroalgae, applicable to laboratory and remote sensing systems.

2 DEVELOPMENT OF METHODS

The primary aim of this thesis is to assess the suitability of using *in-vivo* spectrophotometry to monitor the effect of pollutants in macroalgae. This method, however, must be used in conjunction with other more traditional methods to enable the health status of the algae to be monitored and also to allow comparisons and evaluations to be made. It was therefore decided that in addition to investigating the *in vivo* spectral properties of the algae, other parameters e.g. growth and fluorescence should also be used.

2.1 FLUORESCENCE METHODS AND OPTIMUM EXPERIMENTAL CONDITIONS FOR THE ALGAE

Fluorescence measurements were taken using a Hansatech Photosynthetic Efficiency Analyser (PEA) which is a non-modulated fluorescence meter that requires the samples to be dark adapted before any measurements are taken.

2.1.1 DETERMINATION OF DARK ADAPTATION TIME

In order to ascertain the optimum dark adaptation time and light level to be used when using fluorescence as a parameter, sections of *E.intestinalis* were dark adapted for various times. The following results were obtained:

Dark adaptation time (minutes)	Mean F_v/F_m value
3	0.68
6	0.74
9	0.76
12	0.80
15	0.81
18	0.81

Table 1: Fluorescence induction ratios (F_v/F_m) of *E. intestinalis* after varying dark adaptation times.

These results indicate that the F_v/F_m values of *E. intestinalis* begin to plateau when the dark adaptation time is 12 minutes. It was therefore decided to use a dark adaptation time of 15 minutes for all future experiments, using standard pre-illumination conditions, as this ensured that the macroalgae were always be sufficiently dark-adapted but not stressed unnecessarily.

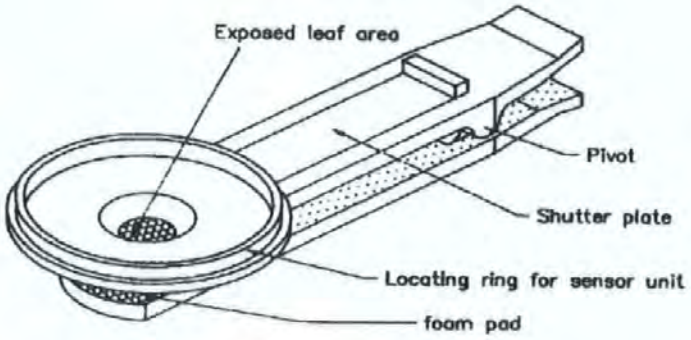

Figure 6: Clip used for dark-adapting the algal samples (taken from Hansateh PEA manual, 1995)

During the dark adaptation time, the algal samples have to remain in the clip (figure 6) designed for use with the Hansatech PEA fluorescence meter. The relatively long dark adaptation time of 15 minutes was found to stress the algae if they were left on the bench, however, the algae appeared to be less stressed (measured by fluorescence) if they were dark-adapted in their respective Instant Ocean solutions (J & K Aquatics, see Appendix 1). The clips were washed carefully afterwards to remove any risk of sample contamination.

2.1.2 FLUORESCENCE – LIGHT LEVEL

The optimum light intensity for *E.intestinalis* when measuring fluorescence was determined by setting the Photosynthetic Efficiency Analyser (PEA) to emit varying light levels. According to the PEA manual, the optimum light intensity is the maximum intensity that can be used without incurring an 'over-scale' error. In the case of *E. intestinalis* 100% light did not cause an over-scale error so it was decided that this should be the level used for all fluorescence measurements taken for each experiment under standard conditions.

2.1.3 USE OF NUTRIENTS AND LIGHTING REGIME

A preliminary experiment was set up to determine the nutrient and lighting requirements of *E. intestinalis*. After 48 hours acclimation, intercalary sections (10 for each treatment) of *E. intestinalis*, 25 mm in length, were incubated in 33 ppt Instant Ocean either with or without nutrients (50 mg/l $NaNO_3$ (BDH) and 10 mg/l Na_2HPO_4 (BDH)). They were exposed to either 24 hour illumination (40-50 µmols $m^{-2}s^{-1}$) or a lighting regime of 16 hours light and 8 hours darkness. The algae were incubated at 15°C and fluorescence induction ratios (F_v/F_m) measured, after 15 minutes dark adaptation, to give a good indication of the health status of the algae.

The following results were obtained:

	24 hour lighting		16:8 hours light:dark	
	With nutrients	Without nutrients	With nutrients	Without nutrients
0 hours	0.812	0.823	0.815	0.812
24 hours	0.805	0.802	0.837	0.810
48 hours	0.756	0.662	0.836	0.738

Table 2: Mean fluorescence induction ratios $(F_v/F_m)_l$ for *E. intestinalis* exposed to different lighting regimes and nutrient conditions.

These results suggested that the algae were healthier when supplied with nutrients and subjected to a regime of 16:8 hour light:dark lighting.

2.1.4 CHOICE OF REGION OF *E.INTESTINALIS* FROND

F_v/F_m values were recorded, using a dark adaptation time of 15 minutes, for different areas of the *E. intestinalis* frond. The results did not show any statistical difference between the different regions ($P<0.05$), therefore, it was decided that intercalary sections should be used. The intercalary section of *E.intestinalis* was chosen, as it is wide enough for the clip used during fluorescence measurements and covered the area required during the *in vivo* spectrophotometry. Use of the tip of the frond was avoided because it could be undergoing cellular changes if the *E.intestinalis* became reproductive during the experiment and the results could consequently be significantly affected. Some *E.intestinalis* sections did become reproductive during experiments and it was observed that these sections changed from their usual green colour to a pale green/yellow. It was also evident that the F_v/F_m values dropped just before the algae became reproductive so, after consultation with a statistician and statistical testing, it was decided that any sections that became reproductive should be removed from the experiment completely.

2.1.5 CHOICE OF LAMPS USED

In order to determine the optimum lamp type and lighting levels for the incubation of the *E.intestinalis*, an investigation into the health status of the algae under different lighting conditions was performed.

Different lamp types have a different spectral output and this output could affect the health status of the algae. A comparison of the emission of different lamp types (figure 7) indicated that the spectral outputs of the different lamps were considerably different. The

emission of the light tubes was measured using a Quantaspectrophotometer with a flat sensor (Techtum Instruments – model QSM-2500).

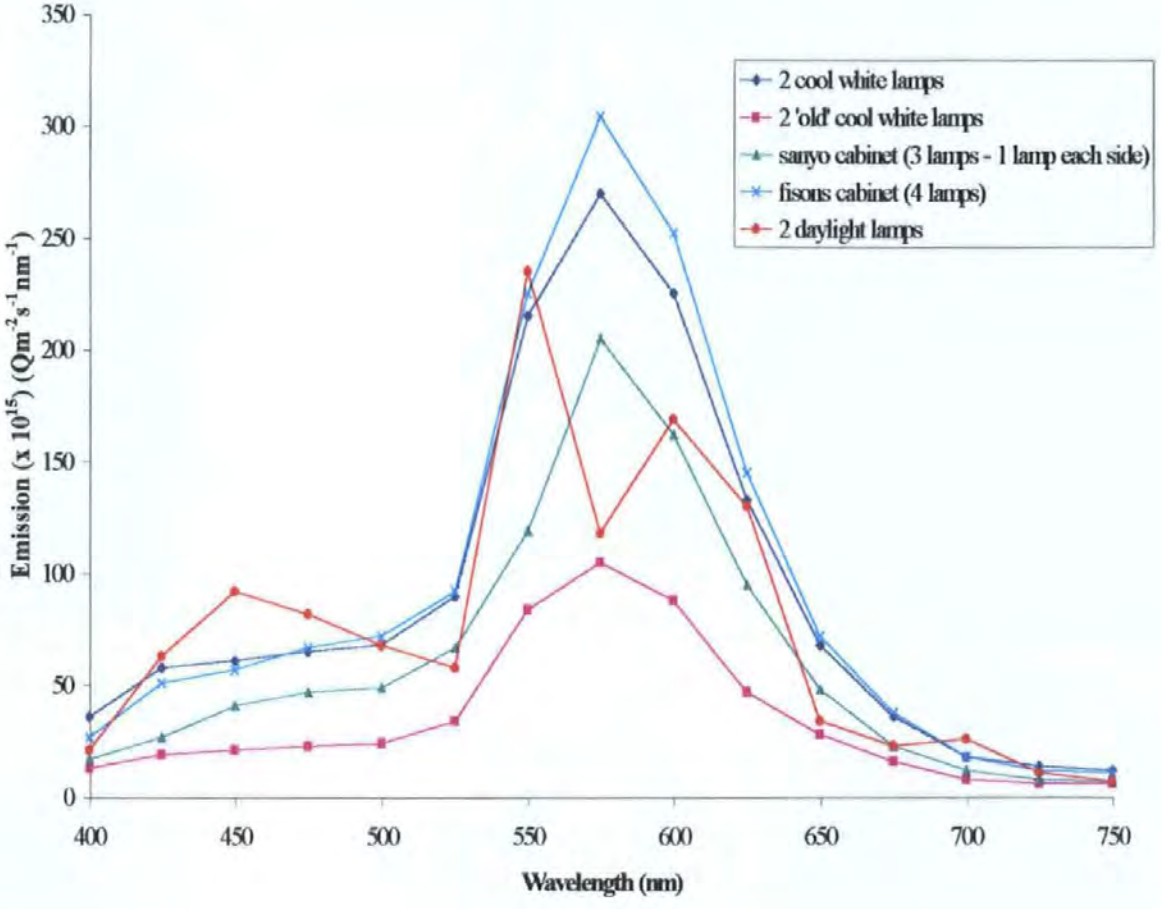

Figure 7: Emission spectra of different types of lamps in different lighting cabinets.

In addition to the type of lamp, it is also evident, by comparing the emission spectra from old and new lamps, that the age of the lamp also affects the spectral output. This must therefore be taken into consideration if successive experiments are carried out over a relatively long time period.

In order to provide optimum conditions for the algae in the laboratory, lighting is extremely important and should resemble the natural environment as much as possible. The predominant algae to be used throughout all experiments was *E.intestinalis* and the

light spectra should therefore resemble daylight or irradiance at relatively shallow depths as *E.intestinalis* is an intertidal algae.

It is apparent in figure 7 that many of the lamp types did not resemble natural daylight as they emit relatively low levels in the region of 400 – 500 nm in comparison to direct sunlight or irradiance at several metres depth in coastal water. The lamp that emitted the most throughout these wavelengths was the 'daylight lamp'. This lamp type also differed from the other lamps tested in that it had much lower levels than all the other new lamps tested at 575 nm. Overall, the 'daylight' lamp appeared to provide the emission spectra that was most appropriate because it had fairly high emission in the blue region and didn't have such a disproportionate peak in the green region as the other lamps. In addition, it also emitted relatively low levels in the red region, which would correspond with the coastal water absorbing much of the red light in the natural environment.

When *E.intestinalis* was exposed to the different light types it was found that they had better fluorescence measurements when kept under the daylight lamps with a 16:8 light:dark ratio. However, in addition to the light quality, the light quantity was also found to be important (figure 8). When the algae were kept under lighting of the same type but different amounts, the algae appeared to remain healthier, according to fluorescence measurements, when the light levels were not above 15 x 10^{18} Q $m^{-2}s^{-1}nm^{-1}$. It was therefore decided that the algae would be exposed to 2 daylight tubes, to ensure maximum coverage on the light bench, but would be placed a sufficient distance away from the lamps to receive 14 x 10^{18} Q $m^{-2}s^{-1}nm^{-1}$.

High levels of light appeared to result in photoinhibition and this is illustrated in fig. 8, which shows the effect of different lighting on fluorescence after 24 hours. A similar effect was also found by Hanelt *et al*, 1997, when the level of photoinhibition of *Laminaria saccharina* was measured using *in vivo* fluorescence changes of photosystem II.

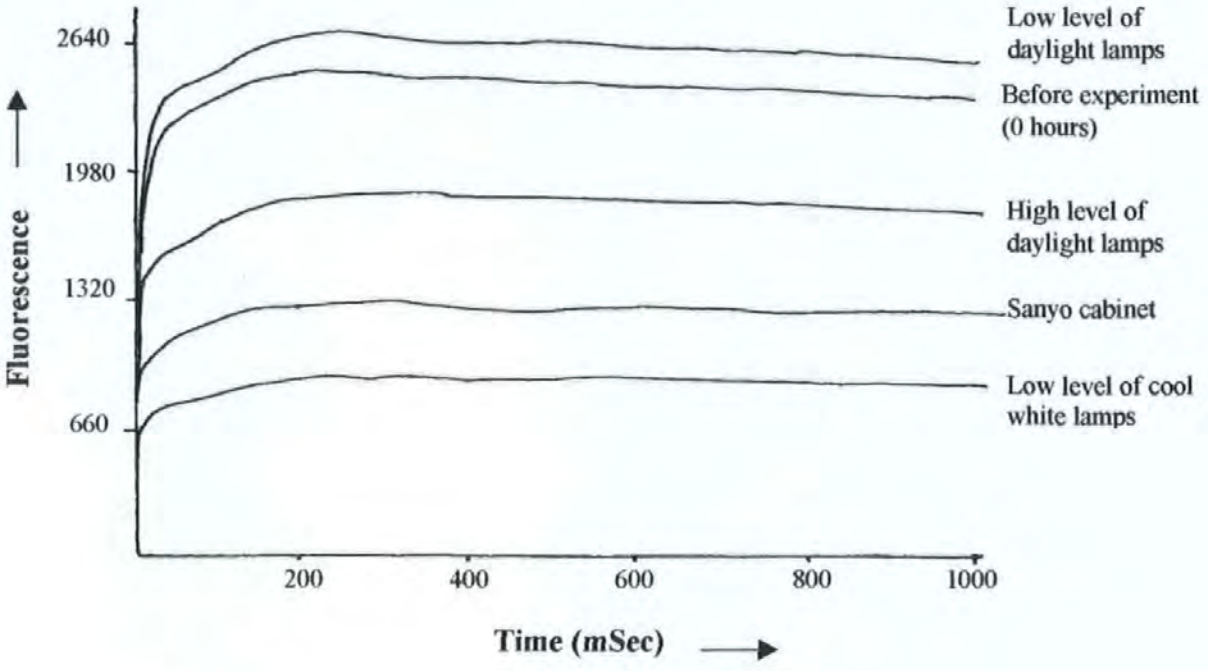

Fig. 8: Fluorescence of *Enteromorpha intestinalis* exposed to different lighting for 24 hours

2.2 DEVELOPMENT OF SPECTROPHOTOMETRIC METHODS

Two different spectrophotometers were used during the course of experiments. The first was the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere, which is a relatively new instrument. The second was the Pye Unicam SP8-100 UV/Vis spectrophotometer with diffuse reflectance accessory 790824, which is an older instrument with a larger sphere than the Labsphere RSA-UC-40.

2.2.1 ATI UNICAM UV4 UV-VIS SPECTROPHOTOMETER EQUIPPED WITH A LABSPHERE RSA-UC-40 INTEGRATING SPHERE

Description of instrument

The ATI Unicam UV4 UV-Vis spectrophotometer is a relatively new instrument with many capabilities. These capabilities were expanded by the use of the Labsphere RSA-UC-40 integrating sphere that allowed the measurement of reflectance and transmittance of samples *in vivo*. The sphere has an internal diameter of 63.5 mm and the internal surface is Spectralon. It is a single beam sphere and the light beam enters the accessory from the UV4 spectrophotometer and hits a mirror inside the sphere. The mirror inside the sphere is movable and can direct the beam to the reference port or the sample port. The original sphere supplied had 5 ports including a specular exclusion port which enabled part of the sphere wall to be removed and replaced with a light trap to allow the measurement of diffuse reflectance. However, after extensive testing the sphere was returned to Labsphere, the designers, because it was found to have several design faults and modifications to the sphere were carried out. This resulted in the sphere only having 4 ports. These were the transmittance sample port (also where the light beam enters the sphere), reflectance sample port, reference port and the output port.

As illustrated in Figure 9, the samples are held on to a wedge that ensures that the light beam is directed to the sample at an 8° angle of incidence. This was a modification from the original sphere to allow the measurement of diffuse (specular excluded) reflectance by the removal of the wedges instead of having a separate specular exclusion port. If the wedges are removed then the light beam is directed to the sample at a 0° angle of incidence and any specular component of reflection is directed out of the sphere through the transmission port and so is excluded from the measurement (Labsphere manual, 1995)

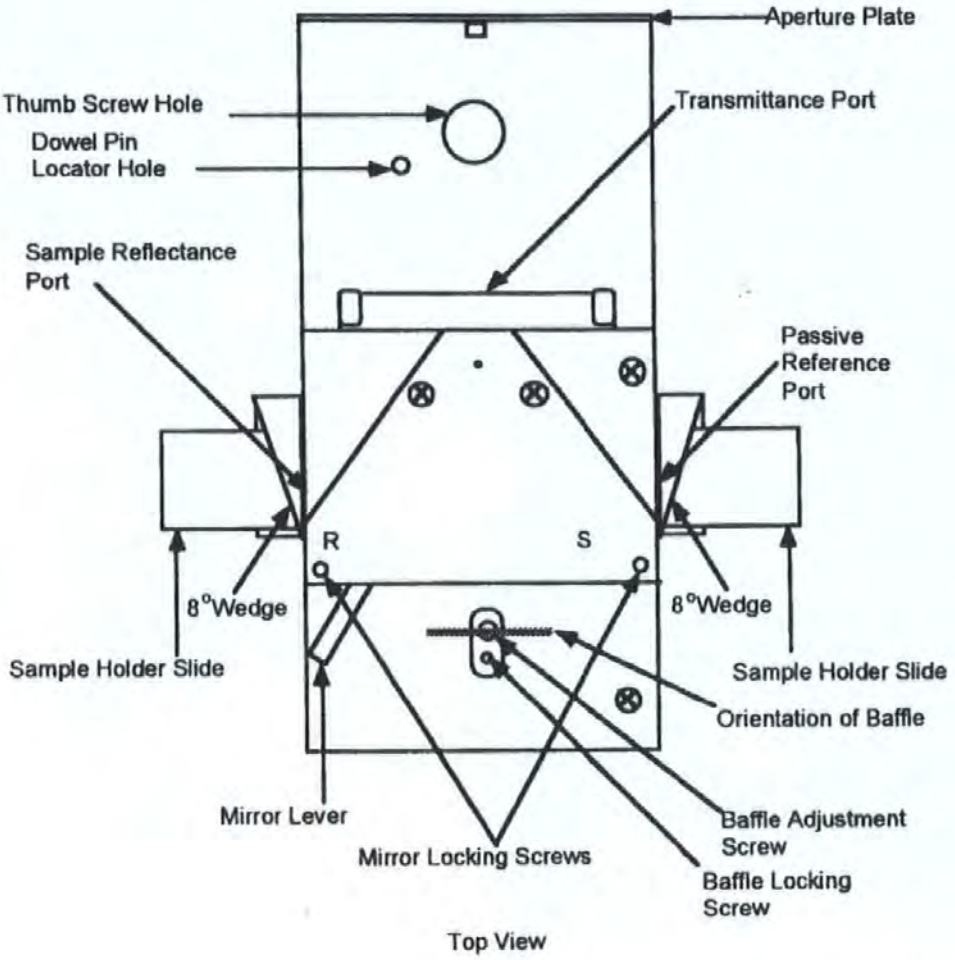

Figure 9: Top view of Labsphere RSA-UC-40 Accessory (Taken from Labsphere manual, 1995)

Setting Up The Instrument

Before the commencement of any experiments the RSA-UC-40 integrating sphere was optically aligned to ensure that there were no problems with the beam alignment (Labsphere RSA-UC-40 manual). The UV4 spectrophotometer was calibrated using a 'high absorbance EHT table' to reduce the noise level of the scans because the sphere absorbs a large amount of the input light. The high absorbance EHT table applies a higher voltage than standard to the Phototmultiplier.

Running the scans

The spectrophotometer was controlled using the Windows-based package 'Vision'. The scans were run over a wavelength range of 225-850 nm with a bandwidth of 4 nm to maximise the energy and minimise the noise of the scan. The scan speed of the instrument was set to '*Intelliscan*', which enhances the signal-to-noise ratio of the peaks without extending the scan duration. The spectrophotometer is able to do this by reducing the scan speed at the peaks of the scans, which increases the time taken to measure each data point, and by increasing the scan speed in areas of the scan which are flat and without peaks. A data interval of 2 nm was used and the changeover point between the deuterium and tungsten lamp occurred at 325 nm. When measurements were taken below 325 nm it was important to ensure that the machine was turned on for 30 minutes prior to use to allow the bulbs to warm up in order to ensure consistent results. The lower limit of the machine is 190 nm but during preliminary tests it was found that the scan had less noise above approximately 210nm.

The use of the *Intelliscan* was found to be important as it ensured that the time duration that the algae was tested was kept to a minimum. Stressing of the algae was, therefore, kept to a minimum and it was also found that the algal samples changed over a period of time if a very long time interval was used. This was confirmed by Dr Art Springsteen (pers. comm.) who tested some *Ulva lactuca* when the original sphere was sent back to the manufacturers. He tested the *U.lactuca* on a Perkin-Elmer Lambda-19 equipped with a Labsphere RSA-PE-10 integrating sphere accessory, identical to that used by the National Research Council Canada for standardisation of artefact standards of diffuse reflectance and transmittance, and found that the samples did change with time. When a relatively thick clump of *U.lactuca* was tested over a 50-minute time period the transmittance increased by 3.5 % at 825 nm. Therefore, as all experiments were using single pieces of *E.intestinalis*, '*Intelliscan*' appeared to be the most appropriate, as exposure to the light

beam would be kept to a minimum and therefore the moisture level of the algae would not change to such an extent that the results would be affected.

All data were collected without any smoothing in order for the data obtained to be as versatile as possible. After collection of the raw data, any smoothing required was done by using the smoothing algorithm employed by the Vision software based on a Savitsky Golay filter modified to reduce high frequency breakthrough so the amount of noise on the spectrum was reduced. The raw data had medium smoothing applied after collection as this was considered to be enough to increase the signal-noise ratio without being at the expense of spectral resolution. The scans that had either no smoothing or low smoothing were still relatively noisy, whereas it was felt that using high or very high smoothing could potentially mask some subtle changes.

Use of masks

The ports in the RSA-UC-40 integrating sphere were considerably larger than many of the samples. This was not a problem with the transmission port (where the light enters the sphere) as a 3 mm aperture plate was already supplied which was to be used when measuring reflectance. It was found that this plate could additionally be used successfully when measuring transmittance of small samples when, as long as it was in place when the baselines were set, there was no effect on the transmittance measurements of the sample.

The reflection port was also considerably bigger than many of the samples to be tested but no masks were supplied with the sphere. It was therefore decided to make masks to reduce the size of the ports. The size and colour of the masks had to be chosen carefully to allow relatively small samples to be measured without reducing the accuracy of the measurements. The use of white and black masks was investigated but it was found that the black masks were most suitable. This was because the black masks did not reflect any

of the incident light and so, ultimately, did not affect the results obtained. The initial masks that were made were circular, but it was found that this affected the reflectance results. This could have been due to the sample not receiving all the incident beam of light or the halo effect caused by the light beam hitting the edge of the mask. To overcome this, a rectangular mask, slightly larger than the actual light beam being directed by the mirror onto the reference port, was used. This was determined by setting up the spectrophotometer to produce a continuous beam of white light that was measured on the port using paper, drawing the size and position of the light beam, and then making a mask that could fix onto each side of the sphere so that its size and position would remain the same for each experiment. The masks were made of a black plastic that was sprayed with matt black paint and the same masks were used for all experiments. Results indicated that, as long as the masks were in place when the baselines were set, the reflectance of the samples was not affected.

When measuring samples of very low reflectance, an integrating sphere is sensitive to small-angle scatter from the sample beam coupling optics. This can strike the wall of the reflectance port and create a 'halo-effect' around the port, which can have a small effect on the reflectance measurements. This may easily be corrected using a procedure known as 'zeroline correction' and this correction should also be applied when a mask is used at the reflectance port. In order to use the zeroline correction, reflectance measurements are obtained in the usual way and, in addition, the reflectance using a light trap is measured. These results are then placed in the following formula: (Labsphere manual, 1995)

$$\rho_s = ((R-Z)\rho_r) / (100\% - Z)$$

where: ρ_s = Corrected reflectance of sample

R = the sample data displayed on the instrument

Z = the data from the light trap measurements

ρ_r = the reflectance value of the reference standard

Measurement of regular transmittance factor

The calibrated CSTM-SRS-99-005 'Spectralon' standard was placed in the reference port holder and the uncalibrated USRS-99-010 'Spectralon' standard was placed in the reflectance sample port. The mirror in the sphere was set to the 'R' (Reference) position *ie.* the input beam of light was directed to the calibrated CSTM-SRS-99-005 Spectralon standard placed in the reference port holder.

A 3 mm aperture plate was placed in front of the transmittance port to allow samples to be used that were smaller than the original sample port and a baseline scan of 100% transmission factor was run. The sample to be measured was then placed in front of the mask in the transmittance port, the scan run and the results recorded and any manipulations, ie. smoothing, were performed. The data was then transferred to an 'Excel 97' spreadsheet for easy storage and handling.

Measurement of total reflectance

The optical paths to the reference port and the sample port should be symmetrical, but it was decided that in order to ensure the optical path was never changed, the baselines and sample reflectance were always performed using just the reference port. This was decided during testing when slight differences were sometimes observed when the different sides of the sphere were used to set the baseline and run the sample. When the same side was used each time, the reflectance results of the same sample were identical. By using the same side of the sphere, the potential problem of substitution error (discussed later) was also avoided.

To allow samples that were smaller than the original sample ports to be used, masks were placed over both the reference port holder and the reflectance sample port. The calibrated

CSTM-SRS-99-005 Spectralon standard was then placed in the reference port holder and the sample was placed in the reflectance sample port. A light-trap was placed behind the sample and the mirror in the sphere was set to the 'R' (Reference) position. A baseline scan was run to record 100 % reflectance. The sample was then placed in the reference port holder with the light trap placed behind it and the calibrated CSTM-SRS-99-005 Spectralon standard was placed in the reflectance sample port and the scan was run. The data was recorded and any manipulations required *ie.* smoothing were performed and the data was transferred to 'Excel 97'.

Substitution error

Substitution error, sometimes known as 'single beam sample absorption error' can be defined as:

"a systematic, predictable and non-random error inherent in single beam integrating spheres measuring reflectance and transmittance."

(Labsphere RSA-UC-40 manual)

It can be a potential problem when using single beam spectrophotometers but can be eliminated by following certain procedures. Substitution error is actually caused by the difference between the throughput of the sphere when the reference makes up part of the sphere wall and the throughput when the sample is substituted for the reference. This change in the throughput of the sphere depends on whether reflectance or transmittance is being measured. During reflectance measurements, the throughput of the sphere will usually be lower when the sample is present because when running the 100 % baseline a reference material of virtually 100 % is used. However, during transmittance measurements the throughput of the sphere is usually higher when the sample is present because the 100 % baseline reference is run with the transmission port open which would result in zero reflectance from the transmission port inside the sphere. This could be one reason why unexpectedly high transmittance values were sometimes observed.

Substitution error is not really a problem if the sample and the reference are similar in their reflectance. However, the reflectance of algae is relatively low compared to the reflectance reference standard, so methods of correction were necessary as even small alterations could have been significant. The Labsphere RSA-UC-40 has 2 identical reflectance ports – the 'reference' port on the left of the sphere and the 'sample' port on the right side of the sphere. By using the 'sample' port as a passive reference port, the problem of substitution error was overcome. When setting the 100 % baseline, the reference standard should be placed in the reference port and the sample, complete with light-trap behind, should be placed in the passive reference port. When measuring the sample reflectance, the positions of the reference standard and the sample should then be switched over. By using this method, the average throughput of the sphere remained unchanged during the 100 % baseline measurement and the sample measurement.

2.2.2 PYE UNICAM SP8-100 UV/VIS SPECTROPHOTOMETER WITH DIFFUSE REFLECTANCE ACCESSORY 790824

Description of instrument

The Pye Unicam SP8-100 UV/Vis spectrophotometer is an older instrument than the ATI Unicam UV4 UV-Vis spectrophotometer but the diffuse reflectance accessory, or sphere, is much larger than the Labsphere RSA-UC-40 integrating sphere. The sphere allows the Pye Unicam SP8-100 UV/Vis spectrophotometer to be used to measure the reflectance and transmittance of samples *in vivo*. One of the main differences between the SP8-100 and the UV4 is that the SP8-100 is a double beam instrument.

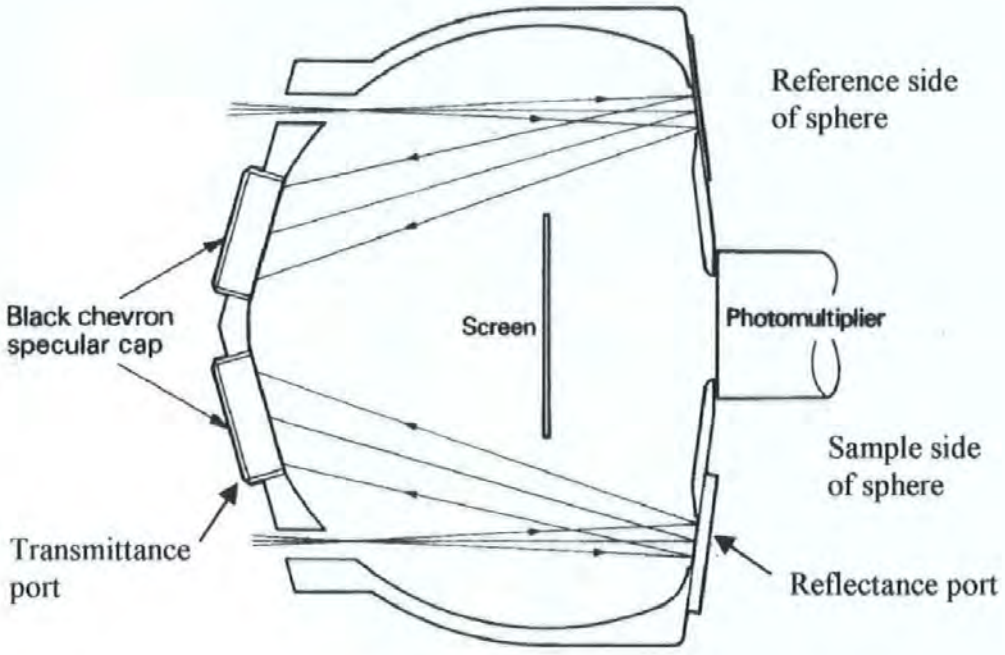

Figure 10: Diffuse reflectance accessory, or sphere, used with Pye Unicam SP8-100 UV/Vis spectrophotometer

Setting Up The Instrument

Before any experiments were performed, the inner surface of the sphere was re-coated using Eastman White Reflectance Paint (Kodak) by the use of a Kodak Laboratory sprayer. This was important as it ensured that the inner surface of the sphere was free from dirt and any bumps/dents so it reflected as much light as possible.

Running the scans

The spectrophotometer was interfaced with a BBC-B computer and was running a program designed by Dr. D.N.Price, University of Plymouth, titled 'Spectral Analysis'. Before any scans were run, the spectrophotometer was turned on for approximately 20 minutes to ensure that the lamps were adequately warmed up to ensure that results obtained were consistent. The data was recorded both by computer and the integral chart recorder.

The scans were run over a range of 300 – 750 nm with a scan speed of 5 nm/sec, data interval of 10 nm and a chart speed of 10nm/cm.

Use of masks

Masks were used when measuring both transmittance and reflectance, as the ports were considerably larger than many of the samples to be measured. These masks were supplied with the original instrument and did not affect the quality of the results as long as the mask was in place when calibrating the spectrophotometer to 100 % transmittance. The algae were always positioned on the inside of the mask throughout all experiments.

Measurement of sample transmittance

The white reflectance standards were placed in the reflectance ports at the rear of the sphere and remained there for all transmittance measurements. The transmission port containing the appropriate mask was left open and a 100 % baseline was recorded. The transmission port was then blocked to prevent light entering and a 0 % baseline recorded. The transmittance of the sample could then be measured by placing it in the transmission port and running the scan.

Measurement of sample diffuse reflectance

Masks for measuring the reflectance of samples smaller than the reflectance ports were supplied with the sphere so these were used throughout all experiments. The white standard reference was used behind the mask to set the 98 % baseline and the light trap was used behind the mask to set the 0 % baseline. A correction has to be made to the data because the standard white reference only gives 98 % reflectance above 400 nm dropping to 95 % reflectance at 300 nm. These corrections were automatically calculated by the computer program that records the results, but had to be performed manually if values were taken from the integral chart recorder.

Calculation of absorptance

The SP8-100 UV/Vis spectrophotometer with its sphere records actual transmittance which is a property of the sample. Therefore, it is possible to work out the *in vivo* absorptance spectra of a sample by using the calculation:

% Absorptance = 100% - (%R + %T).

2.2.3 COMPARISON OF THE 2 SPHERES

Reflectance

The *in vivo* reflectance values obtained by both instruments were extremely similar when using scattering (algae) and non-scattering samples (filters and coloured card).

Transmittance

The transmittance values of both the spectrophotometers with their respective spheres were compared using coloured filters and *E.intestinalis* in order to make comparisons between the performance of the spheres using non-scattering and scattering samples as it was found that the initial comparative results obtained appeared to differ.

Comparison of transmittance values using coloured filters.

When tested using coloured filters, the percentage transmittance values obtained (table 3) were extremely similar with both spectrophotometers and their relative spheres. This is illustrated clearly in figure 11 when the respective percentage transmittance values for each sphere are plotted against the other.

	ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere			Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824		
Wavelength (nm)	**Colour of filter**			**Colour of filter**		
	Yellow	**Blue**	**Red**	**Yellow**	**Blue**	**Red**
750	91.4	72.8	88.8	90	72	87.9
700	92.2	41	88.2	90	41.5	86.5
650	91.5	42.7	82.7	89.9	42.5	81.5
600	91.5	21.5	25.4	89.5	22	27
550	89.8	48.2	0.4	87	46	0.5
500	51	71.5	0.3	51	69	0.5
450	0.5	72.4	5.7	1	71	4
400	0.2	57.4	23.4	0.5	58	21
350	19.7	12.7	7.8	16	13.5	9
300	0	6.5	0.3	0.5	7	0.5

Table 3: Percentage transmittance of coloured filters using both spectrophotometers.

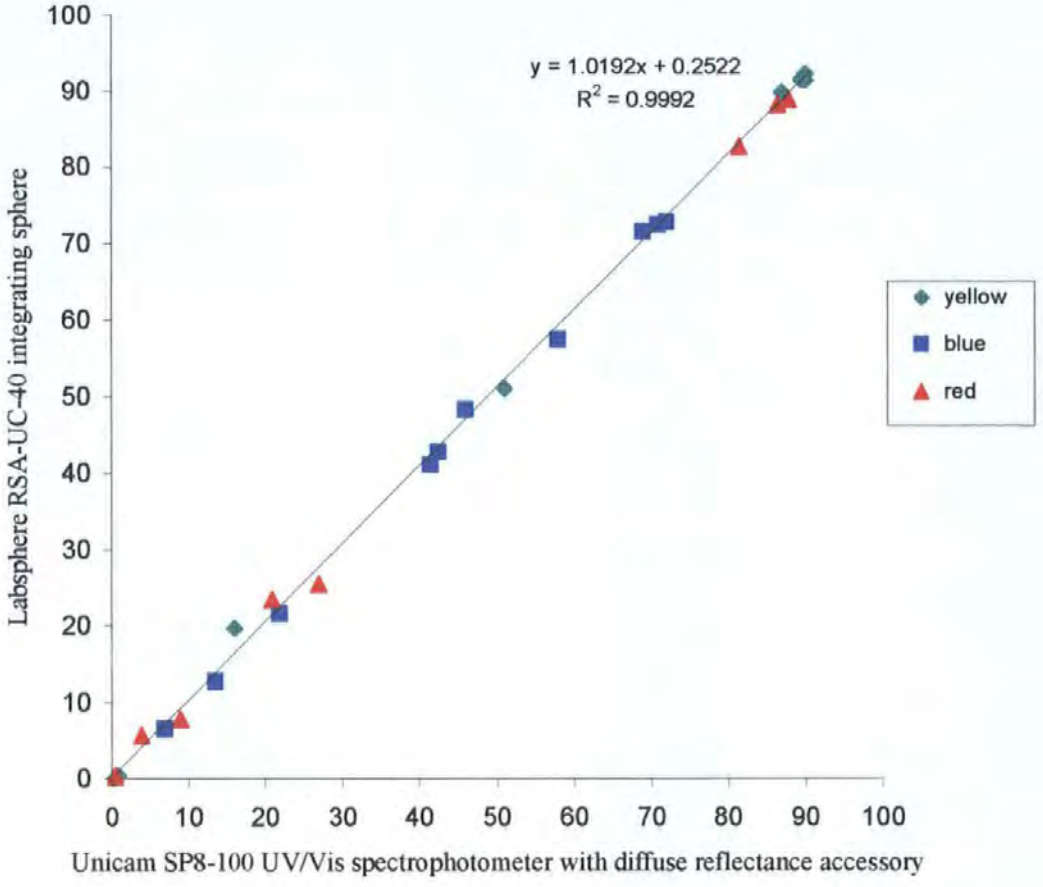

Figure 11: Comparison between ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere and Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824 using coloured filters

However, even though the values are almost identical when using coloured filters which are non-scattering it was also necessary to compare the spheres when samples that scatter light are used. Due to its structure, *E.intestinalis* tended to exhibit a degree of scattering when transmittance was measured.

Comparison of transmittance values using control and treated (Irgarol 1051, 2500μg for 5 days) *E.intestinalis*.

	ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere		Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824	
Wavelength (nm)	**Control (% T)**	**Irgarol treated (% T)**	**Control (% T)**	**Irgarol treated (% T)**
725	97.3	112.6	55.0	75.0
650	15.1	18.0	6.5	12.0
600	39.9	44.0	19.5	29.5
550	58.4	65.7	29.5	44.2
350	11.2	13.1	4.0	8.2

Table 4: Percentage *in vivo* transmittance of treated (Irgarol 2500 μg/l, 5 days) and untreated *E.intestinalis* using both spectrophotometers.

The transmittance values obtained for both the control *E.intestinalis* and the *E.intestinalis* exposed to 2500 μg/l Irgarol 1051 for 5 days appeared to be higher when using the Labsphere RSA-UC-40 integrating sphere than those obtained with the Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824. It was therefore decided to compare some of the transmittance ratios obtained.

	ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere		Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824	
Wavelength ratio	**Control (% T)**	**Irgarol treated (% T)**	**Control (% T)**	**Irgarol treated (% T)**
725/650	7.50	5.77	8.46	6.25
550/650	4.30	3.47	4.60	3.68
600/350	4.35	3.35	4.87	3.59

Table 5: *In vivo* transmittance ratios of treated (Irgarol 1051, 2500 µg/l, 5 days) and untreated *E.intestinalis* using both spectrophotometers.

The calculated transmittance ratios for both spectrophotometers were then used to calculate the percentage change between Irgarol-treated *E.intestinalis* and the control *E.intestinalis* using the formula:

Percentage change (%) = ((Control value – Treated value)/Control value) x 100

The results of the percentage change in transmittance ratio due to exposure to Irgarol 1051 in Table 6 indicate that although the actual transmittance results obtained using the ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere appear to be higher than those obtained using the Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824, the resultant percentage change due to the Irgarol 1051 treatment are similar.

Wavelength Ratio (nm)	**ATI Unicam UV4 UV-Vis Spectrophotometer equipped with Labsphere RSA-UC-40 integrating sphere**	**Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824**
725/650	**23.1**	**26.1**
550/650	**19.3**	**20.0**
600/350	**23.0**	**26.3**

Table 6: Percentage change in *in vivo* transmittance of E.intestinalis treated with Irgarol 1051 (2500µg/l, 5 days) using both spectrophotometers.

Although both spheres can be used to measure the transmittance of a sample, there is a slight difference between the actual measurements that the different spectrophotometers record. The Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824 measures the regular transmittance of the sample, which is a property of the actual sample. The RSA-UC-40 integrating sphere, used with the ATI Unicam UV4 UV-Vis Spectrophotometer, measures 'regular transmittance factor' not actual transmittance of the sample. Regular transmittance factor is defined as:

'the ratio of the flux transmitted by the specimen and evaluated by a receiver to the flux passing through the same optical system and evaluated by the receiver when the specimen is removed from the system'

(Labsphere RSA-UC-40 manual)

This regular transmittance factor is instrument specific and is supposed to closely approximate the total transmittance of a sample. However, it was found that when using samples that had a fairly high degree of scattering, *e.g. E.intestinalis*, the regular transmittance factor was considerably higher than total transmittance. Although this regular transmittance factor is not a quantitative measurement of the transmittance of a

scattering sample, the RSA-UC-40 integrating sphere was still used for many experiments as the readings were still relative and it had the advantage of having other facilities such as the ability to produce $1^{st} - 4^{th}$ derivatives of the scans if required. Derivatives of spectra, which are independent of absolute values, can reveal information that is sometimes difficult to pick up with a standard scan. Examples of derivatives are shown in Fig. 12

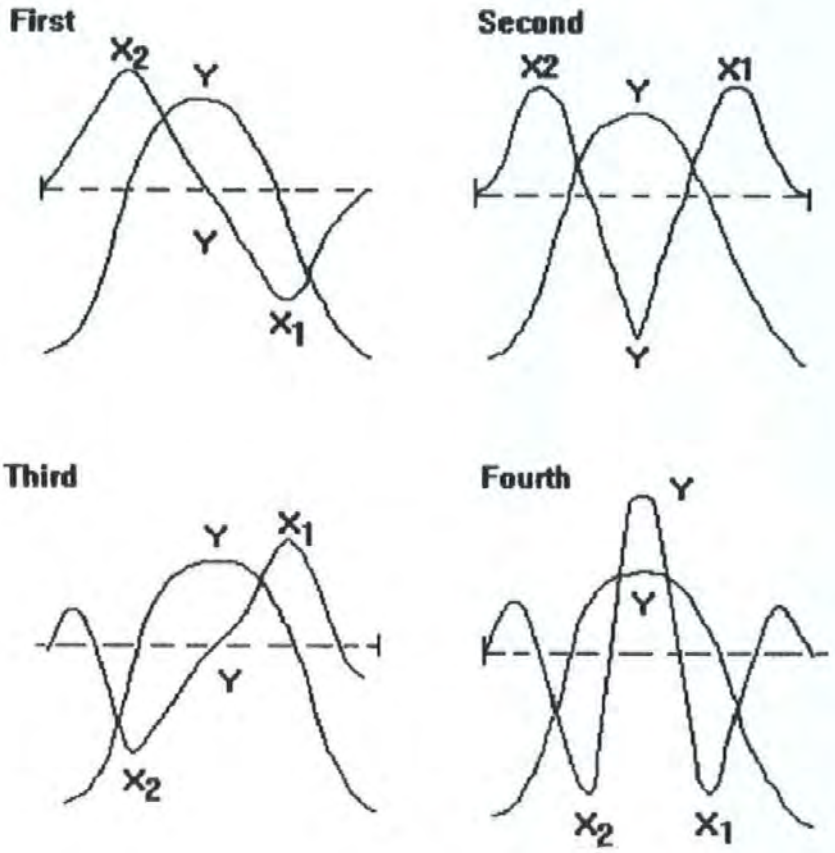

Fig. 12: $1^{st} - 4^{th}$ derivatives of a typical Gaussian curve

It was found when performing various data manipulations that the most useful derivative in this study was the first derivative. The first derivative is basically measuring the rate of change during a scan and hence produces a positive peak during the part of the scan where values are increasing and a negative peak where values are decreasing.

Both spectrophotometers were used during this study as they enabled complementary data to be obtained and used *ie.* use of derivatives with the RSA-UC-40 integrating sphere, used with the ATI Unicam UV4 UV-Vis Spectrophotometer, and calculation of thallus absorptance using the Pye Unicam SP8-100 UV/Vis spectrophotometer with Diffuse Reflectance Accessory 790824.

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL MATERIAL

3.1.1 COLLECTION

Enteromorpha intestinalis was collected at low tide from midshore rockpools in the intertidal region of Wembury Bay (OS SX517484) and other localities (see Fieldwork section) located in the South West of England.

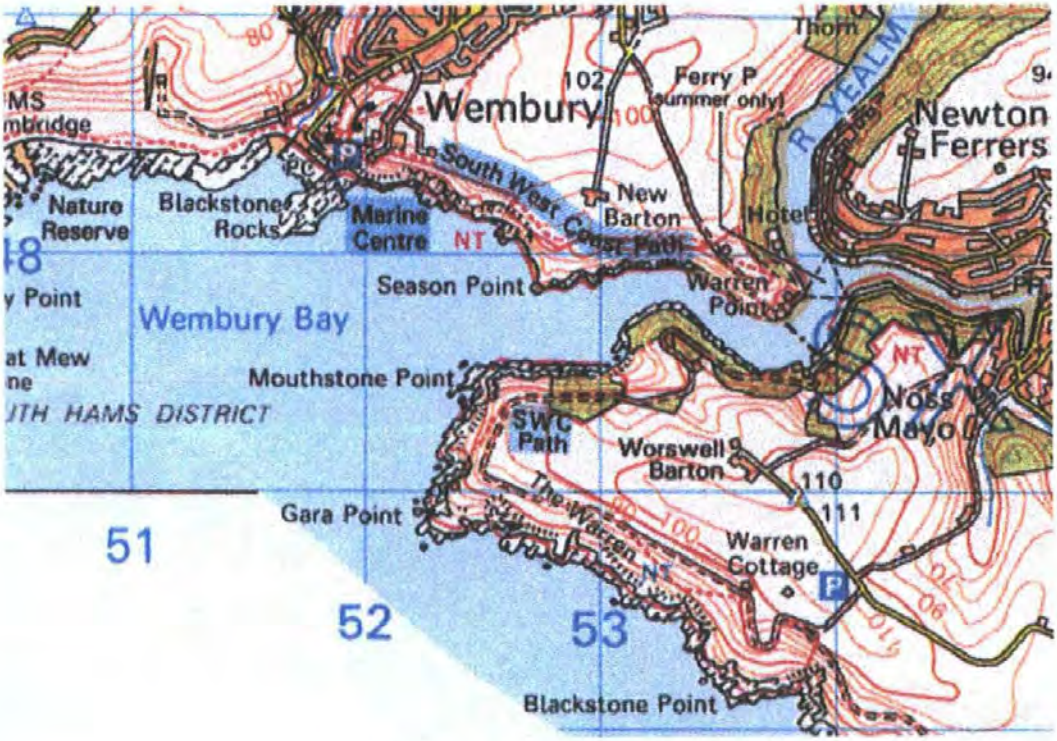

Figure 13: Map of Wembury Bay (OS SX517484) (taken from Ordnance Survey Map)

The environmental parameters of water temperature and salinity were recorded on site at the time of collection. Weather conditions were also noted. In order to minimise the occurrence of sporulation and ensure the macroalgae were in a similar reproductive state for each experiment, collection was avoided either just before or on a spring tide. The *E.intestinalis* was collected from the same rockpools on the beach for each experiment and

was placed in a clean bucket containing seawater to be transported to the laboratory. Wembury Bay is considered to be a 'clean' area as it is a conservation area and is not subjected to heavy boat use or other impacts.

3.1.2 IDENTIFICATION OF THE ALGAE

On return to the laboratory the macroalgae were positively identified as *E.intestinalis* using a Vanox Olympus research microscope, model AHBT. Microscopic identification was essential, as it is only possible to distinguish certain *Enteromorpha* species by cellular characteristics. It is extremely important that the same species of *Enteromorpha* was used throughout this study as there are known metal accumulation differences between even closely related species of macrophytes (Phillips, 1994). *E.intestinalis* and *E.prolifera* are morphologically extremely similar but their cellular arrangement and characteristics are considerably different. *E. intestinalis* cells are irregularly arranged and generally have a 'hook-shaped' chloroplast located at one end of the cell. This is in contrast to *E. prolifera*, which has a regular arrangement of cells and a ring-shaped chloroplast (Burrows, E.M. 1991). Plates **2**and **3** illustrate the differences between *E.intestinalis* and *E.prolifera.*

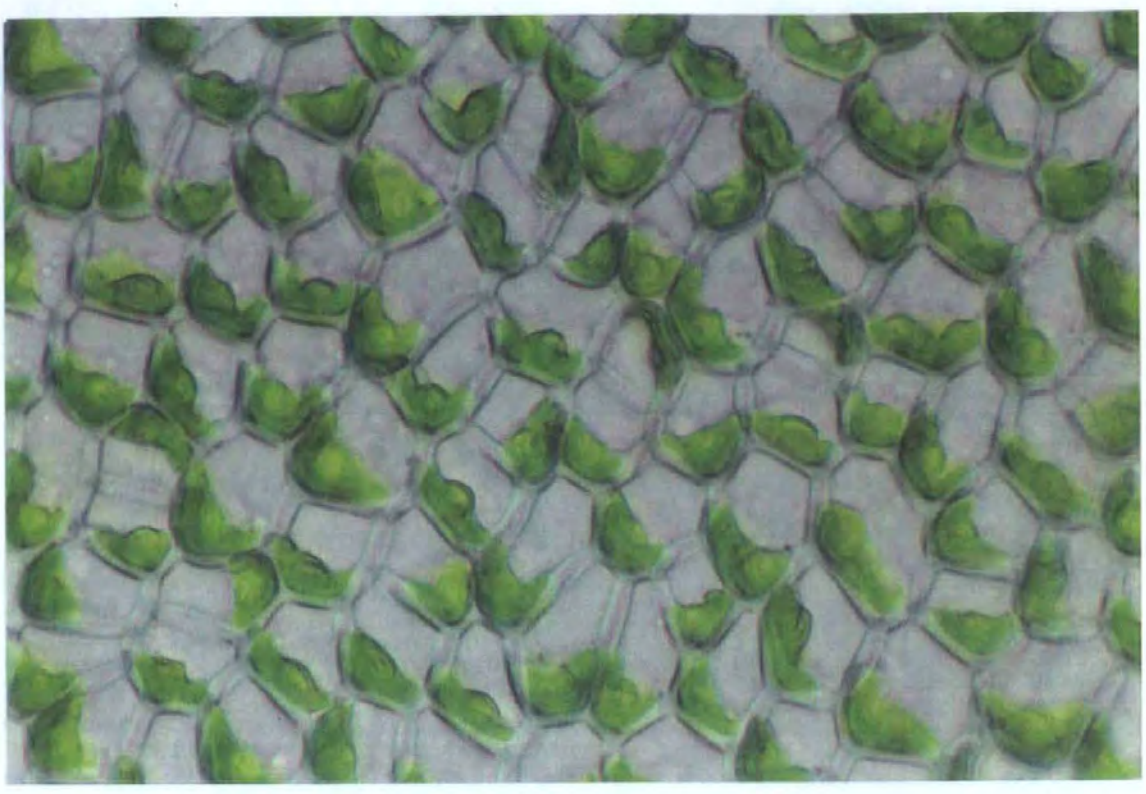

Plate 2 : Cellular arrangement in *E.intestinalis*

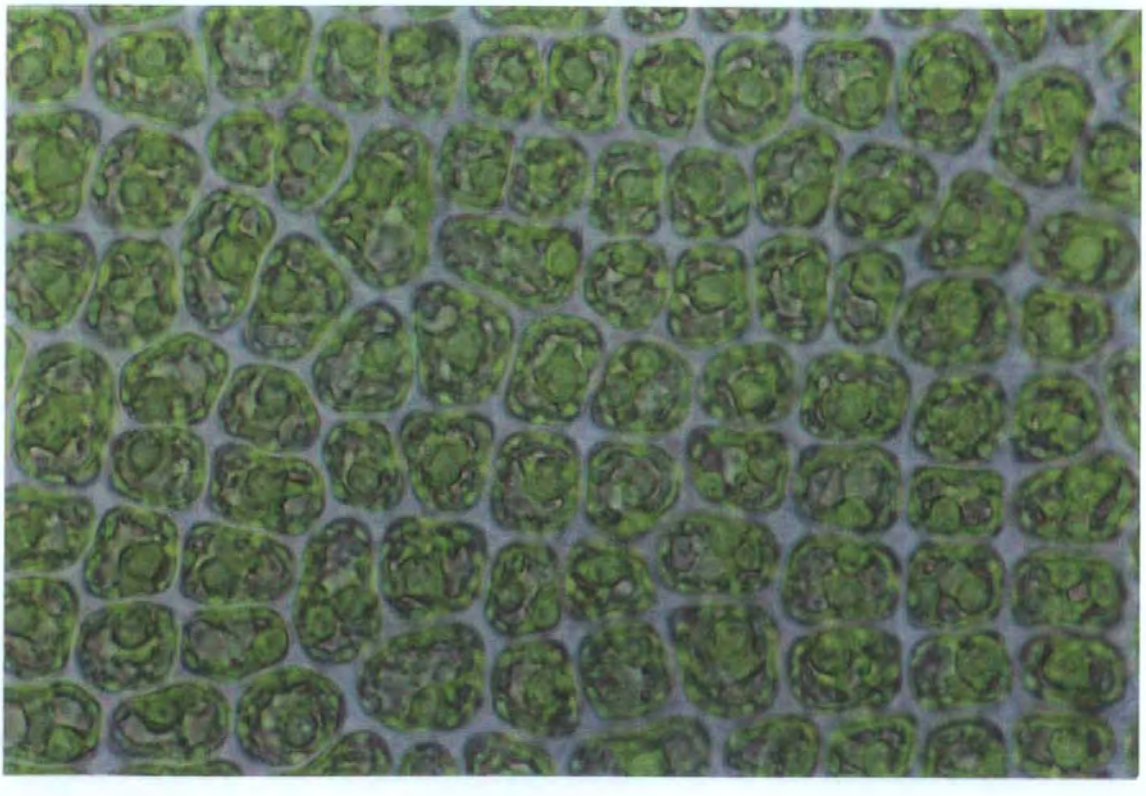

Plate 3 : Cellular arrangement in *E.prolifera*

3.1.3 STORAGE

The *E.intestinalis* was washed in artificial seawater (Instant Ocean, details earlier) with a salinity of 33 ppt. It is important that the algae used is cleaned of any mud because even superficial amounts would lower the reflectance when measuring the *in vivo* spectral properties (Guillaumont *et al*, 1997). The decision to use artificial seawater, as opposed to filtered seawater, was based on keeping variables in all experiments to a minimum. Although seawater could be filtered there could never be complete certainty that the properties of the seawater would not change over time due to seasonal variations. The use of Instant Ocean has the advantages that it has a consistent composition that is known (Appendix I) and does not contain chelating agents that could affect trace metal availability. In addition, the salinity can be controlled as it had been shown that salinity can significantly affect the accumulation of metals by macroalge in certain situations (Phillips, 1994). Although the composition of the Instant Ocean should be uniform throughout batches, the same batch of Instant Ocean was used for each set of experiments to further eliminate any possibility of differences. After washing, the macroalgae were transferred to tanks containing aerated (0.2 l/min) Instant Ocean (salinity 33 ppt) and nutrients – 50 mg/l sodium nitrate ($NaNO_3$) (BDH) and 10 mg/l di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) (BDH). The tanks were then placed under a light bench with 40-50 $\mu E\ m^{-2}s^{-1}$ 16:8 hour light:dark lighting (Phillips day light lamps) in a 15 °C temperature controlled room and the algae were left to equilibrate for 48 hours. The temperature was kept at 15°C because, although *Enteromorpha* species have a high tolerance of temperature they exhibited a maximum growth rate at 15°C (Poole and Raven, 1997).

3.1.4 SECTIONING

After 48 hours acclimation, intercalary sections 25mm in length were cut using a sharp scalpel blade to avoid the cells at the ends of the sections from becoming damaged and

placed in 33 ppt Instant Ocean with nutrients (as above). The length of the sections was measured using Vernier callipers in an attempt to stress the algae as little as possible so that the sections could be cut fairly quickly and in a 15 °C temperature controlled room. Intercalary sections were used to avoid the reproductive area of the frond and to try and ensure a similar area of the frond was used for each experiment so direct comparisons could be made. All handling of the algae was carried out at 15°C in a temperature-controlled room.

3.2 PARAMETERS MEASURED

In addition to *in vivo* spectral properties, other parameters, including fluorescence and growth, were measured to enable comparisons to be made to evaluate the use of the technique. The use of fluorescence and growth also provided an indication of the overall health of the algae. Fluorescence and growth data for the studies using copper and Irgarol 1051 was collected jointly with Stella Lewis, University of Plymouth. Fluorescence and *in vivo* spectral properties were analysed statistically by the use of Analysis of Variance (ANOVA) and Least Significant Difference (LSD) analyses. Lowest observable effect concentrations (LOECs) were recorded as the first treatment concentration that exhibited a significant difference from the control group where $P = 0.05$. Growth was analysed statistically by the use of the non-parametric Kruskal-Wallis test, at the 95 % confidence limit, due to non-normality and non-homogeneity of variance.

3.2.1 FLUORESCENCE

Fluorescence was measured using a Hansatech Photosynthetic Efficiency Analyser (PEA). Fluorescence of the *E.intestinalis* was measured using 100 % light with duration of 1 second after 15 minutes dark adaptation in 33 ppt Instant Ocean.

The Photosynthetic Efficiency Analyser records full fluorescence traces and individual parameters. When measuring fluorescence the full area of the clips was covered with the

algae so that direct comparisons could be made, as some of the fluorescence parameters are area dependent. Fluorescence induction ratios (F_v/F_m) were the main parameters recorded.

3.2.2 GROWTH

Growth was recorded as an increase in length over the experimental period. The final length of the sections was measured by the use of Vernier callipers. Although it is possible that this method may not be as accurate as using an image analyser, it was used because use of the image analyser would have resulted in the algae being more stressed than by the use of Vernier callipers if repeated measurements were to be made. This is because the algae would have had to be dried and placed on a light box for measurement and, in addition, it would not have been possible to control the temperature of the room in which the image analyser was kept. Callipers were therefore used throughout all the experiments to minimise stressing the algae and the same pair of callipers was also used during each experiment to minimise variation of results.

Growth is presented throughout as Percentage Relative Growth which was calculated as follows:

$$\% \text{ Relative Growth} = \frac{(\text{Final length (mm)} - \text{Initial Length (mm)})}{\text{Initial Length (mm)}}$$

Although this method is often associated with exponential growth, it was not considered to be a problem as the sections used were relatively small and the time period (usually seven days) was relatively short. This should enable the method to be used regardless of whether the growth of *E.intestinalis* is linear or exponential. It was also considered important to link the growth with the starting length because, even though all sections used were 25 mm long at the start of all experiments, it was important when comparing studies performed at different times using varying pollutants, that changes could be linked to pollutant exposure rather than other differences, for example change in time of season.

3.2.3 FERTILITY

Even though efforts were made to reduce the chance of the sections becoming fertile during the experiments, it was inevitable that some fertility would occur. Fertility was recorded as the percentage of sections that went fertile during the experiment. Although differences were not observed when statistically tested using F_v/F_m, sections that did eventually become reproductive changed colour before releasing the gametes. It is possible, therefore, that changes in the *in vivo* spectral properties may have occurred in these sections before it was obvious visually. In a study by Ikemori *et al*, 1977, it was found that 3 new green pigments were observed in the reproductive stage of *Enteromorpha linza* by using two-dimensional paper chromatographic separation. This could have a considerable effect on the *in vivo* spectral results so it was decided that any sections that did sporulate should be removed from the experiment completely. Plate 4 illustrates the cellular changes that the algae exhibit during sporulation.

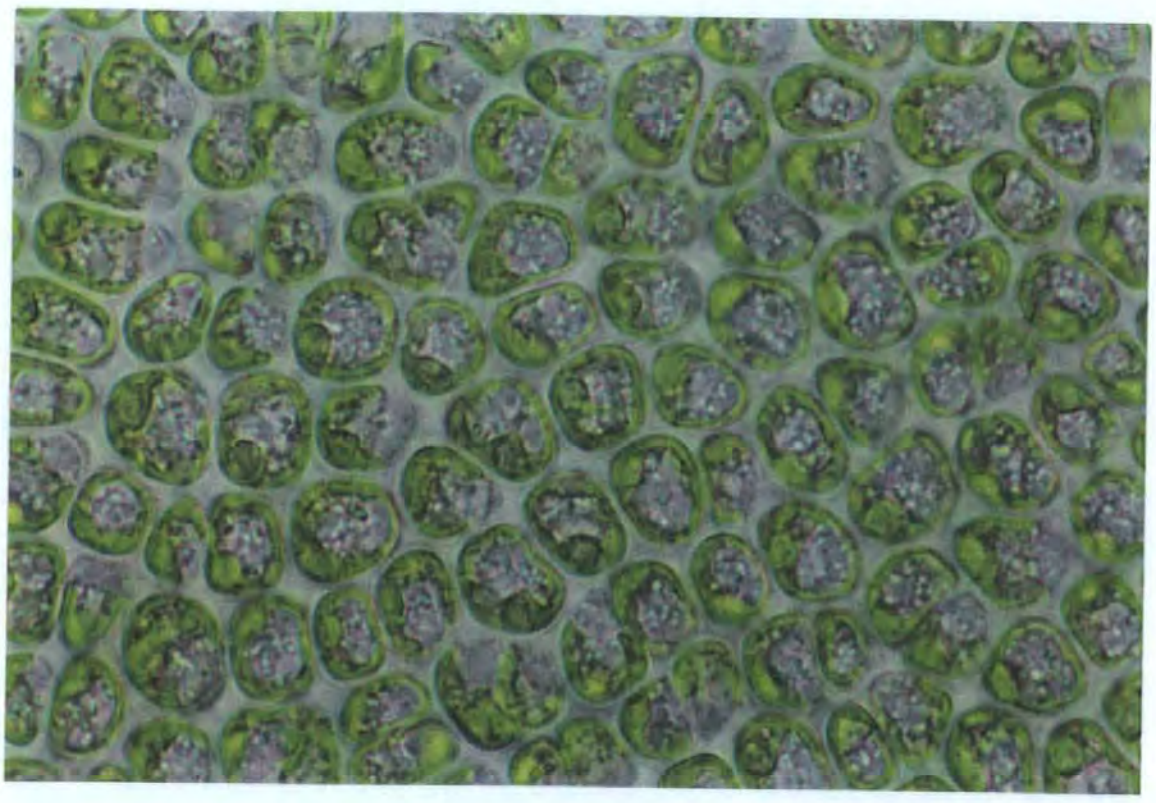

Plate 4 : Sporulation in *E.intestinalis*

3.2.4 SPECTRAL PROPERTIES

Spectral properties of the algae were recorded by *in vivo* spectrophotometry using a spectrophotometer equipped with a sphere as described in Chapter 2. The spectrophotometers used were the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere and the Pye Unicam SP8-100 UV/Vis spectrophotometer with diffuse reflectance accessory 790824. The scans obtained were used in a variety of ways to highlight differences observed after incubation of the macroalgae.

Altered spectra

Any alterations of the spectra after incubation in the treatment solution were recorded.

Wavelength sensitivity

A measure of wavelength sensitivity can be presented by calculating percentage alterations from the control spectra. The sensitivity was calculated by first subtracting the *in vivo* reflectance values obtained with a non-stressed thallus from those of a stressed thallus. The values or differences obtained were then divided by the *in vivo* reflectance of the non-stressed thallus to provide the relative change or sensitivity of reflectance to stress (Carter, 1994). Similar sensitivity ratios were also calculated with *in vivo* transmittance and absorptance values. It has been found that sensitivity maxima often occur between the ranges of 535 – 640 nm and 685 – 700 nm for different plant species (Carter, 1993).

Parameters of damage

Appropriate wavelengths, ratios and relative peak/trough heights can be selected from the sensitivity spectra as parameters of damage. Ratios have been used as indicators of plant stress with some success (Carter, 1994). Ratios have the advantage that they are not so instrument-specific as direct measurements. In addition, it has been found that if leaf

reflectance measured within a stress-sensitive waveband is divided by reflectance measured within a relatively stress-insensitive waveband variations in irradiance, leaf orientation, irradiance angles and shading are largely corrected for (Carter, 1994). This therefore provides a similar parameter to vegetation indices that are commonly employed in remote sensing (Baret and Guyot, 1991), but it has the advantage of being optimised for detecting stress.

3.2.5 METAL ANALYSIS

Metal analysis was used to determine the levels of copper and zinc in the *E.intestinalis* collected from each site. The following method, developed by Paul Williams, a research assistant at the University of Plymouth (pers. comm.), was used to analyse the metal content of the algal tissue. It resulted in a recovery rate for copper of 87.3 % (Std. Error = 1.76, n=5) and zinc of 86.2 % (Std. Error = 1.81, n=5) when a certified reference material (*Ulva lactuca*), supplied by the Community Bureau of Reference, was analysed.

- 10g of material from each site was freeze-dried for 24 hours in acid-washed centrifuge tubes. Freeze dried material (0.5 g) from each site was then put in acid-washed Teflon microwave vessels and had 5 ml nitric acid (Aristar) and 2 ml of 30 % hydrogen peroxide (Analar) added. A blank (no algae) was also prepared. These were left overnight to allow digestion to commence.
- The samples were digested using the MDS-2000 system and the following program:

Power (%)	20	40	60	80	100
Pressure (psi)	20	40	60	80	100
Time (min)	2	2	2	2	2

This program of digestion was repeated after the samples had been cooled for 1 hour and vented.

- The digested material was allowed to cool after which they were filtered and made up to 25ml using 0.1M nitric acid (Aristar). The samples were then analysed on a Varian atomic absorption spectrophotometer.

3.2.6 IRGAROL ANALYSIS

The levels of Irgarol 1051 in the *E.intestinalis* were determined after exposing the algae to varying Irgarol 1051 levels. (results courtesy of Alan Scarlett, pers. comm.)

3.2.7 FIELDWORK

Samples of *E.intestinalis* were collected from various sites in the SouthWest in order to measure their *in vivo* spectral properties and fluorescence. The sites were chosen to include 'clean' sites (Wembury OS SX517484, Mothecombe OS SX610473 and Thurlstone OS SX660435), metal-polluted sites (Restronguet Creek OS SW385810, Pill OS SW 385827, Mylor (OS SW356809), Percuil (OS SW343857) and St. Just (OS SW3618484) in Cornwall and sites with potential organic pollution (Sutton Harbour OS SX486543, Admirals Hard and Pomphlett Creek). Environmental conditions were measured using a PAR meter SKP200 (Skye Instruments Ltd.) and a salinity ATAGO Hand Refractometer. Temperature was also recorded.

Samples of *E.intestinalis* were collected in polythene bags and placed in a cool box for transport back to the laboratory. On return to the laboratory the samples were identified and left to acclimate in 33 ppt Instant Ocean, with nutrients, at 15 °C for 24 hours with a light:dark ratio of 16:8 hours (40-50 μmols $m^{-2}s^{-1}$). After acclimation, fluorescence and *in vivo* spectral properties of the samples were measured.

3.3 EXPERIMENTAL PROTOCOLS

3.3.1 SECTIONS IN PETRI DISHES

The 25 mm intercalary sections of *E.intestinalis*, obtained as in section 2.1.3, were placed in disposable, to prevent contamination, tri-divided petri dishes containing 33 ppt Instant Ocean and the treatment solution. Throughout each experiment the algae were kept at 15°C with a light:dark ratio of 16:8 hours (40-50 μmols $m^{-2}s^{-1}$). Using petri dishes had various advantages including requiring less space and algal material, therefore enabling more replicates to be used, and reducing the need for aeration due to a high surface area:volume ratio. The petri dishes were arranged at random in the cabinet and a range of light readings were taken inside the cabinet to ensure that some areas were not exposed to more light than others. The pollutant concentrations are nominal and so adsorption to the petri dishes or possible uptake by the algae was not taken into account. However, solutions were changed every 48 hours to minimise potential errors. The sections were incubated for 7 days, the solutions being changed every 48 hours and the lids of the petri dishes were removed and the solutions gently swirled to ensure they were aerated every 24 hours.

Number of samples

Twenty-four sections of *E.intestinalis* were used for controls (both standard and carrier) and twelve sections were used for each concentration of pollutant. This was because preliminary results from trial runs indicated that more sections from the controls were likely to sporulate than the sections that were exposed to pollutants. Any sections that did sporulate were subsequently removed from the experiment completely to avoid any possible potential effect on the data.

Preparation of solutions and controls

The copper stocks were made by using copper chloride in MilliQ ultrapure water. This was added to Instant Ocean (33 ppt) containing nutrients (50 mg/l $NaNO_3$ (BDH) and 10 mg/l Na_2HPO_4 (BDH)). The control used during these experiments was Instant Ocean containing nutrients (see above).

To make the triazine stock solutions, the triazines, both Irgarol 1051 and atrazine, were dissolved in ethanol. Treatment solutions were then made by adding 100 µl of each stock solution to Instant Ocean containing nutrients (50 mg/l $NaNO_3$ and 10 mg/l Na_2HPO_4) to give the required concentrations. The 100 µl of stock solution was added to the Instant Ocean in a vortex and stirred for a minimum of 2 hours to ensure the triazine was dissolved in the solution. In addition to a solution containing no triazine or ethanol, a carrier control was also prepared containing 100 µl ethanol to ensure that the ethanol did not have a detrimental effect on the algae. For all experiments, the control used for analysis of data was the carrier control containing ethanol as all the treatment solutions also contained 100 µl ethanol. The Instant Ocean solutions were made fresh every 48 hours and the triazine stocks were kept in the dark at –20 °C in order to prevent degradation.

3.3.2 SECTIONS IN SEALED CONTAINERS

Intercalary sections (25 mm long) of *E.intestinalis* were cut from healthy fronds (as described in the methods section) and placed in 400 cm^3 glass airtight, screwtop jars, each containing 300 cm^3 solution (Instant Ocean containing nutrients and respective alcohol). These were primarily used for the experiments involving alcohols which are very volatile. Throughout the experiment the algae were kept at 15 °C with a lighting regime of 16:8

hours light:dark (40-50 μmols $m^{-2}s^{-1}$). Six sections of *E.intestinalis* were used for each concentration of each alcohol. Screwtop jars were used to prevent loss of the alcohols as they are extremely volatile but the jars were not filled completely and were placed on their sides on a shaker table to provide constant movement to ensure continuous aeration. The experiment ran for 96 hours but all solutions were changed every 48 hours.

4. TRACE METALS

4.1 COPPER

Copper can exist in many different chemical forms, but the levels of dissolved copper in seawater are usually relatively low with an average of 4μg/l in waters with a salinity of over 35% (Chester 1990). However, levels of copper can increase due to various factors, including natural and anthropogenic sources, and levels as high as 176 μg/l have been reported (Bryan and Langston 1992) in Restonguet Creek in the South West of England where levels are high due to previous mining activities in the region.

Copper is an essential micronutrient but it can also be extremely toxic to algae and is, for this reason, often used to control algae in fresh water (see Lobban and Harrison 1994). The effects of copper on macrophytes have been studied more extensively than any other trace metal (Lobban and Harrison 1994), but its physiological effects are still unclear. Copper can exist in different forms, free ions or complexes, in the marine ecosystem and this can affect its toxicity. Generally, it is the bioavailability of the metal that has most effect on the toxicity with free ions being the most bioavailable form (Gledhill et al 1997). The main aim of the following studies involving copper are to assess whether the effects of copper exposure can be determined by examining the *in vivo* spectral properties of *E.intestinalis*. These will be compared with more traditional parameters including growth and fluorescence techniques in order to assess the health status of the algae and the suitability of the technique in assessing copper pollution.

4.1.1 EXPERIMENTAL DESIGN

Collection and acclimatisation of algae

E.intestinalis was collected from Wembury Bay, identified and acclimatised as described in the Method section (Chapter 3).

Preparation of solutions

Copper stocks were made, as described in the Methods chapter, and was added to Instant Ocean (33 ppt) containing nutrients (50 mg/l $NaNO_3$ (BDH) and 10 mg/l Na_2HPO_4 (BDH)) to give concentrations of 0, 25, 50, 100, 200 and 500 µg Cu/l. The glassware used was acid washed before the experiment to ensure there was no metal contamination.

Experimental setup

Intercalary sections (2.5 cm long) of *E.intestinalis* were cut from healthy fronds and placed in tri-divided petri dishes each containing 50 ml of the relevant copper solution (as described in the Methods section).

Parameters measured

Growth was measured on day 7 and was recorded as Relative Growth Rate (RGR). Fertility was also recorded as the percentage of sections that sporulated during the experiment. Any sections that did sporulate were removed from the experiment for reasons discussed earlier.

Fluorescence measurements were recorded on days 0, 1, 2, 4 and 7 as F_v/F_m for one experiment and both F_v/F_m and area above the fluorescence curve for another experiment.

The *in vivo* spectral properties of the algae were also measured on days 0, 1, 2, 4 and 7 using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere.

4.1.2 RESULTS AND DISCUSSION

Growth

Figure 14 illustrates the effect of 7 days copper exposure on the growth of the algae *E.intestinalis*. It is evident that copper does have a significant ($P<0.05$) effect on

E.intestinalis with the fronds exposed to the highest concentration of 500 µg/l exhibiting virtually no growth and a LOEC of 50 µg/l calculated using 95 % confidence limits. An EC_{50} for growth of the alga, when treated with copper, of approximately 65 µg/l was calculated from the dose response graph (figure 14).

This is relatively low because in a study by Correa (1996), it was found that copper concentrations varied from 0.05 µg/l in clean coastal waters in the U.K. to 176 µg/l in polluted U.K. estuaries. Therefore, if the EC_{50} for growth is 65 µg/l, this is lower than some levels of copper in polluted U.K. waters and so *E.intestinalis* could be adversely affected. This EC_{50} for growth was calculated from adult plants but other life stages of the alga, e.g. reproductive stages, may be even more susceptible to copper exposure. However, when dealing with the toxicity effects of copper, the chemical form of the copper must also be taken into account as the copper species can affect the toxicity of the metal to the algae (Gledhill 1997). It is possible, although unlikely at the concentrations used (Gledhill, pers. com.), that the copper present in the seawater may have been complexed by dissolved organic matter which would decrease the amount of free copper concentrations. It is this free ion concentration that directly affects the toxicity and uptake of the copper to the algae (Gledhill 1997) and so care must always be taken in interpreting results involving copper exposure.

Fluorescence

The effect of copper on fluorescence (F_v/F_m) of *E.intestinalis* can be seen in figure 15. The Fv/Fm response of the algae appears to fall into three groups when exposed to copper. It appears that there is no effect on the F_v/F_m of the algae exposed to fairly low levels, up to 50 µg/l of copper, even though growth was beginning to be affected by these concentrations. The algae exposed to 100 and 200 µg/l of copper suffered an initial decrease in Fv/Fm levels until it was exposed for 4 days (96 hrs) after which time it

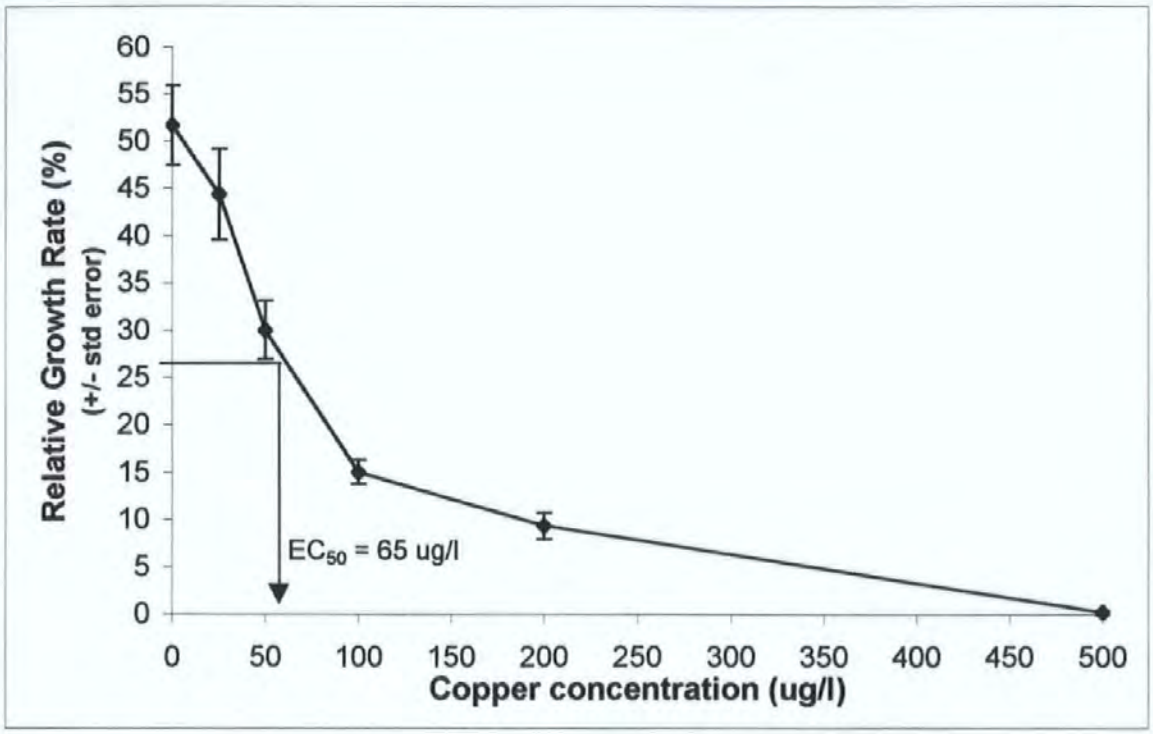

Fig. 14: Relative growth rate of *E.intestinalis* exposed to copper (ug/l)

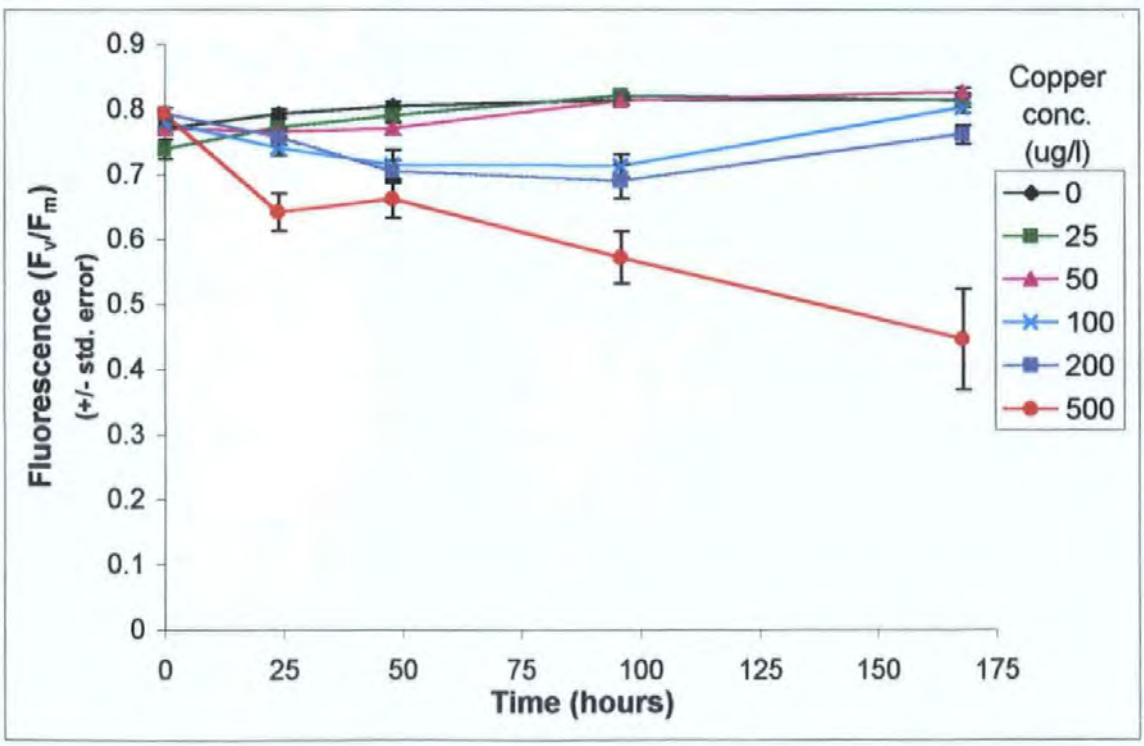

Fig 15: Effect of copper (ug/l) on fluorescence (Fv/Fm) of *E. intestinalis*

appeared to recover to levels approaching those of the control and lower concentrations. The algae that were exposed to 200 µg/l copper appeared to have a fairly high Fv/Fm value after 7 days (mean value of 0.76) even though the mean relative growth rate (%) at this concentration was reduced to 9.32 % as opposed to the control value of 51.67 %. The algae could be tolerating the copper exposure by possibly detoxifying the copper ions or excluding their uptake. If the algae managed to bind the copper ions in some way this would make the copper non-toxic. It is also likely that the energetic cost of this might cause a decrease in growth while having little effect on F_v/F_m. However, the copper did not significantly ($P<0.05$) affect fluorescence until levels of 500 µg/l were used, with the algae exposed to this concentration exhibiting a steady decrease in F_v/F_m values each day. However, although it is evident that copper is having an effect on the fluorescence of the algae at high concentrations, an EC_{50} could not be calculated from the dose response curve because even the highest concentration (500 µg/l) was not high enough to reduce mean F_v/F_m levels to 50 % of the control values.

The fluorescence induction ratio (F_v/F_m) is a sensitive indicator of damage to the photosynthetic system of the algae, especially that of photosystem II. It is possible that a different fluorescence parameter than Fv/Fm may be more sensitive in detecting effects induced by the copper exposure. This may be due to the copper affecting a different part of the algae than photosystem II. Figure 16 illustrates the effect of copper on the fluorescence parameter of Area above the fluorescence curve between F_0 and F_m (refer to figure 1 in Introduction) of *E.intestinalis*. The fluorescence parameter of Area is proportional to the pool size of the electron acceptors Q_A on the reducing side of Photosystem II (Hansatech manual). If the area is dramatically reduced, this indicates that the electron transfer from the reaction centres to the quinone pool would be blocked (Bolhar-Nordenkampf, 1993). However, this is not likely to be the case when exposing *E.intestinalis* to copper because although the copper exposure did result in some decrease

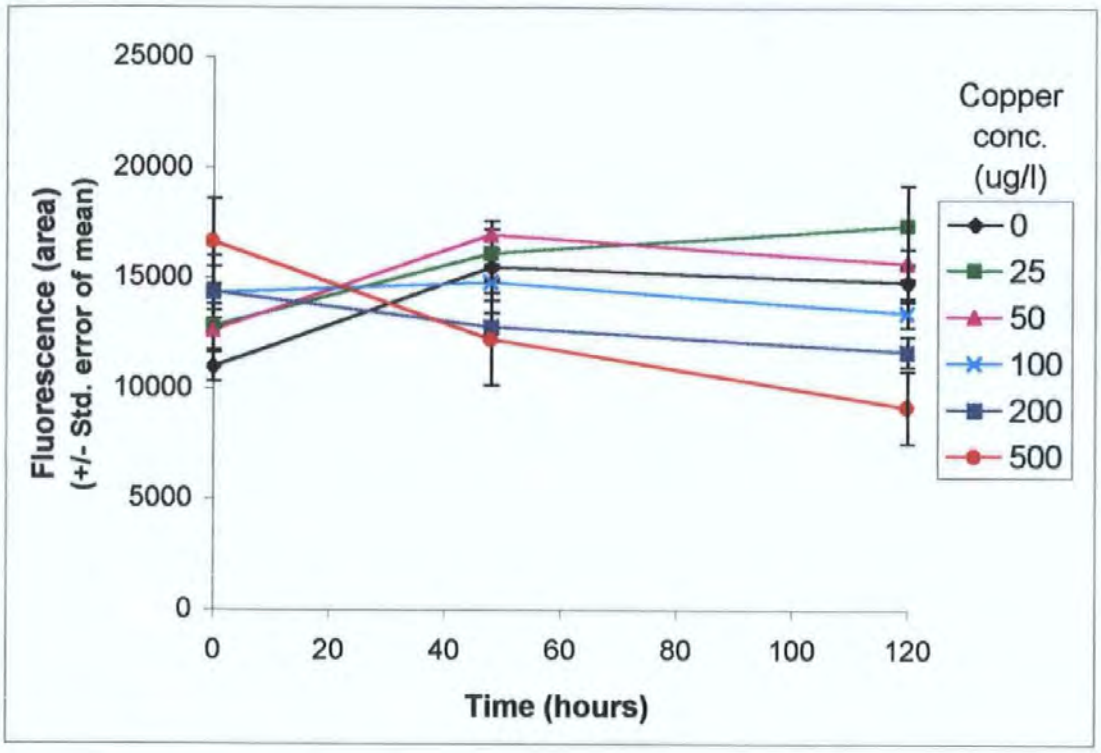

Figure 16: **Effect of copper (ug/l) on fluorescence (area over the) fluorescence curve) on *E.intestinalis***

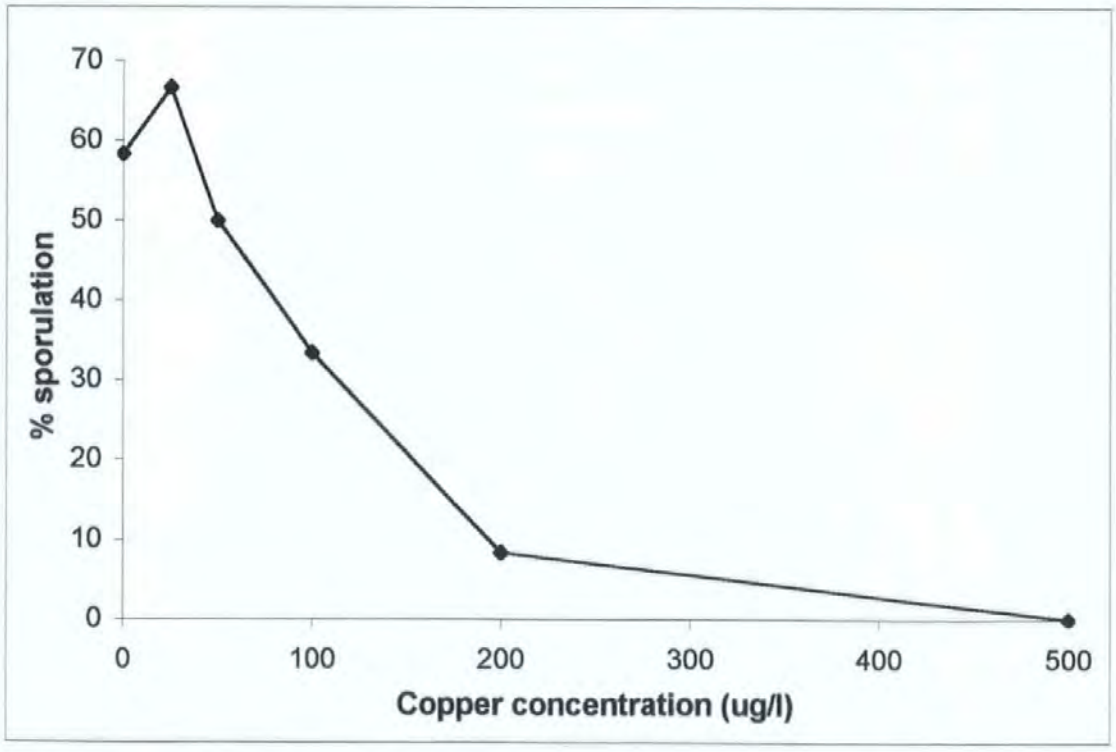

Figure 17: **Effect of copper (ug/l) on percentage sporulation of** *Enteromorpha intestinalis*

in area at 500 µg/l it did not decrease sufficiently to calculate an EC_{50}. Further experiments using increased levels of copper were not performed because growth was affected at copper levels that had either minor or no effect on F_v/F_m and area. This is important when monitoring different parameters to detect stress in algae, and indeed other organisms, as even if one aspect is altered due to a stress it may not necessarily have a detrimental effect on the ultimate survival of the algae. Certain parameters such as growth and reproductive stages may be important in determining the health status of the algae but it is possible that other parameters may be useful in perhaps detecting changes before the health/performance of the algae is threatened.

Fertility

It was noted that copper also had an effect on the percentage of *E.intestinalis* that became reproductive and sporulated during the experiment (Figure 17) as there appeared to be some correlation between the inhibition of sporulation of the algae and copper concentration. Percentage sporulation decreased dramatically due to exposure to copper with levels falling from nearly 60% in the control sections to 0% in the sections exposed to 500 µg/l copper. Copper may therefore be interfering with the reproductive physiology of the algae. This is important as many tests are carried out on adult macroalgae when other life stages should also be considered because if they were affected then the ecological success of the algae would consequently be affected. Pollutants have been reported to affect spore liberation and germination with reports of 0.1 ppm crude oil resulting in a decrease of 90 to 100 % with *Gracilaria sps* but reproduction was still observed at 80 – 100ppm for *Enteromorpha compressa* (Premila and Rao, 1997). However, it must be noted that the fertility results obtained were only a secondary observation, as the experiment was not designed to investigate fertility, and so should consequently be treated with caution.

IN VIVO SPECTRAL PROPERTIES OF *E.INTESTINALIS*

IN VIVO TRANSMITTANCE FACTOR

From the results of the effect of copper on the *in vivo* spectral properties of *E.intestinalis* it is apparent that significant changes are evident as a result of the copper treatment. When the algae was treated with copper, an increase in certain regions of both the *in vivo* transmittance factor and reflectance spectra were observed.

Figure 18 shows the effect of copper on the *in vivo* transmittance factor of *E.intestinalis* after exposure for 7 days. It can be seen that after treatment, a dose response is apparent with copper exposure resulting in an increase across the entire *in vivo* transmittance factor spectrum up to 720 nm. Above 720 nm there does not appear to be any difference between the *in vivo* transmittance factor of the algae treated with 500 µg/l copper and the control. (figure 19)

The difference between the control *in vivo* transmittance factor and the *in vivo* transmittance factor of *E.intestinalis* when treated with 500 µg/l copper for 7days can be seen in figure 20. From this, it appears that transmittance is affected across most of the spectrum but especially from 350 nm to 700 nm. This could suggest that the increases in *in vivo* transmittance factor may be due to changes in pigment turnover in the algae. Chlorophyll a has *in vivo* absorption peaks at 440 nm and 675 nm, with smaller peaks at 385, 418, 590 and 625nm, whereas chlorophyll b has *in vivo* absorption peaks at 470 nm and 650nm. The *in vivo* transmittance factor of algae exposed to copper is increased at these regions which suggests that these changes are due to a reduction in these, and possibly other, pigment levels because if more light is being transmitted and reflected

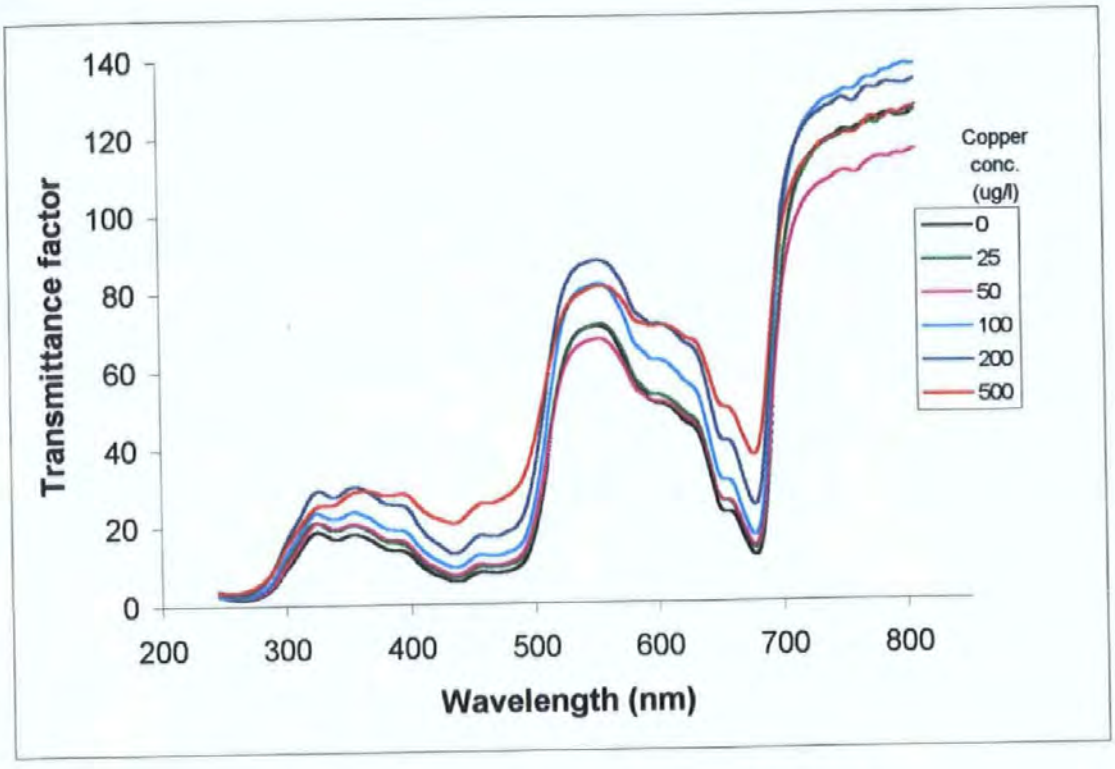

Figure 18: **Effect of copper (ug/l) on *in vivo* transmittance factor *of E.intestinalis* after 7 days exposure**

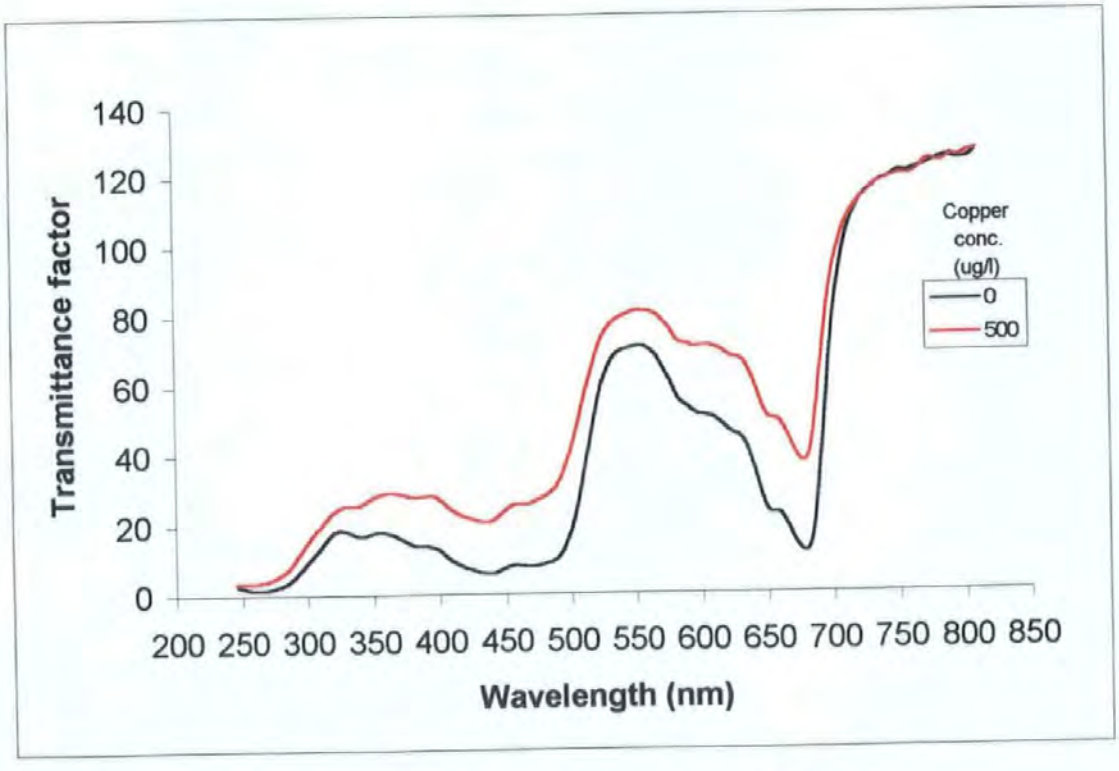

Figure 19: **Effect of 500 ug/l on *in vivo* transmittance factor of *E.intestinalis* after 7 days.**

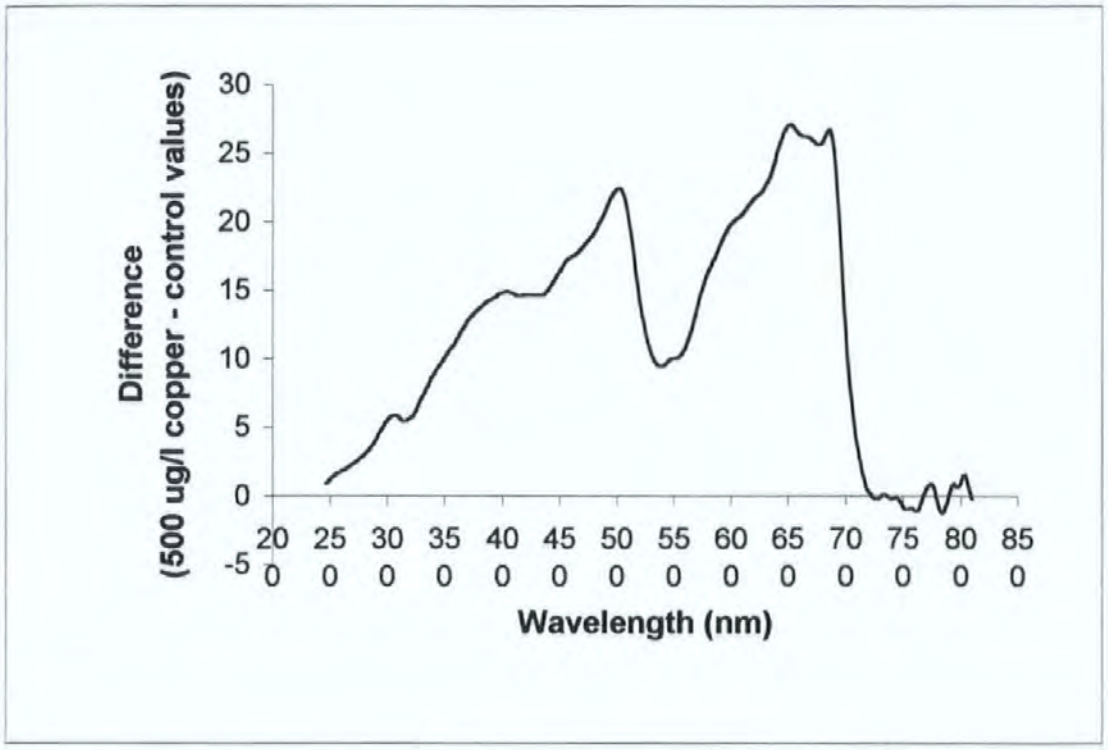

Figure 20: **Difference between *in vivo* transmittance factor of *E.intestinalis* control and when treated with copper (500 ug/l)**

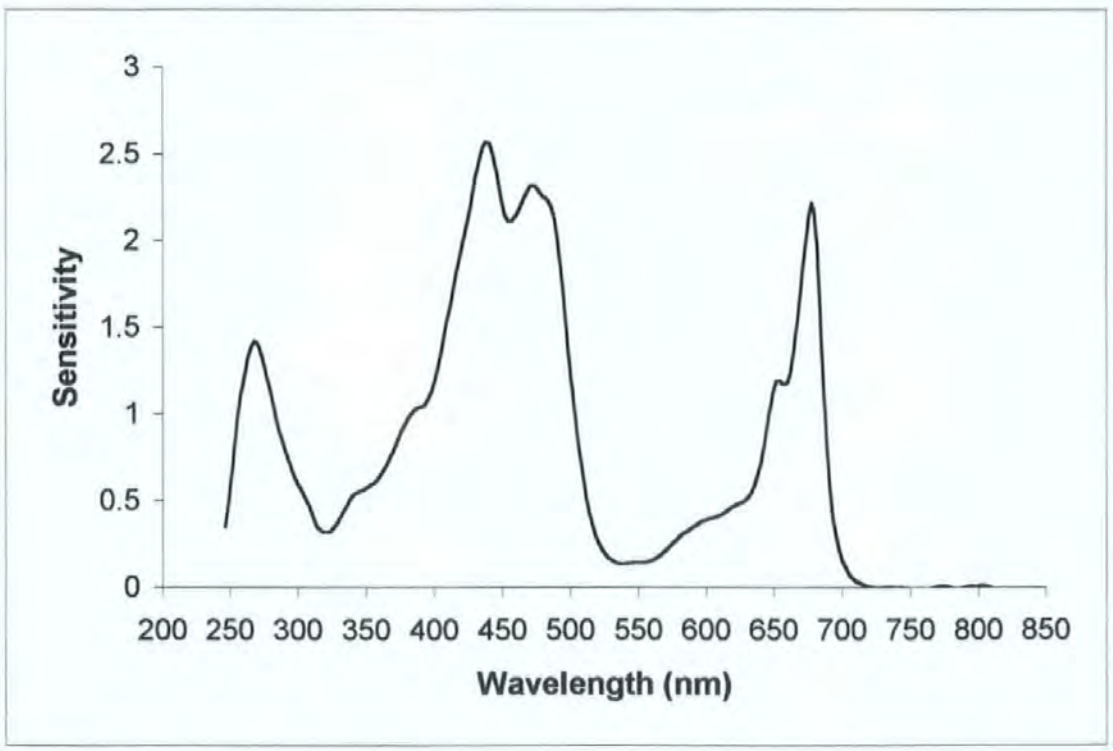

Figure 21: **Sensitivity of *in vivo* transmittance factor of *E.intestinalis* to copper exposure**

(figure 26) then less light is being absorbed at these wavelengths. When a plot of wavelength sensitivity is calculated from these differences *i.e.* mean difference divided by mean control values (Carter 1994), figure 21, it appears that there are particular wavelengths that are more sensitive to copper exposure. The major peaks of sensitivity appear at 270, 440, 472 654 and 680 nm. Many of these wavelengths correspond with the *in vivo* absorption peaks of chlorophyll a, 440 and 675nm, and chlorophyll b, 470 and 650 nm. This indicates that the copper may be decreasing the chlorophyll content of the algae either by degradation or by preventing the resynthesis of the chlorophyll. Lewis *et al*, 1998, found that exposure to copper did have an effect on the chlorophyll a content of *Enteromorpha intestinalis* resulting in decreased chlorophyll a levels with $LOEC_{50}$ (lowest observable effect) levels of 150 µg/l copper. A decreased level in chlorophyll due to exposure to metals was also found in a study by Horler *et al*, 1980, when they investigated the effect of heavy metals on the absorptance and reflectance of terrestrial plants. The overall increase across the rest of the transmittance spectrum up to 700 nm may be due to decreasing levels in other pigments in the algae such as the carotenoids. This could have an additional effect on the algae, as well as possibly reducing photosynthesis, as many of the carotenoids are photoprotective and so reduction in carotenoid levels could reduce the ability of the algae to screen out potentially harmful radiation especially in the blue and UV. The *in vivo* transmittance factor above 700 nm did not appear to be affected by the copper treatment. This could suggest that the differences are not likely to be due to structural changes in the algae.

The plot of wavelength sensitivity, figure 21, allows wavelengths that are more sensitive to copper exposure to be selected and plotted with their respective error bars (figure 22). Copper had a significant ($P<0.05$) effect on *in vivo* transmittance at 442, 474, 550, 656, 680 nm (LOEC = 200 µg/l) and 718 nm (LOEC = 500 µg/l). From this, it can be seen that a dose response is observed for the *in vivo* transmittance factor, after copper exposure for 7

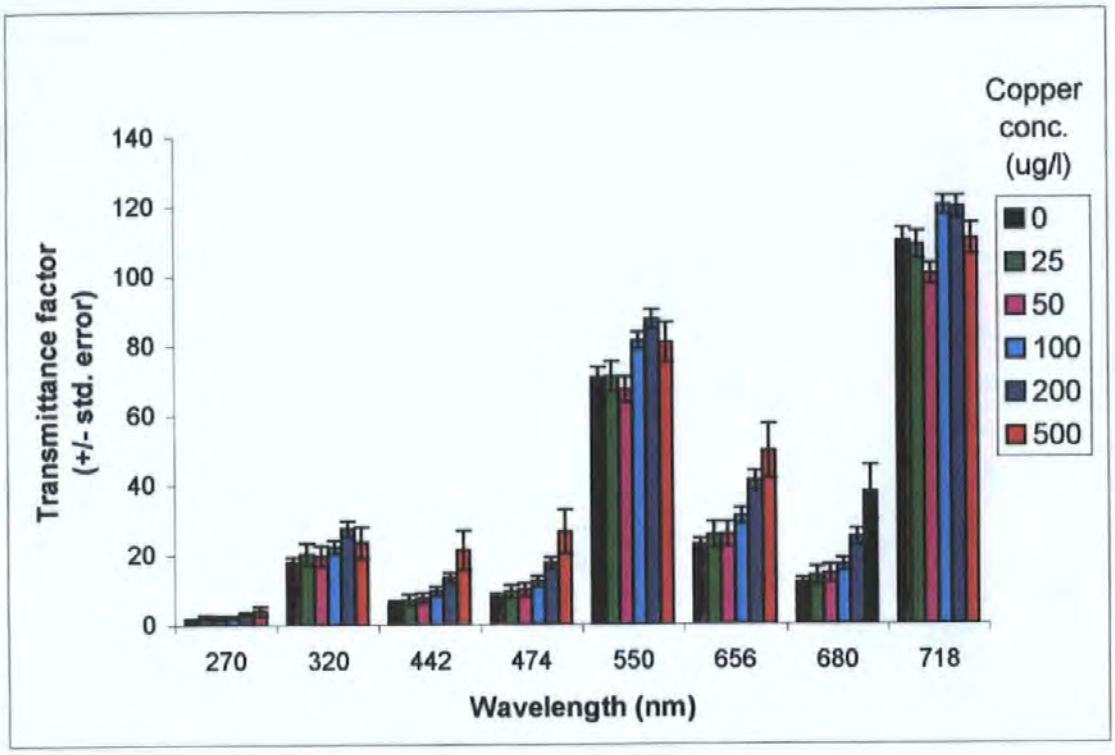

Figure 22: Effect of copper exposure on individual *in vivo* transmittance factor wavelengths

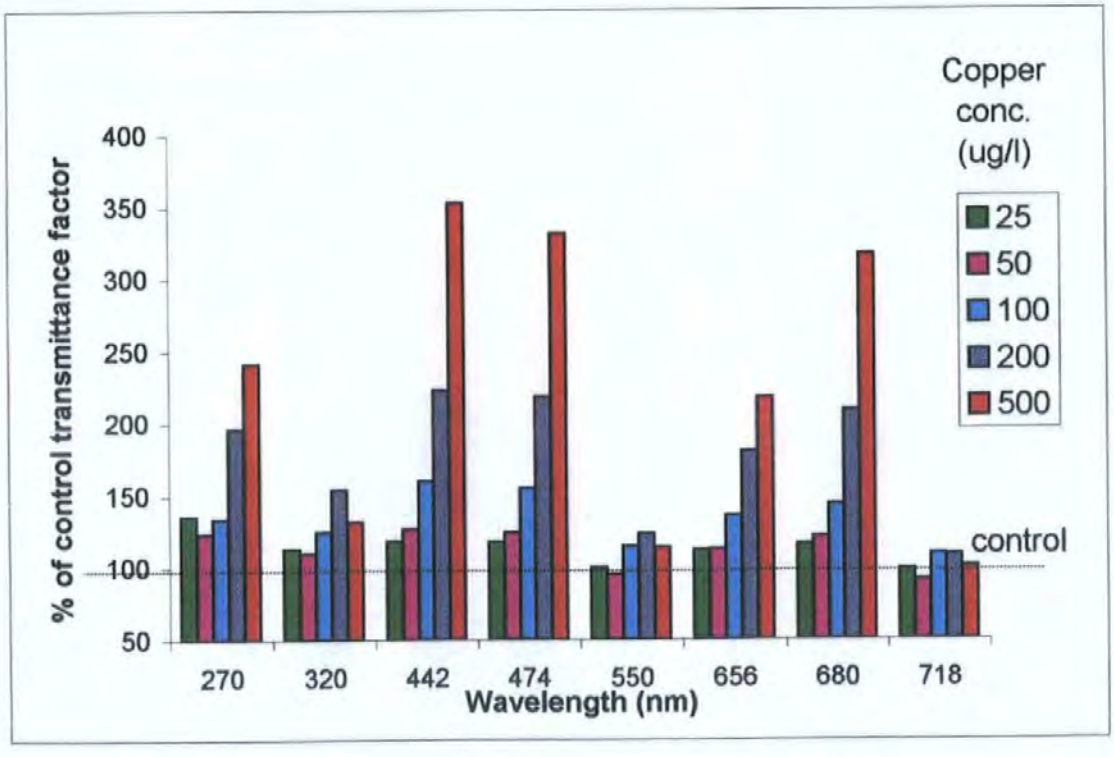

Figure 23: Percentage of control *in vivo* transmittance factor at wavelengths of *E.intestinalis* exposed to copper for 7 days

days, at certain wavelengths *i.e.* 270, 442, 474, 656 and 680nm. When these changes are plotted as a percentage of the control value, figure 23, comparisons can be made *i.e.* with the algae treated with other pollutants and changes that would be difficult to detect due to initial low levels can be observed more clearly *i.e.* the increase in *in vivo* transmittance factor at 270 nm is displayed more clearly in figure 23 than figure 22.

Dose response curves for the *in vivo* transmittance factor of *E.intestinalis* are shown in figure 24. It seems that copper has a similar effect on transmittance factor at 442, 474, 656 and 680 with the increase in transmittance factor at 270 being less apparent. These could be suitable wavelengths to plot to detect stress of the algae by copper exposure but care would have to be taken as it may be that this displays the effects of a reduction in pigment levels which is a response that may be caused by other pollutants that affect the biosynthesis of pigments in some way.

The calculation of ratios is another useful way of analysing the data to highlight copper effects. For algae treated with copper, the ratio of 718/680 nm was chosen since the *in vivo* transmittance factor at 718 nm remained fairly constant with increasing copper concentration, whereas the *in vivo* transmittance factor at 680 increased with an increase in copper concentration. As with the single wavelengths in figure 24 plotted as dose response curves, ratios such as shown in figure 25 may also have the potential to detect stress, caused by exposing the algae to copper. This would be particularly useful if they prove to be specific to copper exposure. From this it is evident that the EC_{50} is 80 µg/l. When compared with growth (EC_{50} 65µg/l) and F_v/F_m (no apparent EC_{50} at concentrations used) it appears that using ratios obtained from the *in-vivo* transmittance factor is a much more sensitive indicator of damage than F_v/F_m and is nearly as sensitive as growth for copper exposure.

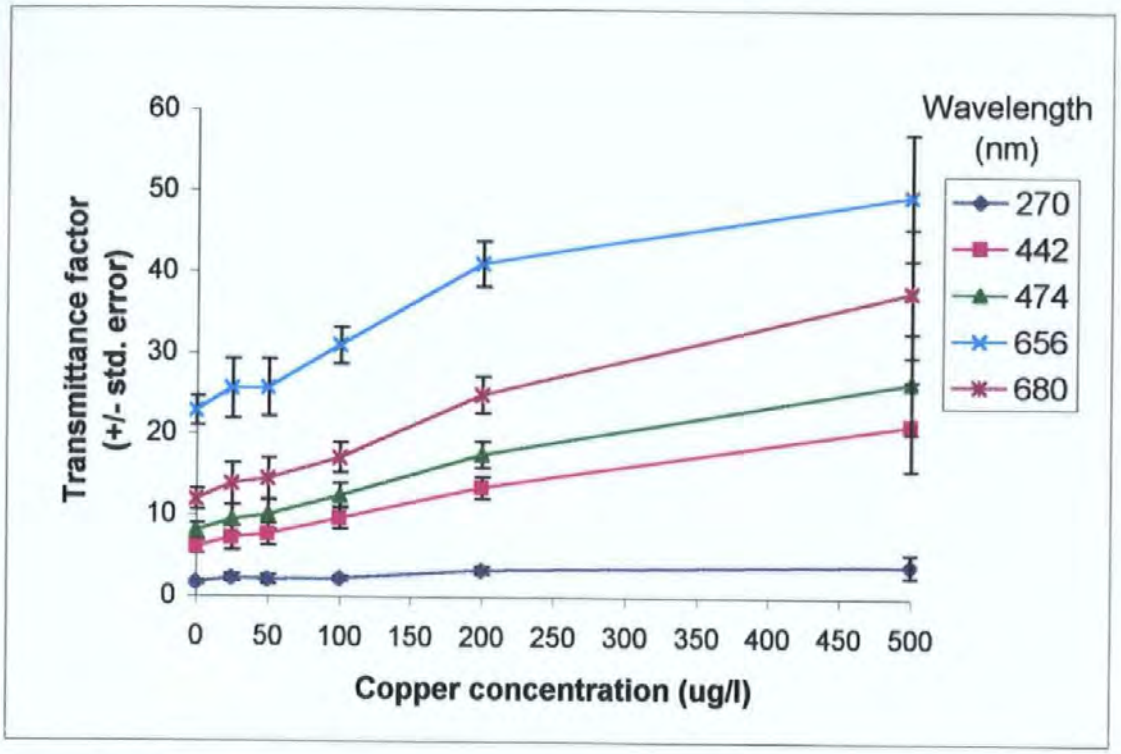

Figure 24: **Effect of copper on the 270, 442, 474, 656 and 680 nm *in vivo* transmittance factor of *E.intestinalis***

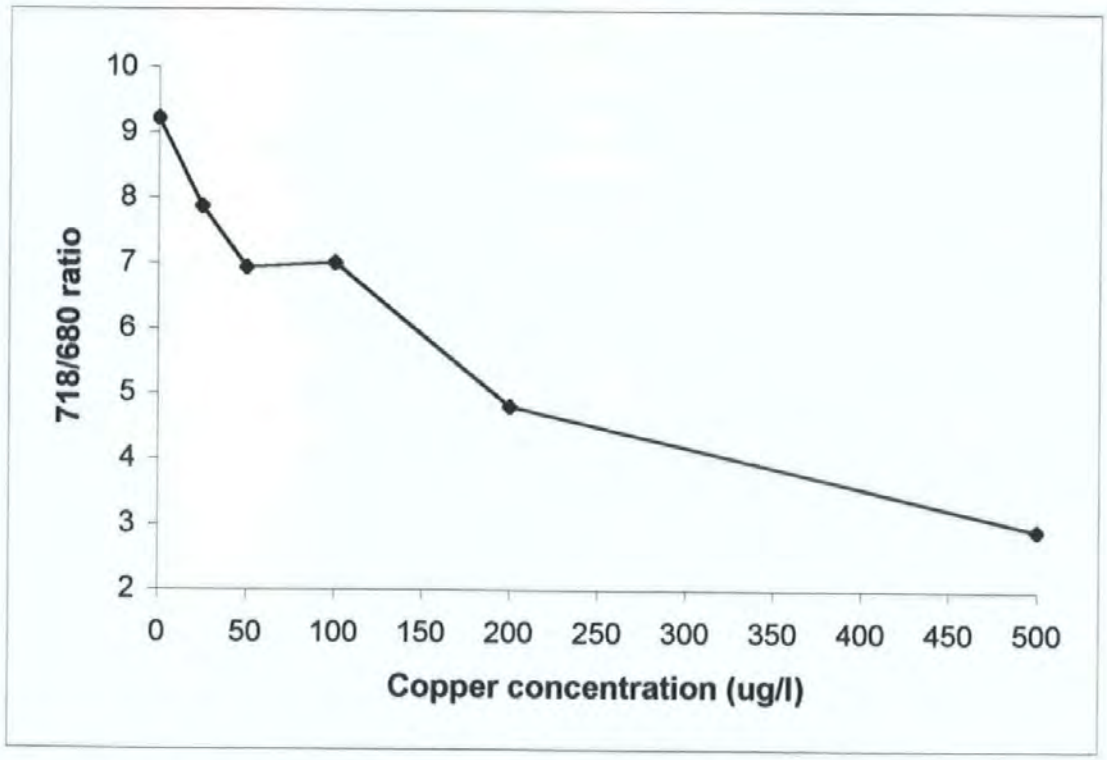

Figure 25: **Effect of copper on the 718/680 *in vivo* transmittance factor of *E.intestinalis***

IN VIVO REFLECTANCE

Figure 26 shows the effect of copper exposure on the *in vivo* reflectance of *E.intestinalis*. It appears that treatment with copper results in an increase in the algal *in vivo* reflectance across most of the spectrum until 720 nm. An increase in copper concentration seems to result in an increase in the *in vivo* reflectance of the algae except for the algae exposed to 50 µg/l. For some reason the reflectance levels of the algae treated with this concentration are lower than those of the control but it is interesting to note that the *in vivo* transmittance factor results did not appear to be 'out of sequence' with the other concentrations.

Figure 27, illustrating the difference for *in vivo* reflectance with copper exposure, indicates that there does not appear to be any particular wavelengths that are more sensitive than others to copper exposure. Certain small peaks are evident and these were used to produce figure 28 to illustrate the significant ($P<0.05$) effect of copper on *in vivo* reflectance of *E.intestinalis* at wavelengths 392, 510, 538, 680 and 690 nm. It is interesting to note that copper does not appear to have a significant ($P>0.05$) effect on *in vivo* reflectance at 740 nm but does have an effect, an increase with increasing concentration, at 680 nm. This is in contrast to the *in vivo* reflectance of *E.intestinalis* when treated with Irgarol 1051 (figure 55) which shows no change at 680 nm and a decrease with increasing concentration at 740 nm. This may provide some evidence that certain pollutants may induce spectral changes that are pollutant-specific and so detection of specific stresses may be possible by the monitoring of *in vivo* spectral properties.

As with the *in vivo* transmittance factor results, selection of individual sensitive *in vivo* reflectance wavelengths and calculation of appropriate ratios are useful ways to analyse the results. Certain wavelengths selected from figure 29 are plotted as dose response curves in

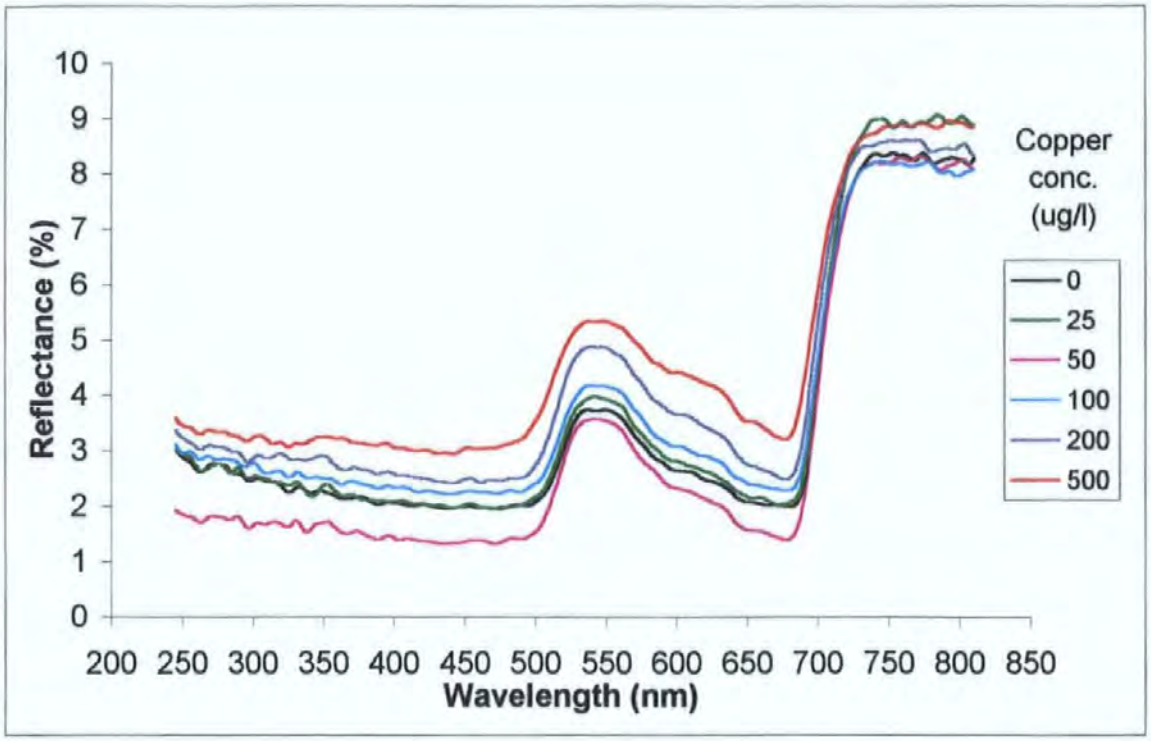

Figure 26: **Effect of copper (ug/l) on *in vivo* reflectance (%) of *E. intestinalis* after 7 days**

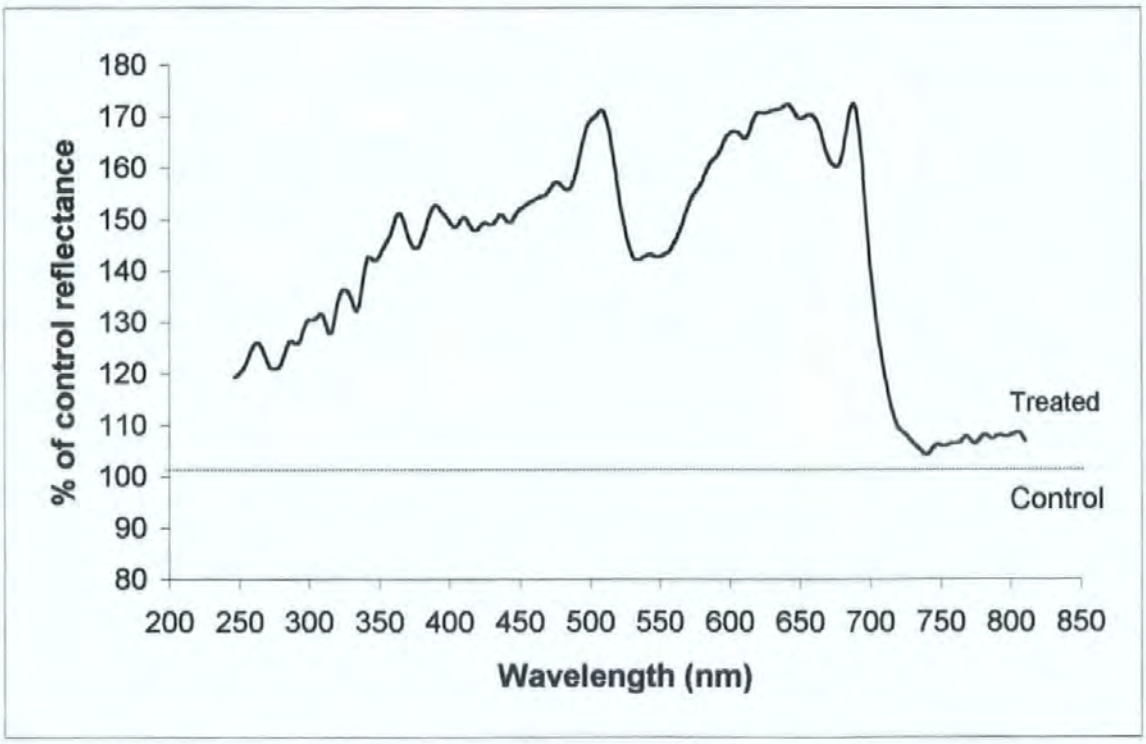

Figure 27: **Copper alteration of *E. intestinalis in vivo* reflectance after 7 days exposure to 500 ug/l**

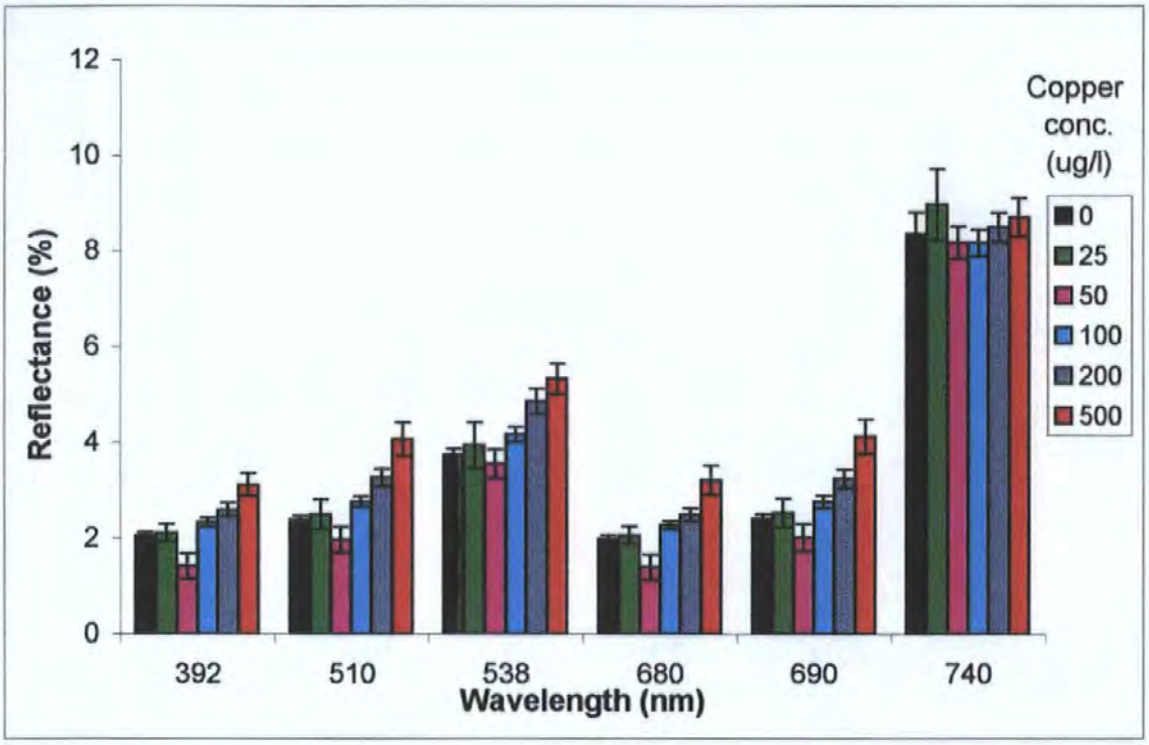

Figure 28: **Effect of copper (ug/l) on *in vivo* reflectance of *E. intestinalis* at selected wavelengths after 7 days**

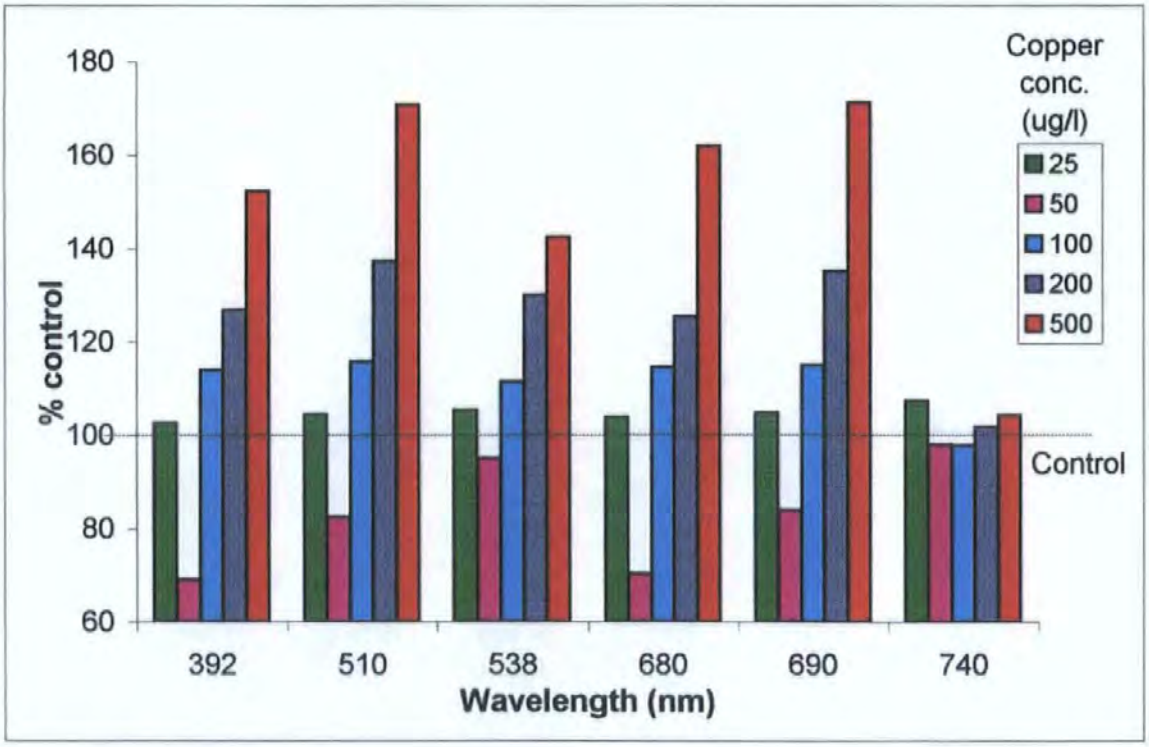

Figure 29: **Effect of copper (ug/l) on the % control *in vivo* reflectance of *E. intestinalis* at selected wavelengths after 7 days**

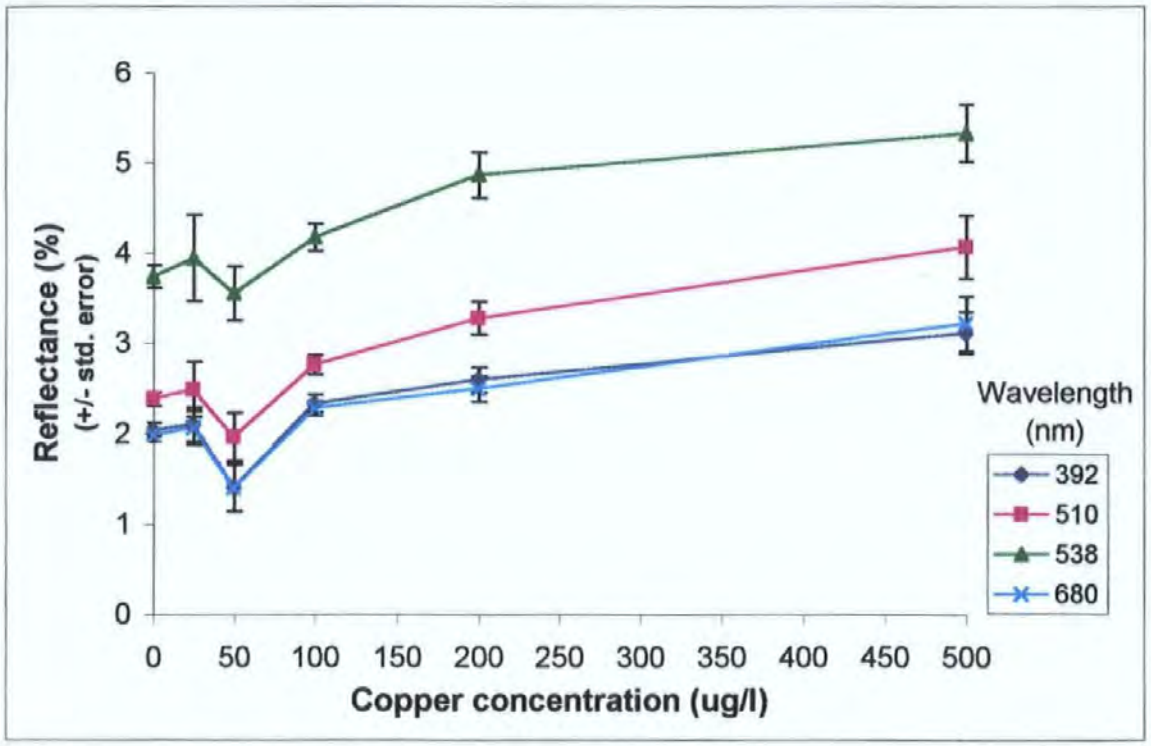

Figure 30: ***In vivo*** **reflectance at specific wavelengths (nm) of** ***E. intestinalis*** **exposed to copper (ug/l) for 7 days**

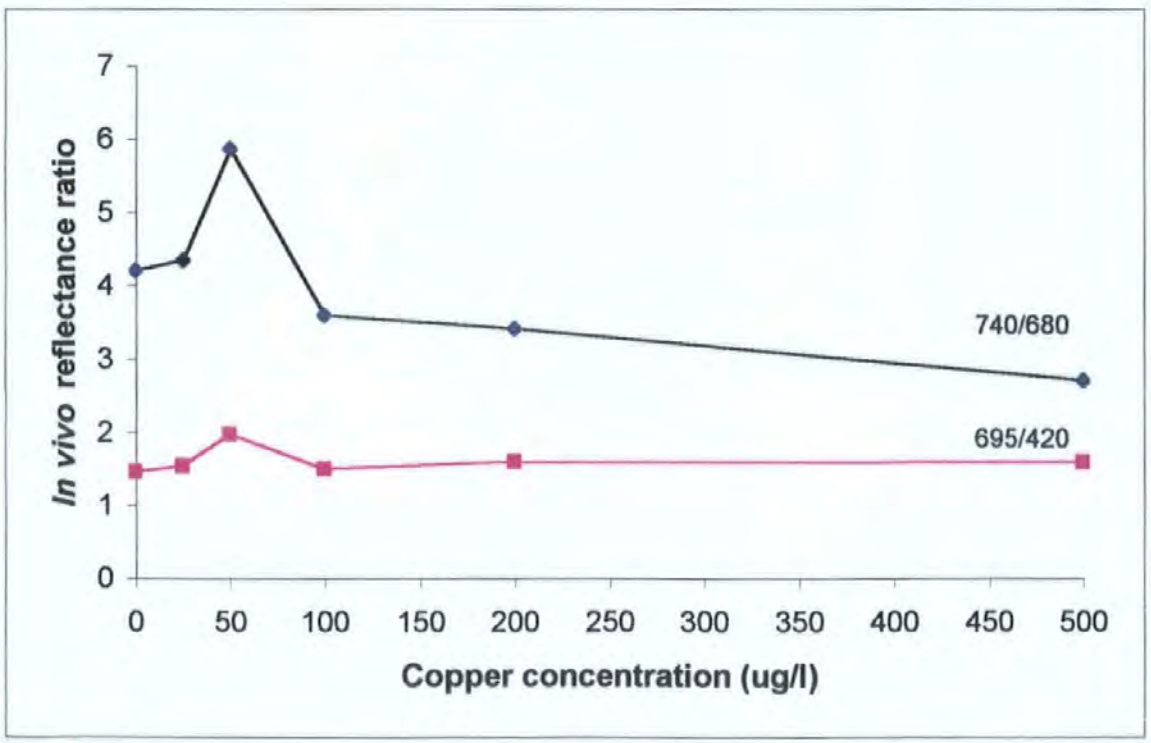

Figure 31: **Effect of copper (ug/l) on the 740/680 and 695/420** ***in vivo*** **reflectance ratios of** ***E. intestinalis*** **after 7 days exposure**

figure 30. This shows that the *in vivo* reflectance of the algae is sensitive to copper exposure at various wavelengths including 680 nm. This increase in the *in vivo* reflectance at 680 nm indicates that the higher the concentration of copper, the higher the reflectance of the algae at this wavelength and therefore, possibly, the lower the levels of chlorophylls. Reflectance ratios are often used to detect whether a plant is stressed and Carter (1994) devised certain reflectance ratios for different plant species and stresses. He found that certain ratios e.g. 695/420 were affected by all the stress agents used and that others were only affected by specific ones. However, when the 695/420 nm ratio was applied to the *in vivo* reflectance data for *E.intestinalis* treated with copper, it appears that this ratio is not as sensitive as certain other ratios devised by choosing suitable wavelengths from the sensitivity figure (figure 27). The 740/680 nm *in vivo* reflectance ratio is plotted with the 695/420 nm ratio devised by Carter. It can be seen that there is a greater response by using the 740/680 nm ratio.

In vivo reflectance ratios and sensitive individual wavelengths may offer potential not only in monitoring pollution in the laboratory but also in monitoring stress effects by remote sensing. If certain reflectance ratios and wavelengths prove to be specific to the copper exposure, then *in vivo* spectrophotometry could be extremely useful in detecting stress in algae in the field caused by copper pollution. However, care would have to be taken in analysing the results because, as with other parameters, other stresses could possibly induce the same response.

Although copper is essential for algae, too much is known to be toxic and copper is widely used as an antifoulant to prevent unwanted colonisation of algae on boat hulls etc. Copper has various toxic effects on the algae but one is that it binds to chloroplast membranes and other cell proteins. This causes the degradation of chlorophyll and other pigments (see Lobban and Harrison 1994) which seems to confirm the results from the *in vivo*

spectrophotometry that pigment levels, especially chlorophyll, are being affected. If the chloroplast thylakoids are damaged irreversibly this would eventually cause the death of the algae because photosynthesis would be prevented.

From the results it is evident that copper does have an effect on the alga, *E.intestinalis*. It appears to effect *in vivo* spectral properties and growth and, at high concentrations, the F_v/F_m values. It must also be remembered that the copper concentrations quoted during these experiments are approximate because, although all the solutions were made up accurately, there is a small amount of copper present in the instant ocean that the algae were kept in (see Appendix 1). This amount of copper is very small (approximately 10 µg/l) and so should not have a detrimental effect on the health status of the algae as this is equal to levels of copper found in relatively unpolluted estuarine waters (Law *et al* 1994). It should also be noted that the concentrations of copper used during all experiments are nominal *i.e.* levels in the Instant Ocean were not checked and losses due to adsorption to the glassware were not taken into account.

4.2 ZINC

Zinc can enter the soil and water by a variety of ways including mining activities and as a component of industrial waste and sewage sludge (Jackson *et al* 1990). It is also used in certain 'phytopharmaceutical' products that are applied to crops (Magalhaes *et al* 1995). Levels of zinc can be relatively high and in SouthWest England, levels of 5820 µg/l and 285 µg/l were found in the river Carnon and Restronguet Creek respectively (Klumpp and Peterson 1979).

Zinc is an essential micronutrient for algae and, although it is toxic in excess, it is less so than copper (Newman, 1998). It is taken up by algae and the features of zinc uptake in the brown algae, *Ascophylum nodosum*, were that uptake was a slow, constant process of accumulation that was irreversible and persisted for long periods of time (Skipnes *et al*, 1975).

4.2.1 EXPERIMENTAL DESIGN

Collection and acclimatisation of algae

E.intestinalis was collected from Wembury Bay, identified and acclimatised as described in the Methods section.

Preparation of solutions

Zinc stocks were made using zinc chloride in MilliQ Ultrapure water. This was added to Instant Ocean (33 ppt) containing nutrients (50 mg/l $NaNO_3$ (BDH) and 10 mg/l Na_2HPO_4 (BDH)) to give concentrations of 0, 25, 50, 100, 200 and 500 µg Zn/l. The level of zinc in the Instant Ocean is quoted to be approximately 18 µg/l which is more than in natural seawater but, as with the copper concentration, it is equal to levels found in relatively unpolluted estuarine waters (Law *et al* 1994). Concentrations of zinc in all experiments are also nominal *i.e.* losses by adsorption of the glassware not being taken into account and levels in Instant Ocean not being checked.

Experimental setup

Intercalary sections (2.5 cm long) of *E.intestinalis* were cut from healthy fronds (as described in the Methods section) and placed in tri-divided petri dishes each containing 50 ml of the relevant zinc solution. 24 sections were used for controls (no zinc present) and 12 sections were used for each concentration of zinc. This was because preliminary results from trial runs indicated that more sections from the control were likely to sporulate than

the sections that were exposed to zinc. The sections were left for 7 days and the solutions were aerated and arranged as described in the Methods section.

Parameters measured

Growth was measured on day 7 and was recorded as Relative Growth Rate. Fertility was also recorded as the percentage of sections that sporulated during the experiment. Any sections that did sporulate were consequently removed from the experiment completely as discussed previously.

Fluorescence measurements were recorded on days 0, 1, 2, 4 and 7 as F_v/F_m.

The *in vivo* spectral properties of the algae were also measured on days 0, 1, 2, 4 and 7 using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere.

4.2.2 RESULTS AND DISCUSSION

Growth

Figure 32 illustrates the effect of 7 days zinc exposure on the growth of *E.intestinalis*. It appears that zinc affects growth but this is not statistically significant (P=0.014) until *E.intestinalis* is exposed to concentrations of 500 µg/l zinc. Growth was not affected sufficiently by the zinc concentrations used to calculate an EC_{50}.

Fluorescence

The effect of zinc exposure on fluorescence (F_v/F_m) of *E.intestinalis* is illustrated in figure 33 and there was no significant ($P > 0.05$) effect on F_v/F_m values with the concentrations used. There is a slight drop after 7 days exposure to 500 µg/l zinc but the control values also dropped slightly and F_v/F_m values for both groups are still in the range that is considered to be 'healthy'.

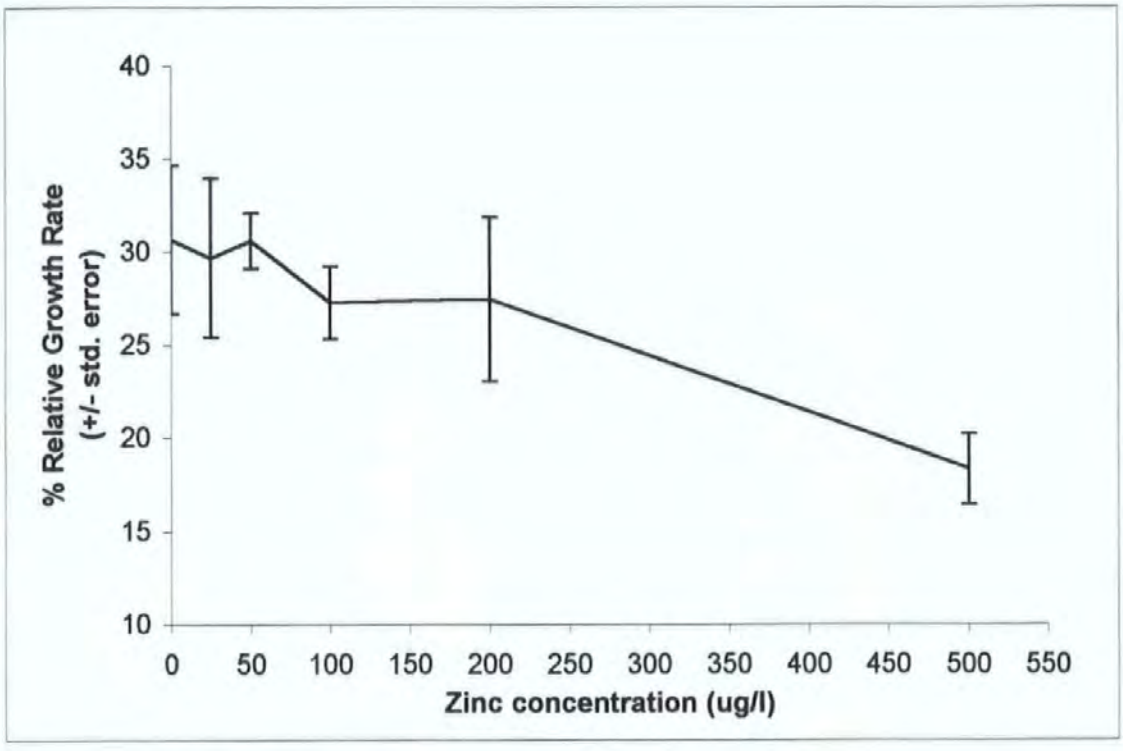

Figure 32: **Effect of zinc (ug/l) on growth of *E.intestinalis* after 7 days**

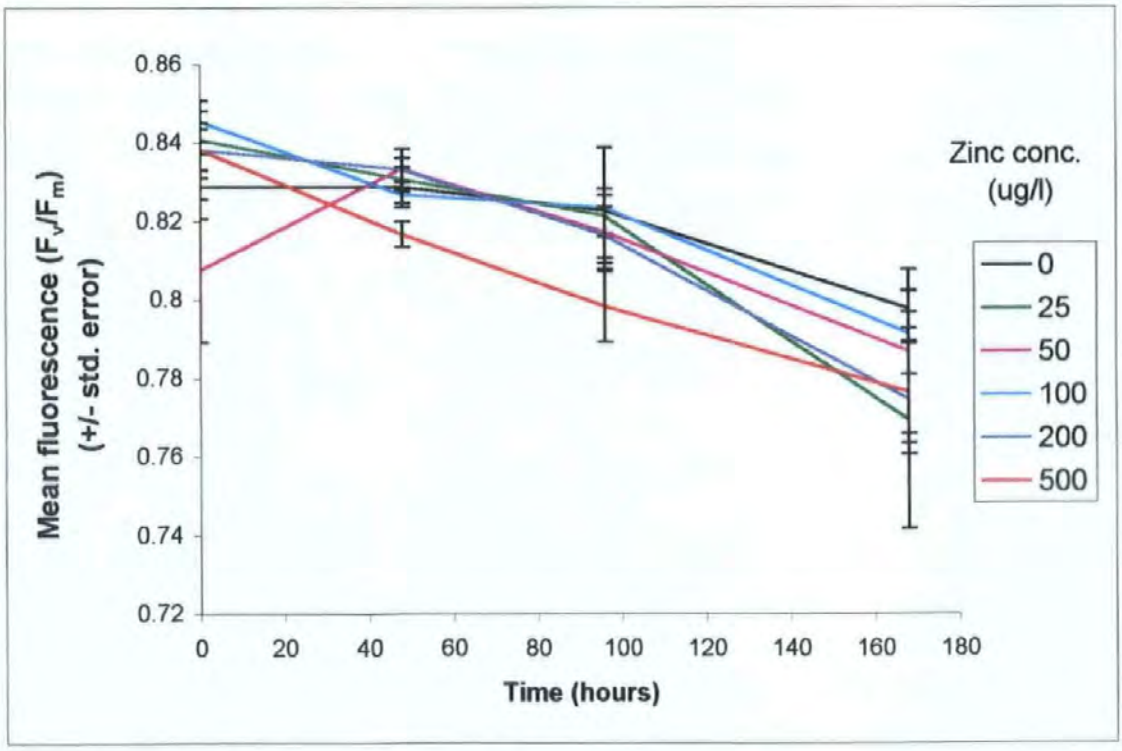

Figure 33 : **Effect of zinc (ug/l) on fluorescence (Fv/Fm) of *E. intestinalis***

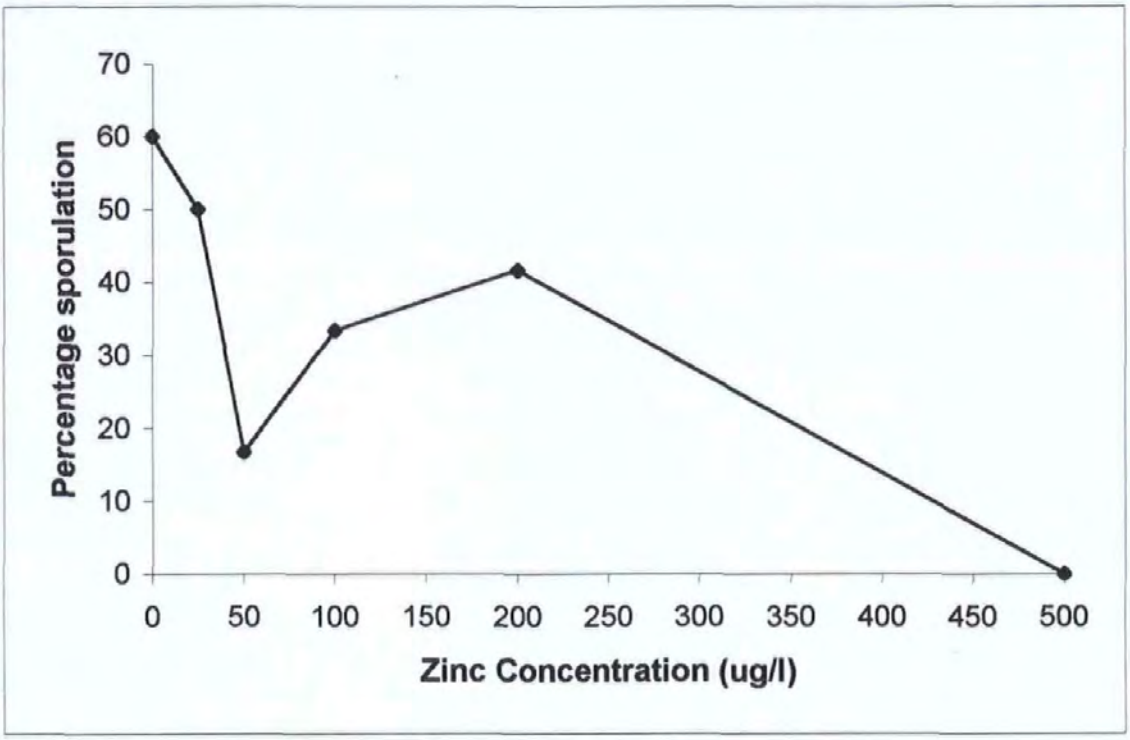

Figure 34: **Effect of zinc (ug/l) on percentage sporulation of** *E.intestinalis*

Fertility

As with copper exposure, there was a correlation between exposure to zinc and inhibition of sporulation, figure 34. Approximately 60 % of the control sections sporulated in comparison to 0 % of the sections exposed to 500 μg/l zinc. This could indicate that although the fluorescence (F_v/F_m) and growth parameters are not severely affected, that the zinc still has a detrimental effect on the algae.

In vivo spectral properties of *E.intestinalis*

In vivo transmittance factor

The results (figures 35 – 39) of the effect of zinc on the *in vivo* transmittance factor of *E.intestinalis* after 7 days indicate that zinc does not have a marked effect on any particular wavelengths. Figure 35 shows that there does not appear to be a clear effect of zinc on the *in vivo* transmittance factor as the control sections and the sections exposed to 500 μg/l zinc are fairly similar. When comparing the control and zinc-treated sections (figure 36) it appears that the *in vivo* transmittance factor is decreased when *E.intestinalis* is exposed to concentrations of zinc ranging from 25 – 200 μg/l but increased when exposed to 500 μg/l zinc. This is slightly confusing and the spectra that was most different (500 μg/l zinc) was used to create a plot of sensitivity (figure 37) in order to assess whether a dose response relationship was apparent at certain wavelengths. The wavelengths selected using figure 37 were plotted in figure 38 but it was found that differences were not significant at the wavelengths chosen, except for at 700 nm ($P<0.05$). This is also apparent when the results are plotted as a percentage of the control (figure 39) and it was decided that there was no particular wavelength where a clear dose response effect could be observed.

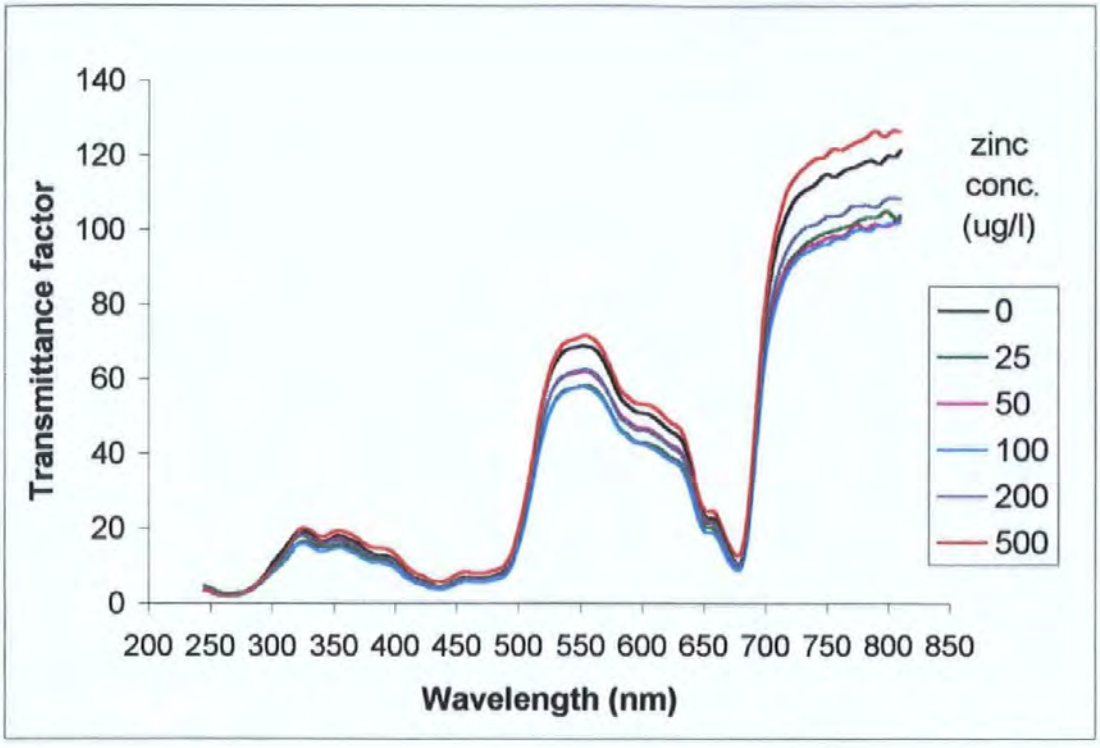

Figure 35: **Effect of zinc (ug/l) on *in vivo* transmittance factor of *E.intestinalis* after 7 days exposure**

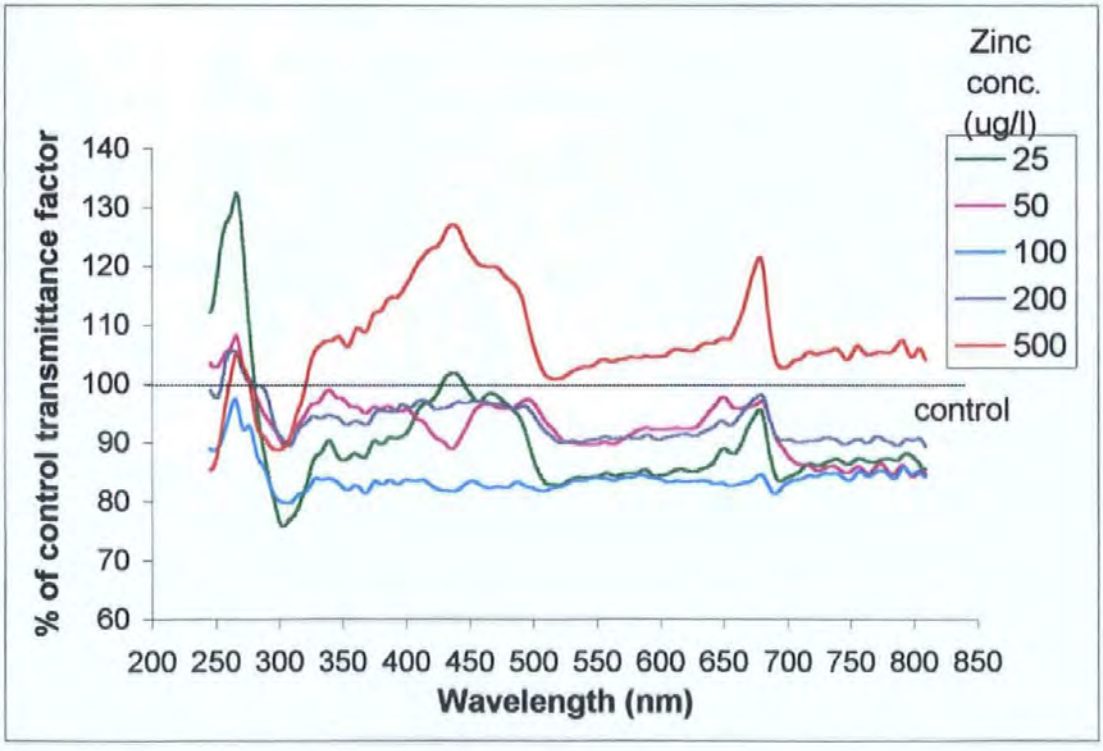

Figure 36: **Zinc alteration of *in vivo* transmittance factor of *E.intestinalis* after 7 days exposure**

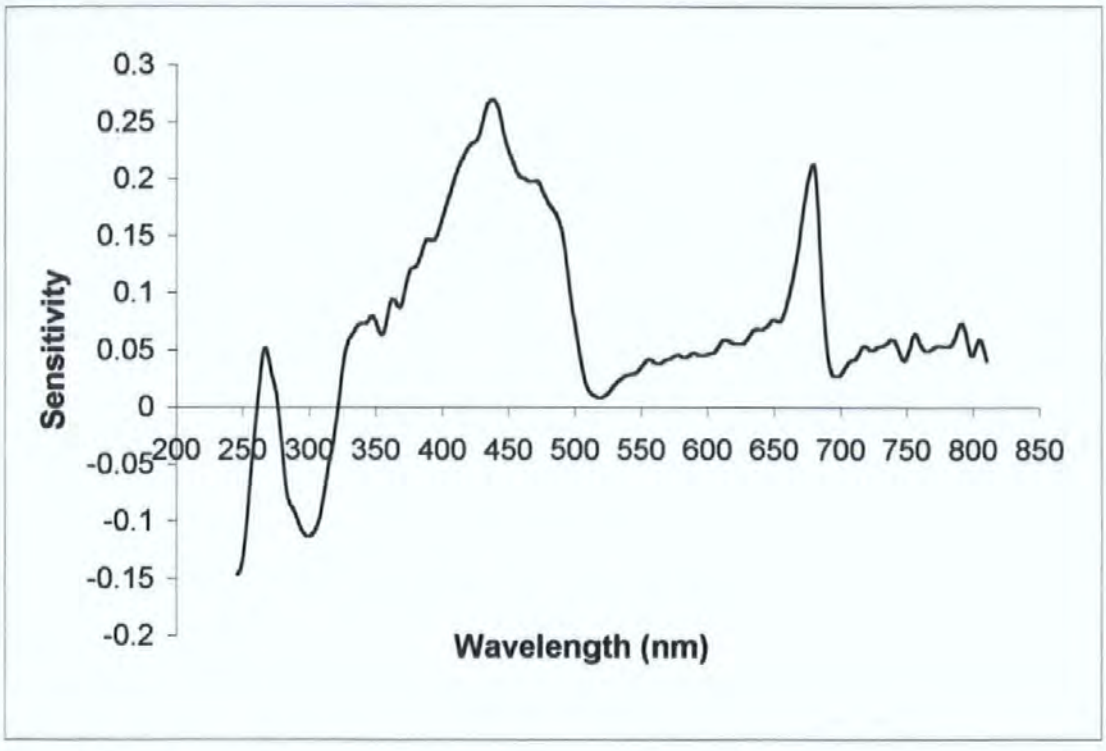

Figure 37: **Sensitivity of *in vivo* transmittance factor of *E.intestinalis* to zinc (500 ug/l)**

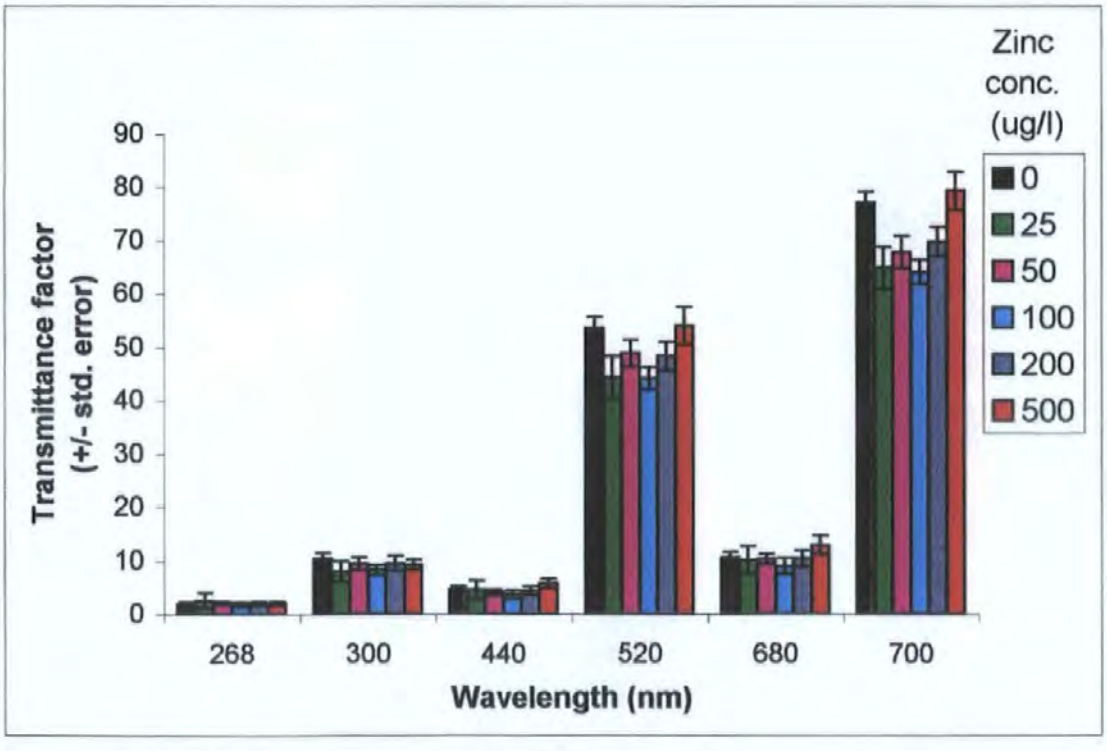

Figure 38: **Effect of zinc exposure on individual *in vivo* transmittance factor wavelengths of *E.intestinalis***

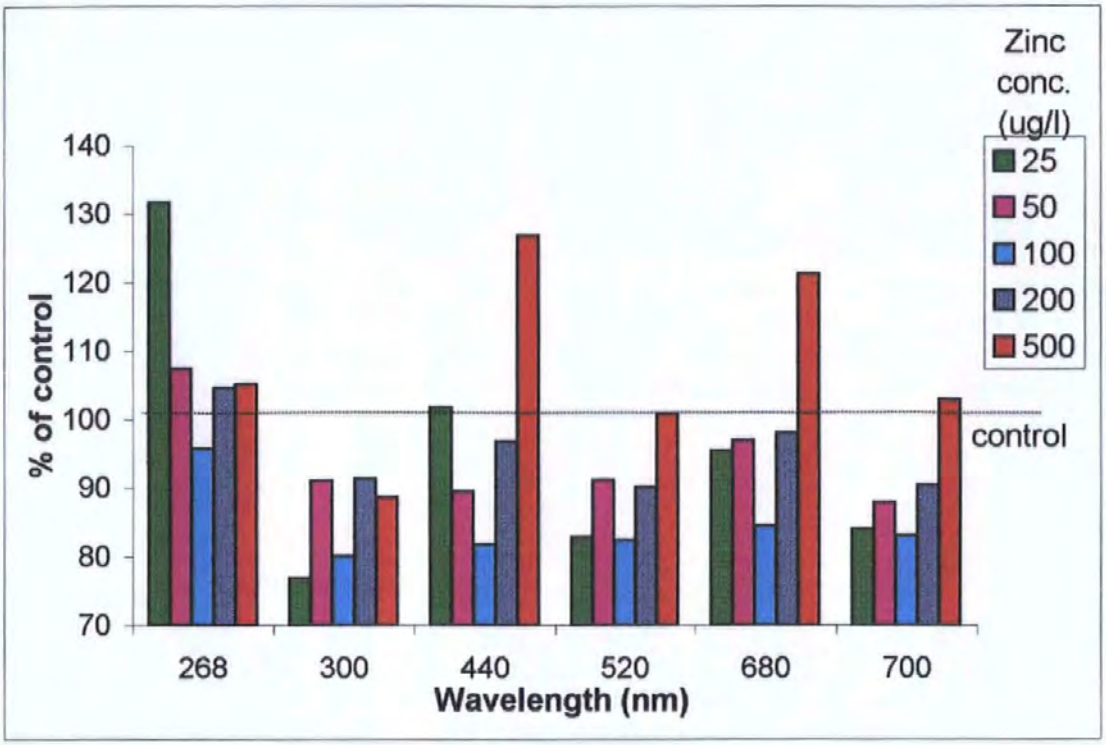

Figure 39: **Percentage of control *in vivo* transmittance factor at set wavelengths of *E.intestinalis* exposed to zinc (ug/l) for 7 days**

In vivo reflectance

The effect of zinc exposure on *in vivo* reflectance of *E.intestinalis* after 7 days is illustrated in figure 40. Zinc appears to have an effect of slightly lowering the *in vivo* reflectance of *E.intestinalis* when concentrations of 25, 50, 100 and 500 μg/l are used but this is in contrast to 200 μg/l which resulted in a slightly higher *in vivo* reflectance. When a plot of wavelength sensitivity (figure 41) was produced, there did not appear to be any definite wavelengths that were affected compared to the exposure of *E.intestinalis* to copper. As with the transmittance data, the wavelengths that had any possible potential were selected and plotted for each concentration of zinc (figure 42) but the standard error bars were fairly close, and in some cases overlapping, for the different concentrations of zinc. Differences were significant ($P<0.05$) for *in vivo* reflectance at 390, 510, 550 and 680 nm but production of figure 43 indicated that there was no particular wavelength where a clear dose response effect could be observed.

Although the use of *in vivo* spectral analysis was not as useful for zinc as it was for copper this in itself is interesting as it shows that effects of certain trace metals may be able to be identified from 'general' metal effects. It could be potentially useful that zinc does not appear to elicit any obvious signature in *the in vivo* optical properties as this, in itself, could possibly be a basis for using *in vivo* optical properties for distinguishing between exposure to different metals. This technique could, therefore, have considerable use in ecological monitoring.

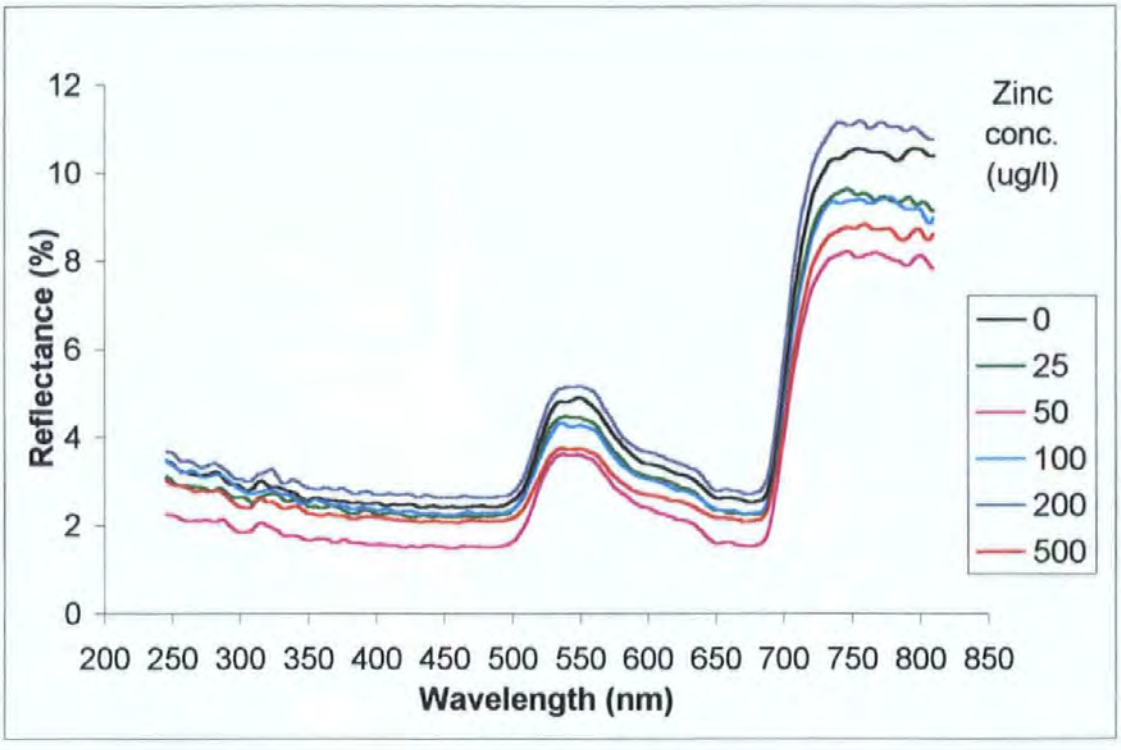

Figure 40: **Effect of zinc (ug/l) on *in vivo* reflectance (%) of *E.intestinalis* after 7 days exposure**

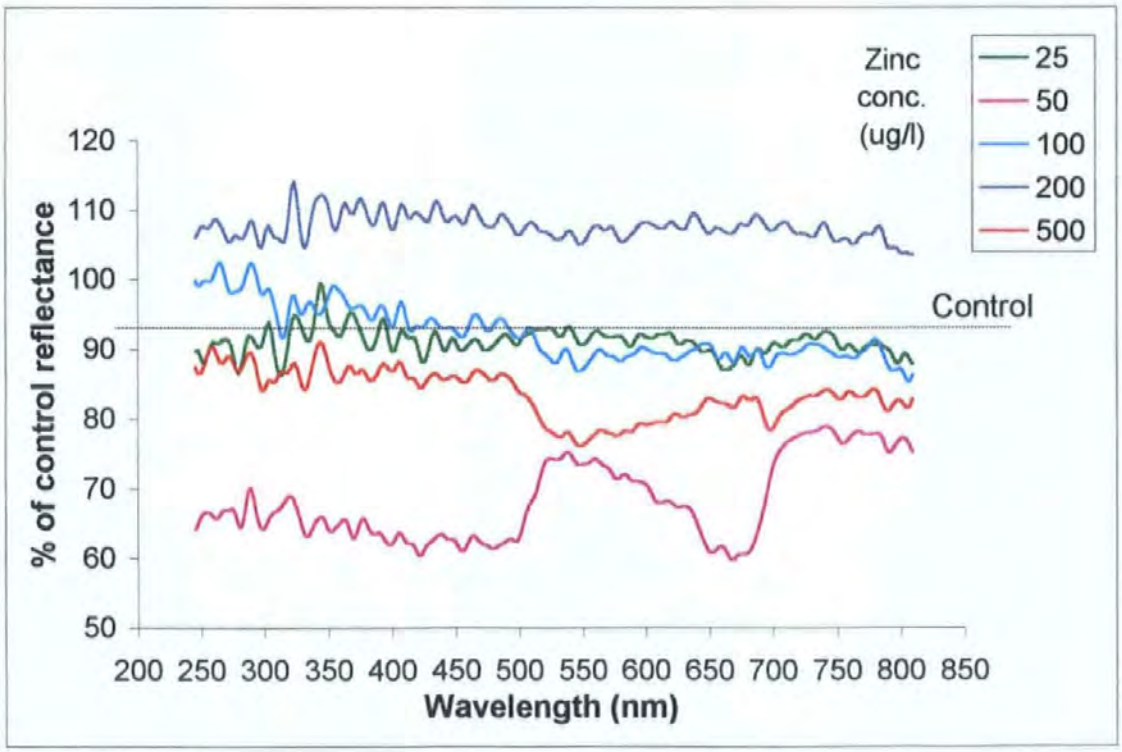

Figure 41: **Zinc alteration of *in vivo* reflectance of *E.intestinalis* after 7 days**

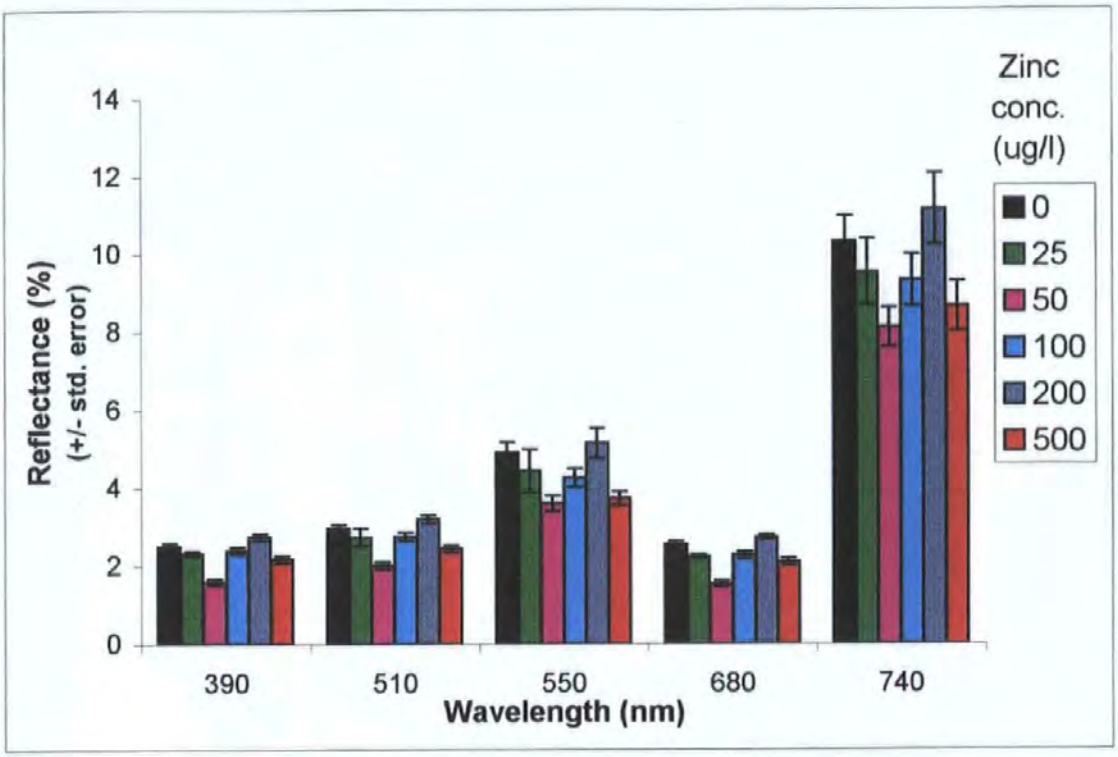

Figure 42: **Effect of zinc (ug/l) exposure on individual *in vivo* reflectance wavelengths of *E.intestinalis***

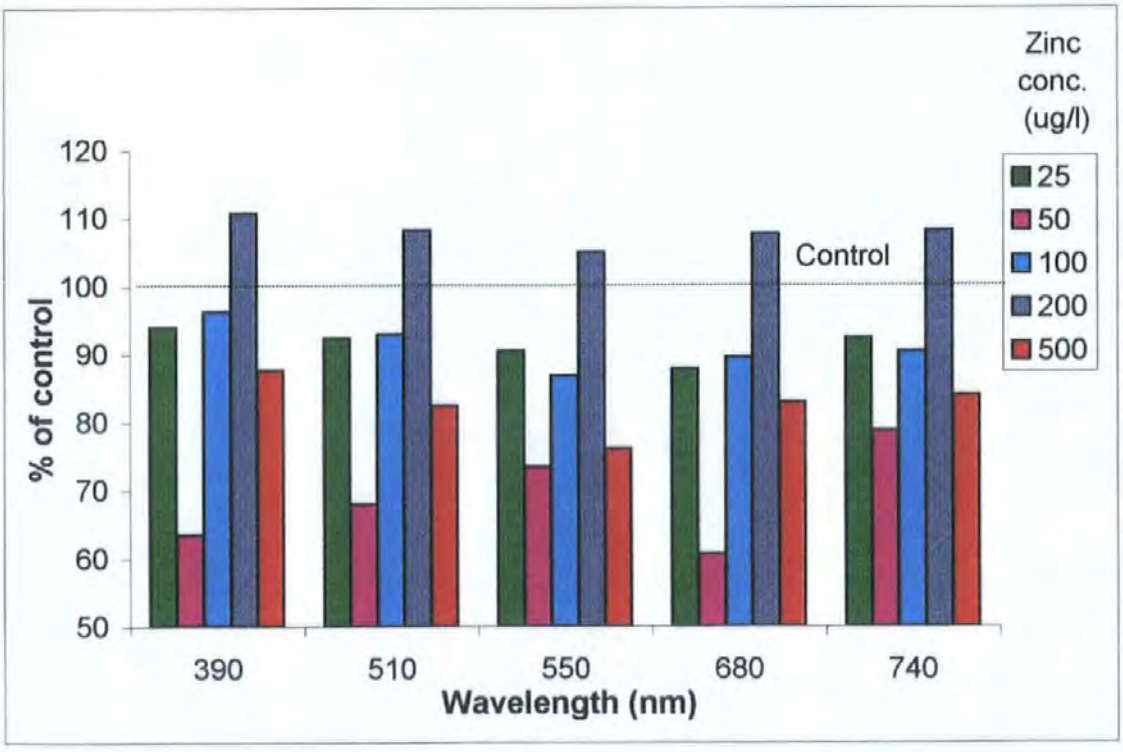

Figure 43: **Percentage of control *in vivo* reflectance values of *E.intestinalis* exposed to zinc (ug/l) for 7 days**

5 TRIAZINES

Triazines are a group of herbicides which are known to inhibit the photosynthetic activity at Photosystem II (PS-II) and have differences in activity caused by various lipophilic side chains which result in slight variations of structure (Somerville 1990). Triazine herbicides are not added directly to the marine environment but they can enter it by various human activities including runoff and leaching from boats. The main effect of triazine herbicides is to inhibit photosynthetic electron transport by competing with the mobile electron carrier, plastoquinone, for binding to the reaction centre polypeptide (Somerville, 1990). This then results in inhibition of Photosystem II activity.

The triazine herbicides chosen for this study were Irgarol 1051 and atrazine.

5.1 IRGAROL

The herbicide Irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine), figure 44, belongs to the triazine group of herbicides and is manufactured by Ciba Geigy as an antifoulant. Irgarol 1051 is frequently added to antifouling paints to prevent the primary colonisation of hull surfaces by microalgae which would lead to the subsequent attachment and growth of seaweeds (Liu *et al,* 1999). Although the majority of the green macroalgae attached to boat hulls dies back in the winter months, some turfs become established and it is then very difficult to monitor corrosion of the boat hull (Terry and Edyvean, 1983).

Plate 5: *Enteromorpha intestinalis* on a boat (Pomphlett Creek)

Irgarol 1051 has become a popular constituent of antifouling paints since problems with the use of marine antifouling paints containing tributyl-tin (TBT) compounds were highlighted. TBT compounds were introduced in the mid-1960s and their use had become widespread by the 1980s leading to high levels of TBT leachate in coastal waters (Gibbs, 1994). TBT was found to be toxic to non-target organisms including inducing the 'imposex' response in neogastropods and its use was subsequently restricted for small boats in UK waters in 1987 (Gibbs 1994). An alternative to TBT therefore had to be found resulting in the use of Irgarol 1051 in antifouling paints. The antifouling paints that Irgarol 1051are used in are normally tin-free and based on copper and zinc metal oxides and so the herbicide is used to improve the efficacy of the paint by inhibiting the growth of copper resistant algae (Gough, 1994). It is used in over 80 products (HMSO 1993) and the

majority of these are available for use by the general public so its use may be potentially widescale. This has therefore resulted in Irgarol 1051 being used directly in the marine environment and there is increasing concern about possible effects on the marine ecosystem, in particular, algae.

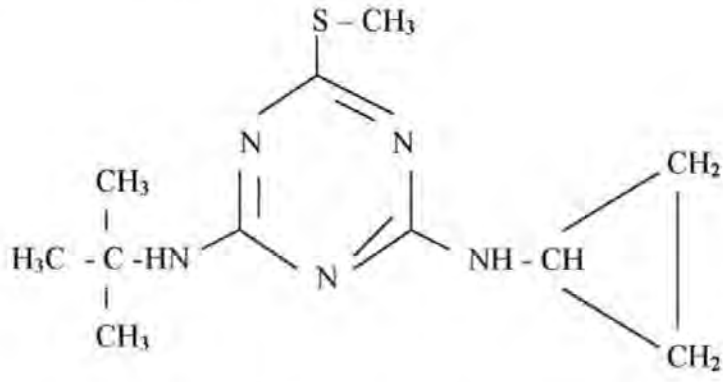

Figure 44: Structure of Irgarol 1051 (Ciba Geigy, 1995)

Irgarol 1051 is highly stable in the marine environment, with a half-life of 273 days in artificial seawater irradiated with a Xenon lamp, and is therefore a persistent compound with a low rate of photolysis (Ciba-Geigy, 1995). It is often detected in waters close to intense boating activity (Scarlett *et al*, 1997) and is an extremely effective algicide. There is, therefore, concern over the levels reported (Readman *et al* 1993, Gough *et al* 1994, Tolosa *et al* 1996 and Scarlett *at al* 1997) in the environment and its possible effects on non-target organisms.

The aim of this study was to investigate if *in vivo* spectral properties of *E.intestinalis* could be used to detect stress caused by exposure to Irgarol 1051 using fluorescence (mainly F_v/F_m) and growth as additional comparitors to assess algal health. An additional part of the study was to investigate the effect of combined copper and Irgarol 1051 solutions on the algae as currently used in antifouling paints in the marine environment.

5.1.1 EXPERIMENTAL DESIGN

Collection of algae

E.intestinalis was collected from Wembury Bay, Southwest England, identified and acclimated as described in the Methods section.

Preparation of solutions

Irgarol 1051 stock solutions were made as described in the Methods section. Treatment solutions were then made by adding 100 µl of each stock solution to Instant Ocean containing nutrients (50 mg/l $NaNO_3$ and 10 mg/l Na_2HPO_4) to give concentrations of 0.25, 2.5, 25, 250 and 2500µg/l (equivalent to 0.001, 0.01, 0.1, 1 and 10 µM).

Experimental setup

Intercalary sections (2.5 cm long) of *E.intestinalis* were cut from healthy fronds and placed in tri-divided petri dishes each containing 50 ml of the relevant Irgarol 1051 solution (as described in the Methods section).

Parameters measured

Growth was measured on day 7 and was recorded as Relative Growth Rate.

Fertility was also recorded as the percentage of sections that sporulated during the experiment. Any sections that did sporulate were subsequently removed from the experiment for reasons discussed earlier.

Fluorescence measurements (F_v/F_m) were recorded before exposure (0 hours) and at 24, 48 96 and 168 hours.

The *in vivo* spectral properties of the algae were measured at the same times as fluorescence *i.e.* 0, 24, 48, 96 and 168 hours using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere.

5.1.2 RESULTS AND DISCUSSION

Growth

Figure 45 shows that Irgarol 1051 has a significant ($P < 0.05$) effect on the growth of *Enteromorpha intestinalis* and a LOEC of 2.5 μg/l was observed. It can be seen that relatively low levels of Irgarol 1051 affect growth of the algae with the highest concentration (2500 μg/l) exhibiting virtually no growth. An EC_{50} for growth of the algae, when treated with Irgarol 1051, of 7.2 μg/l (figure 46) was calculated from the dose response curve. The manufacturers of Irgarol 1051 (Ciba Geigy) have published data relating to the acute toxicity of the compound (Ciba Geigy Technical bulletin) and the minimal inhibition concentration for *E.intestinalis* is reported as 10 μg/l. The EC_{50} of 7.2 μg/l and LOEC of 2.5 μg/l are therefore smaller than the reported minimal inhibition concentration. This could have serious implications on the environmental impact of Irgarol 1051 on *E.intestinalis* as the EC_{50} for growth was based on data obtained from adult plants. There could be other deleterious effects either at the sublethal level or on other life stages of the algae that may be more sensitive.

Readman *et al* (1993) reported on levels of Irgarol 1051 contamination, from the use of antifouling paints, in coastal waters and found substantial levels (up to 1.7 μg/l) in water taken from yacht marinas in the Mediterranean. This concentration of Irgarol 1051 in the water is likely to have an effect on growth of *E. intestinalis* as from the growth results in figure 45 it is evident that Irgarol 1051 concentrations as low as 2.5 μg/l had a considerable effect on the growth (RGR = 10.2% compared with control value of 37.43%) of the algae.

Although the difference is unlikely to be statistically significant, the mean relative growth rate of the algae exposed to 0.25 μg/l is slightly higher (41.2%) than that of the control sections (37.43%). It has been reported that in some cases certain triazine herbicides

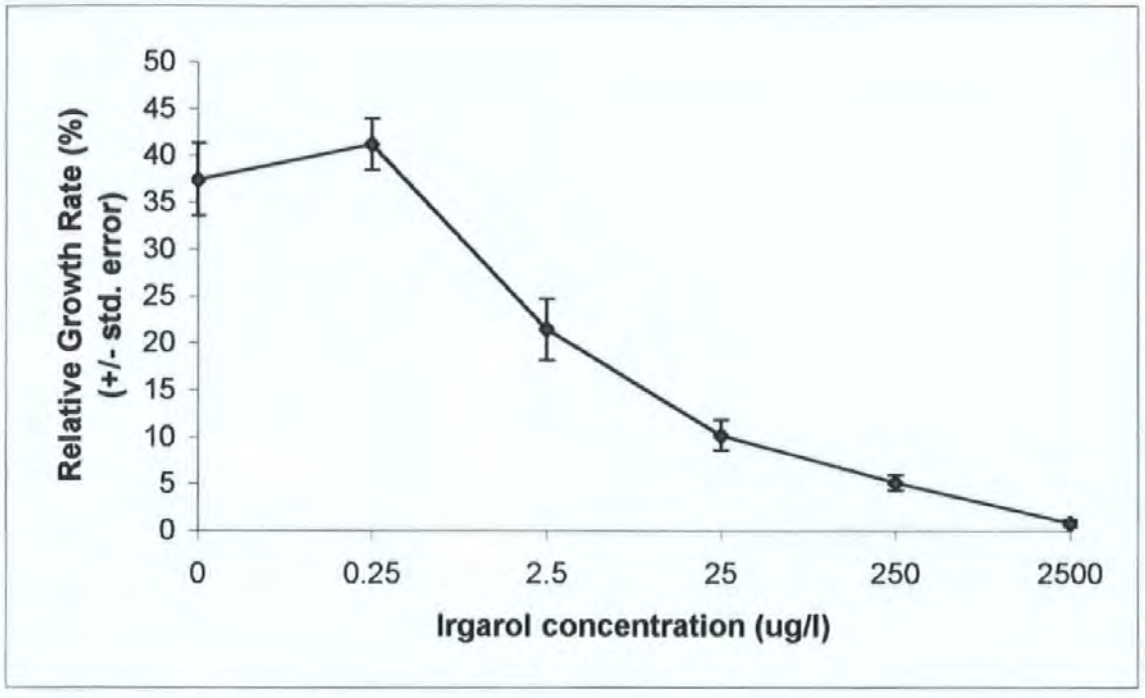

Figure 45: **Relative growth rate of *E.intestinalis* exposed to Irgarol 1051 (ug/l)**

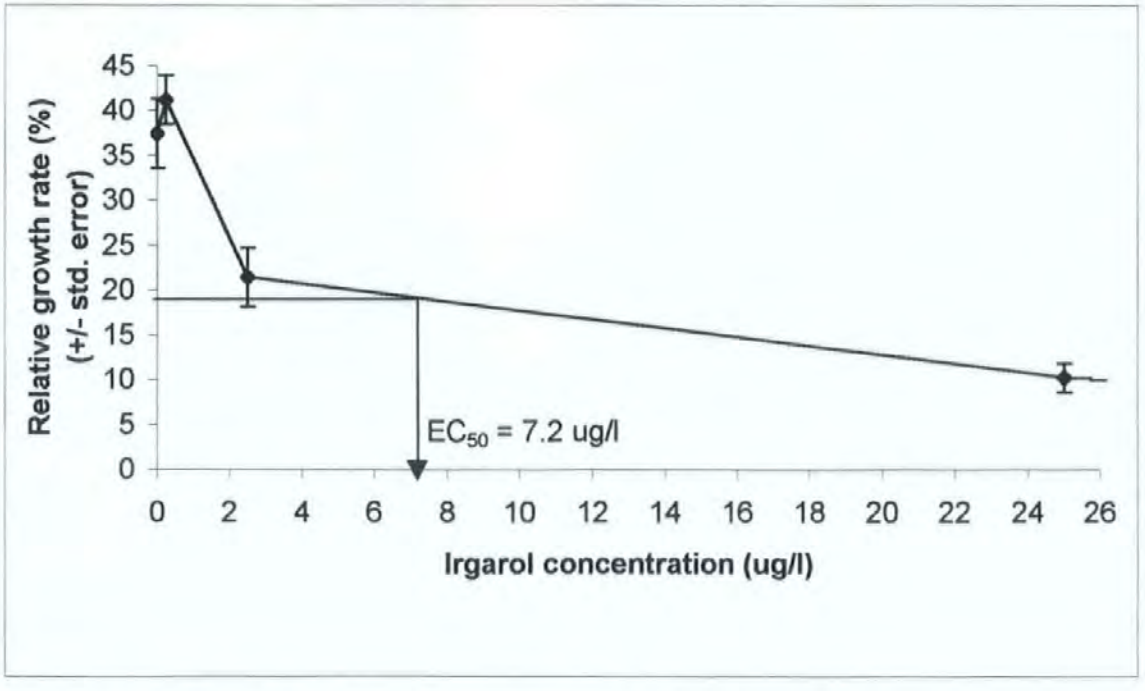

Figure 46: **EC_{50} for growth of *E.intestinalis* when treated with Irgarol 1051 (ug/l)**

stimulate growth at subtoxic concentrations (Ashton and Crafts, 1981). The growth of maize was found to increase when treated with low concentrations of simazine (Lorenzoni, 1962 and Bartley, 1957: cited in Ashton and Crafts, 1981) and so this could account for the increase observed.

Fluorescence

Figure 47 illustrates the effect of Irgarol 1051 on the fluorescence (F_v/F_m) of *E.intestinalis*. Fluorescence induction ratios (F_v/F_m) are a sensitive indicator of specific damage to photosynthetic system II of the algae and results indicate PS II activity is significantly ($P < 0.05$) affected by exposure to Irgarol 1051 with the LOEC calculated as 2.5 μg/l at the 95 % confidence limit.

The results show that Irgarol 1051 has a considerable effect both within 20 hours and at a relatively low concentration (25 μg/l). The calculated EC_{50} for fluorescence (F_v/F_m) for the algae, when treated with Irgarol 1051 for 7 days, was 16.8 μg/l (figure 48). This is higher than the EC_{50} that was calculated for growth, 7.2 μg/l. However, although growth was affected at lower levels of Irgarol 1051 exposure, as mentioned above, fluorescence techniques have some advantages in that they are non-invasive. They can also give good indications of the health of the algae in the environment as the technique can be used in the field to provide immediate results. However, growth provides an ultimate assessment of the health of the algae.

The effect of the Irgarol 1051 on the fluorescence of the algae appears to fall into three groups. The lowest concentration (0.25 μg/l) does not appear to have any effect on the fluorescence of the algae even after an exposure of 7 days (no effect concentration, NOEC). The next highest concentration (2.5 μg/l) does not appear to have an effect for the

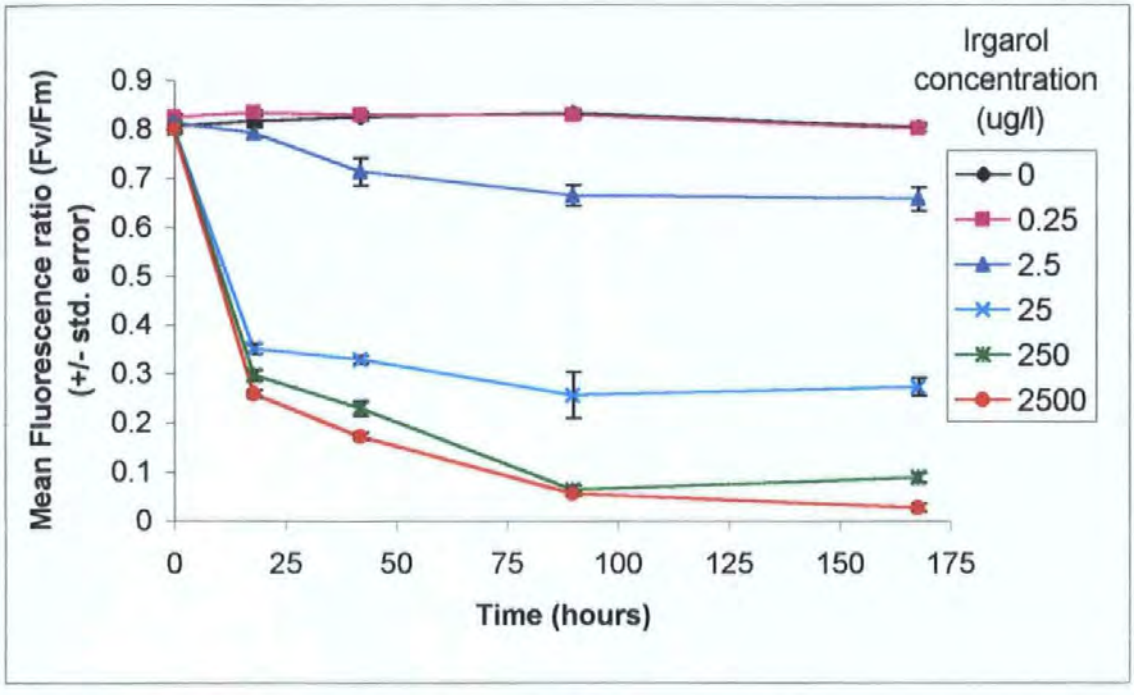

Figure 47: **Effect of Irgarol 1051 (ug/l) on fluorescence (Fv/Fm) of *E. intestinalis***

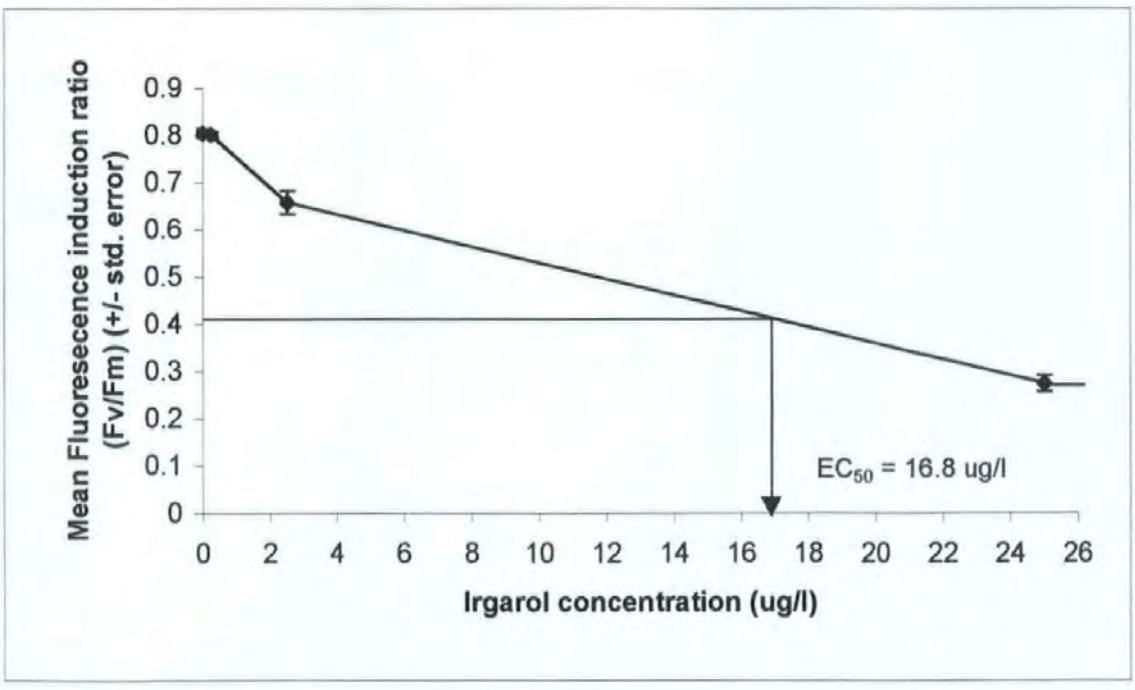

Figure 48: **EC_{50} for fluorescence (Fv/Fm) of *E. intestinalis* exposed to Irgarol 1051 (ug/l) for 7 days**

first 20 hours of exposure and then lowers the F_v/F_m of the algae to 0.66. The F_v/F_m of the algae treated with this concentration then levels out indicating that the Irgarol 1051 is having a detrimental effect on the algae and is presumably preventing some action at PS II but that the algae is able to continue to photosynthesise enough to survive adequately. Irgarol 1051 is known to inhibit PS-II activity by displacing plastoquinone from the D-1 Protein. This prevents the electron carrier quinone binding and so electron flow is prevented resulting in the chain of events being halted. When *E.intestinalis* was treated with 2.5 µg/l Irgarol 1051 it is possible that the Irgarol 1051 is not present in high enough quantities to entirely outcompete the plastoquinone from the D-1 Protein so the electron flow is not halted but is, instead, slightly inhibited. When the fluorescence data is compared to the growth data it is apparent that growth at this concentration is also affected but that the algae is still able to grow to a certain extent.

The third main group of Irgarol 1051 concentrations is from 25 – 2500 µg/l. All these concentrations have an effect on the F_v/F_m of the algae within a short space of time. The first readings were taken after 18 hours exposure and all 3 concentrations had dramatically lowered the F_v/F_m values of the algae to values between 0.258 – 0.356 by this time. This indicated that the PS II activity of the algae was being inhibited severely. At the top 2 concentrations, 250 and 2500 µg/l, the F_v/F_m values of the algae were extremely low, less than 0.06, after 96 hours. This indicates that electron flow must have been almost completely halted in all PS II sites. This corresponds with the growth data, as very little growth was apparent at these concentrations with the top concentration exhibiting virtually no growth.

IN VIVO SPECTRAL PROPERTIES

On examining the *in-vivo* spectral properties (figures 49 and 50) of the algae it was apparent that treatment with Irgarol 1051 resulted in an increase in transmittance factor across most of the spectrum and a decrease in certain areas of reflectance.

In vivo Transmittance factor

Figure 49 is the effect of Irgarol 1051 on the *in vivo* transmittance factor of *E.intestinalis* after treatment for 7 days. A clear dose response is apparent with treatment of the algae with Irgarol 1051 resulting in an increase in transmittance factor across certain regions of the spectrum: 320 – 400, 500 – 670 and 700 – 820 nm but a plot of wavelength sensitivity can highlight the regions of the spectrum that are most sensitive to treatment with Irgarol 1051.

Figure 50 shows the wavelength sensitivity of the transmittance factor after 7 days of treatment with Irgarol 1051. From this, it can be seen that most of the wavelengths across the spectrum appear to be sensitive to treatment with Irgarol 1051. A symptom of triazines is foliar chlorosis followed by necrosis (Ashton and Crafts, 1981) but this does not really seem to be apparent here. Chlorophyll *a* has *in-vivo* absorption peaks at 440nm and 675 nm with smaller peaks at 385, 418, 590 and 625 nm and chlorophyll b has *in-vivo* absorption peaks at 470 and 650 nm. If the primary effect of the Irgarol 1051 were to degrade the chlorophyll, or prevent the re-synthesis of it, it would be expected that the main regions of increased transmittance factor would be at these wavelengths. If more light were being transmitted in these regions this would indicate that less chlorophyll was present as less light would be absorbed in these regions. However, the transmittance factor results do not show this. Instead, although some of the above regions are affected, the other effects

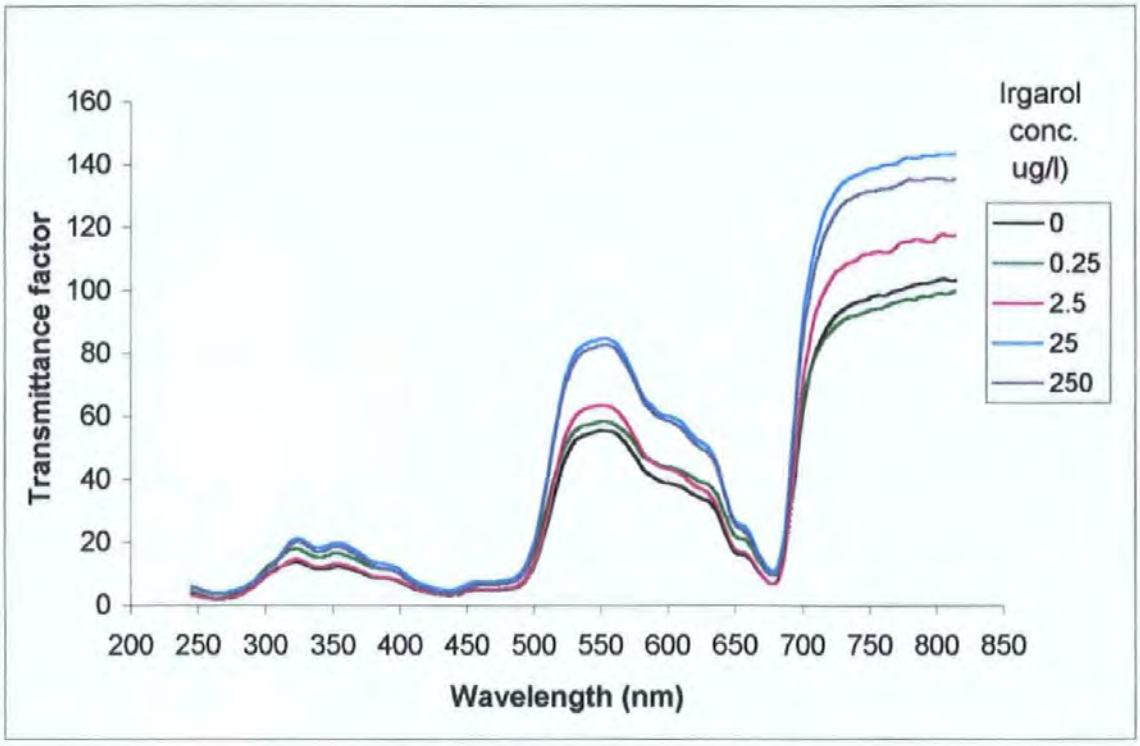

Figure 49: Effect of irgarol 1051 (ug/l) on transmittance factor of *E. intestinalis* after 7 days exposure

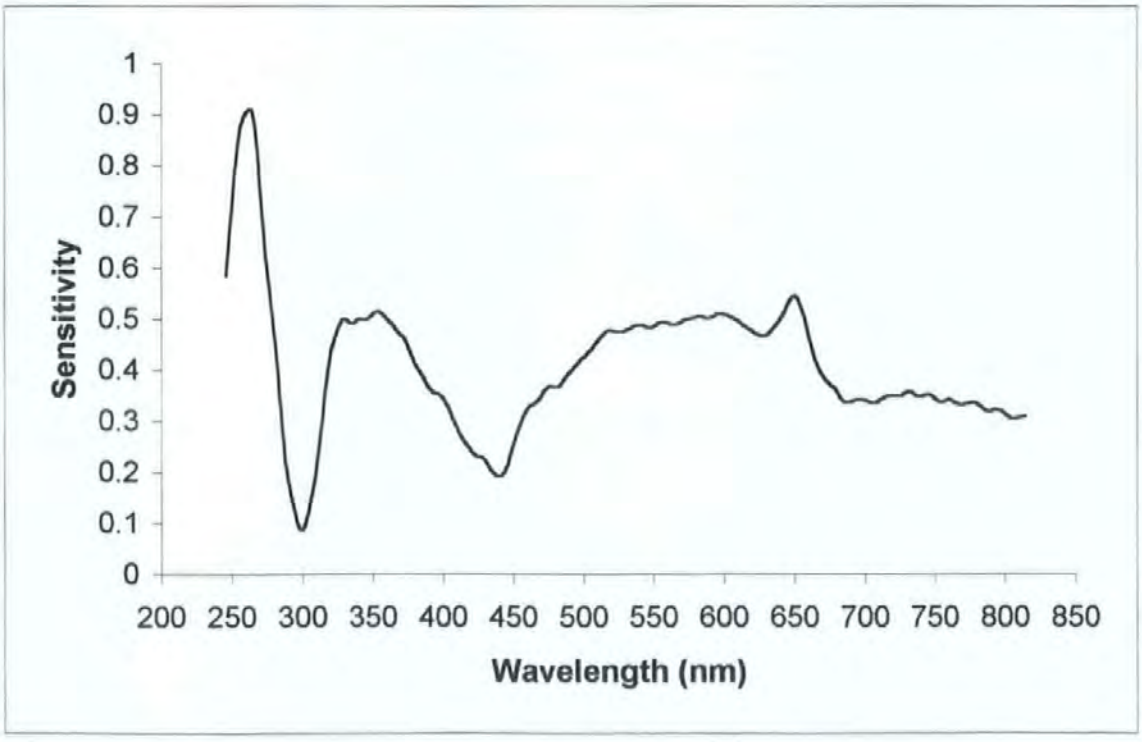

Figure 50: Sensitivity of transmittance factor of *E. intestinalis* to Irgarol 1051 (250 ug/l) exposure for 7 days

include considerable increases in transmittance factor between 500-670 and from 710 nm and above. In fact, figure 51 illustrates the difference between the control and algae treated with 250 µg/l Irgarol 1051 after 7 days and from this it appears that 2 of the main chlorophyll regions, 440 and 680 nm are less affected. The increases in transmittance factor could therefore be due to structural changes in the algae as opposed to changes purely in pigment turnover. It was noted that, on handling, the algae treated with the higher concentrations felt thinner than the controls did but the treated samples still appeared to look fairly green to the eye. This could support the fact that Irgarol 1051 was having an effect on the structure of the algae as opposed to the chlorophyll directly.

It could also be possible that some of the carotenoids could be affected due to exposure to Irgarol 1051. Carotenoids absorb light in the blue/green part of the spectrum (see Salisbury and Ross, 1985) and there is an increase in the transmittance factor in parts of this region. However, this is probably not the primary result of treatment with Irgarol 1051 because from the fluorescence results it is evident from the reduction with Fv/Fm that photosynthesis is affected. The role of many of the minor carotenoids is photoprotective rather than light harvesting and they are thought to screen out potentially harmful blue and near-UV radiation (see Dennis and Turpin, 1990) but they are also accessory pigments in photosynthesis. It could, therefore, be that if the carotenoids are degraded or the resynthesis of them is prevented then this could have an additional effect on the health of the algae as well affecting photosynthesis in some way.

Figure 52 is a measure of transmittance factor for individual wavelengths. These wavelengths were selected by using the plot of wavelength sensitivity (figure 51) that was produced by dividing the values for difference between the control and the treated transmittance factor by the transmittance factor of the control (Carter, 1994). This

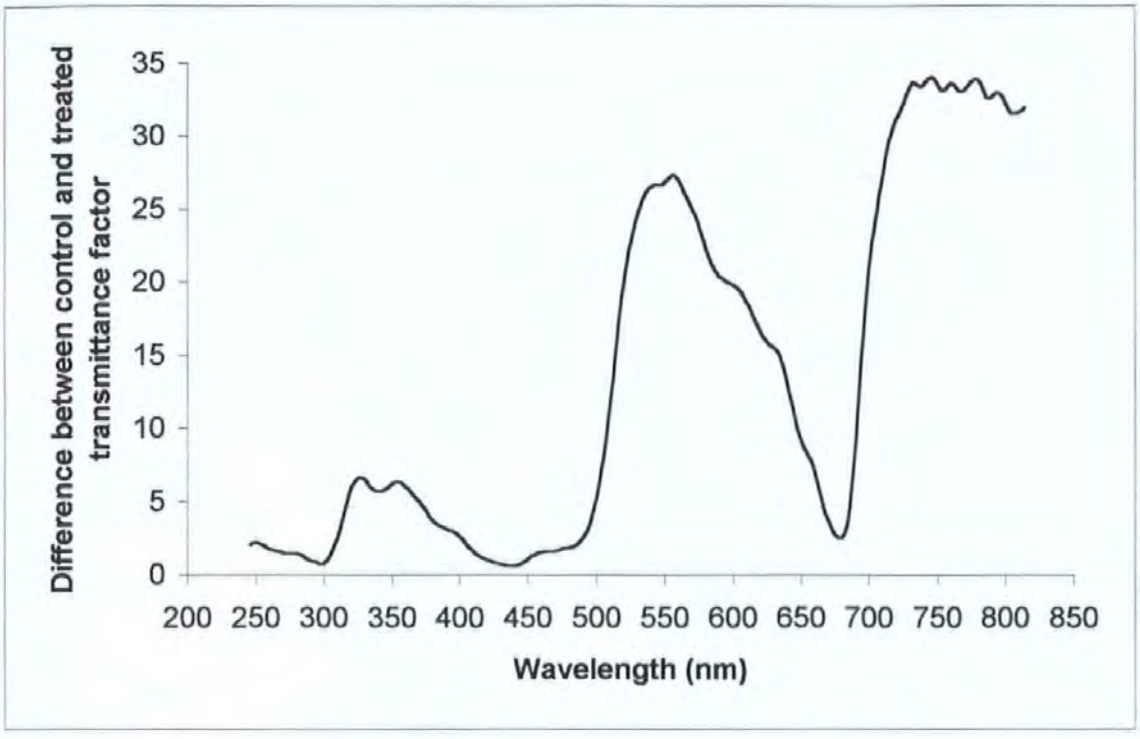

Figure 51: Difference between the control transmittance factor and *in vivo* transmittance factor of *E.intestinalis* when treated with 250 ug/l irgarol 1051 for 7 days

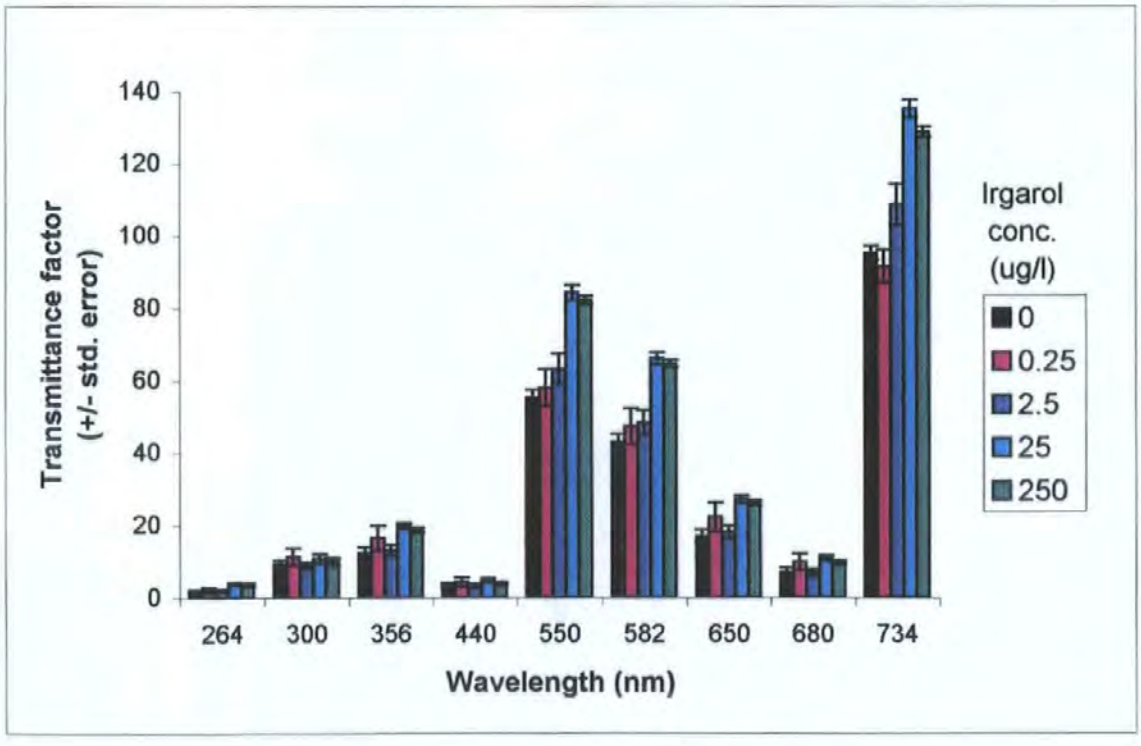

Figure 52: Effect of Irgarol 1051 on transmittance factor of *E.intestinalis* at selected wavelengths (nm)

indicates the sensitivity of the wavelengths to the stress of the treatment. Figure 52 illustrates that the wavelengths of 264, 550 (LOEC = 25 μg/l) and 734 nm (LOEC = 2.5 μg/l) increase significantly ($P<0.01$) in response to treatment with Irgarol 1051 whereas there appears to be no difference between the transmittance factor of the treated or control algae at 300 and 440 nm. Some differences can be observed between the control and treated *in vivo* transmittance sections of the algae at 680 and 264 nm. These differences at 680 are not as significant ($P<0.05$) as those at 264, 550 or 734 but they may indicate that some changes in chlorophyll levels are occurring.

By plotting the results of the *in vivo* transmittance factor wavelengths which appear to be sensitive against Irgarol 1051 concentration, a dose response curve was obtained (figure 53). These individual wavelengths may be useful indicators of stress by Irgarol 1051 if they prove to be specific to that pollutant.

A further useful method of analysis of the data is to calculate ratios of suitable wavelengths. From the *in vivo* transmittance factor results, a ratio of 582/440 and 550/440 nm was selected because the *in vivo* transmittance factor at 582 and 550 nm increases whereas the *in vivo* transmittance factor at 440 nm remains fairly constant for all concentrations of Irgarol 1051 and the control. Figure 54 illustrates that there appears to be a dose response relationship between both ratios and the Irgarol 1051 concentration. This, as with some of the single sensitive wavelength *in vivo* transmittance factor results, could be extremely useful if they are specific to stress of the algae by Irgarol 1051.

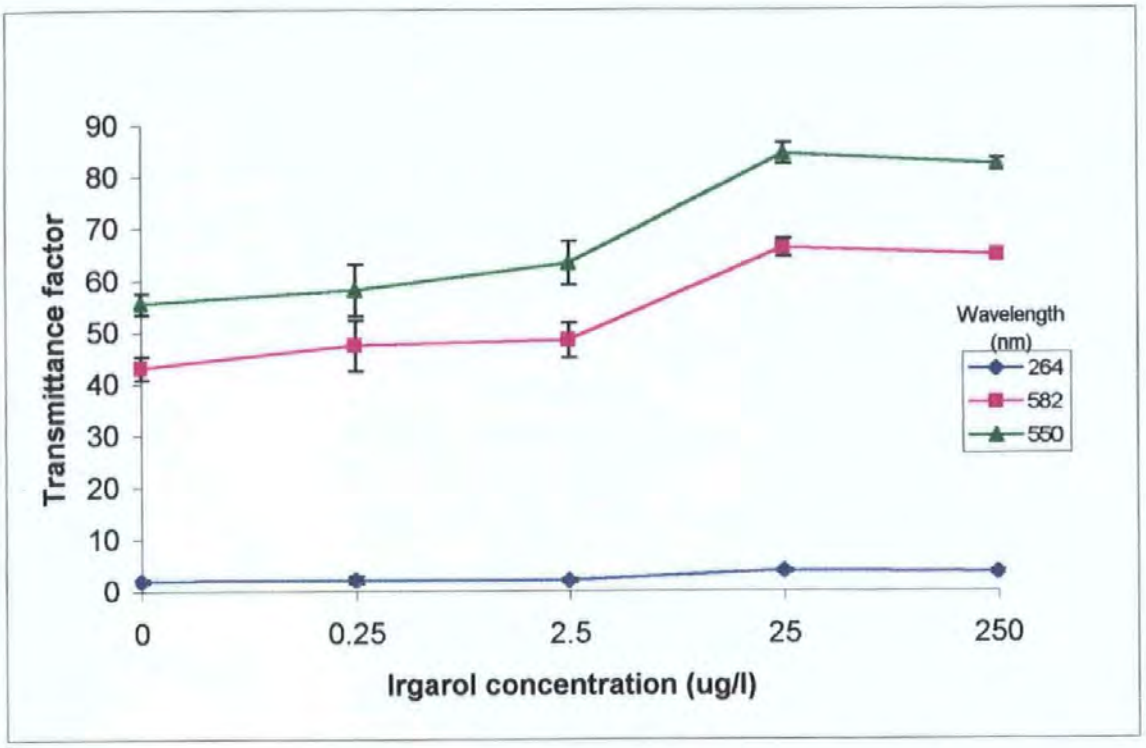

Figure 53: **Effect of Irgarol 1051 (ug/l) on *in vivo* transmittance factor, at 264, 582 and 550 nm, of *E. intestinalis* after 7 days**

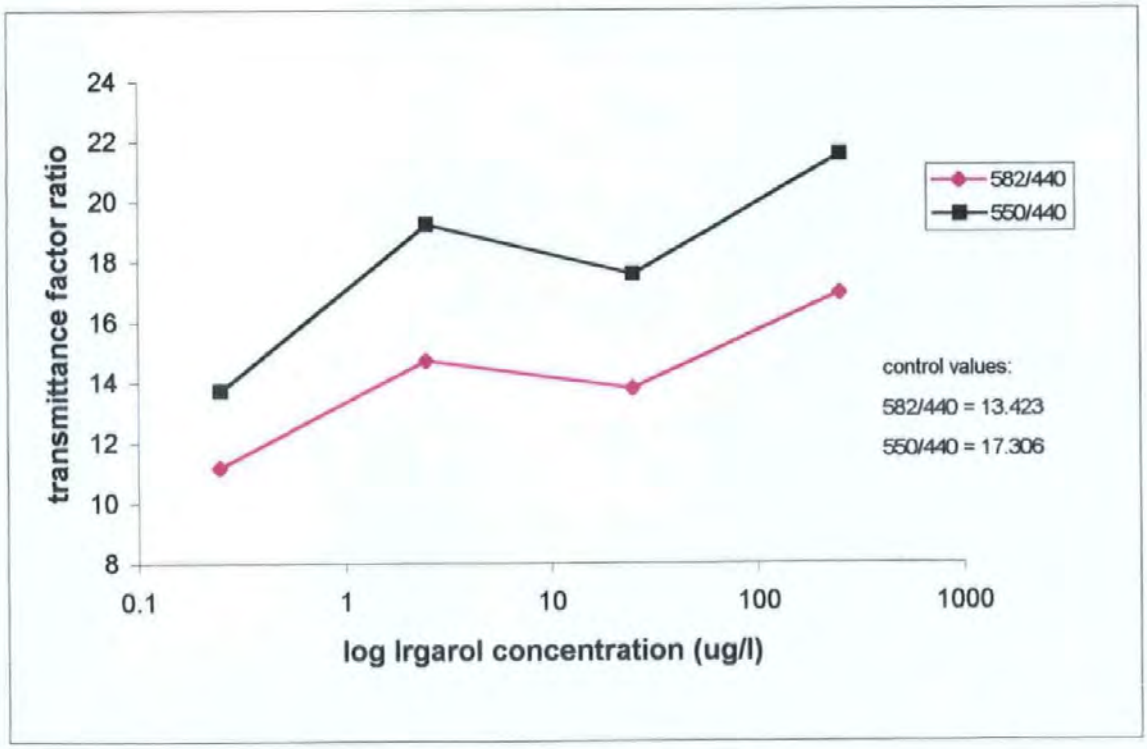

Figure 54: **Effect of Irgarol 1051 (ug/l) on the 583/440 and 550/440 nm *in vivo* transmittance factor ratio of *E. intestinalis***

***In vivo* reflectance**

Figure 55 shows the effect of treatment with Irgarol 1051 on the *in vivo* reflectance of the algae after 7 days. The *in-vivo* reflectance of the algae was decreased with the addition of Irgarol 1051 especially in the regions from 530 – 570 and 700 nm and above. As with the transmittance factor results, there appears to be a clear dose response relationship between reflectance and treatment with Irgarol 1051.

Figure 56 is a plot of wavelength sensitivity of *in vivo* reflectance of *E.intestinalis* when treated with Irgarol 1051. From this, it is clear that the reflectance is decreased considerably in 2 main regions: 520 – 650 and 700 nm and above. As with the transmittance factor results, it is apparent that there is very little effect at 680 and 440 nm, indicating that differences may not be primarily due to changes in chlorophyll.

Reflectance often increases with an increase in the number of air spaces in a leaf or thallus as diffused light passes more often from highly refractive hydrated cell walls to lowly refractive intercellular air spaces (Barrett and Curtis, 1992). *E.intestinalis* has internal air spaces which tend to increase the backscatter of light (Lobban and Harrison, 1994). Refraction and reflectance increase the light path within a thallus so enhancing the chance of photon capture. It may be possible that Irgarol 1051 had an additional effect of altering the structure of the algal thallus in some way as well as inhibiting photosystem II. This could account for the reduction in *in-vivo* reflectance of the algae.

As with the *in vivo* transmittance factor results, it is useful to select individual sensitive reflectance wavelengths and also to calculate appropriate ratios. Figure 57 shows the reflectance values at various wavelengths for *E. intestinalis* when treated with Irgarol 1051. These wavelengths were selected from the sensitivity graph (Figure 56). From this, it can be seen that *in vivo* reflectance at 550 and 740 nm decreases significantly ($P<0.01$,

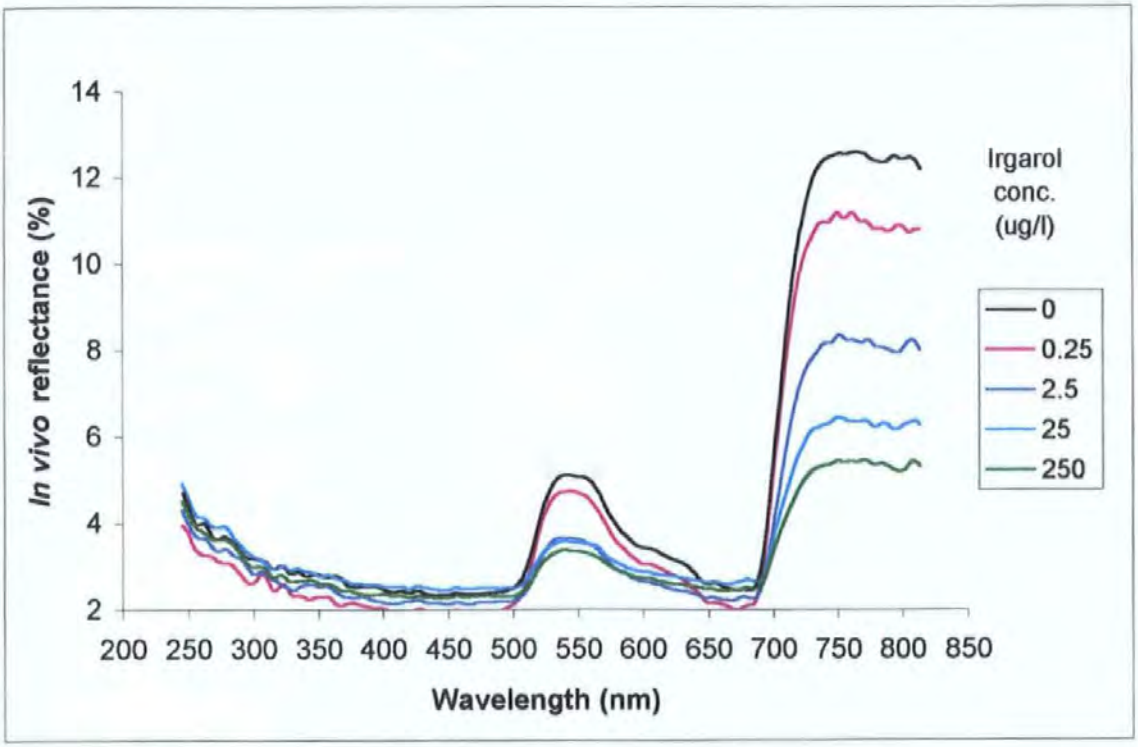

Figure 55: Effect of Irgarol 1051 (ug/l) on *in vivo* reflectance of *E. intestinalis* after 7 days exposure

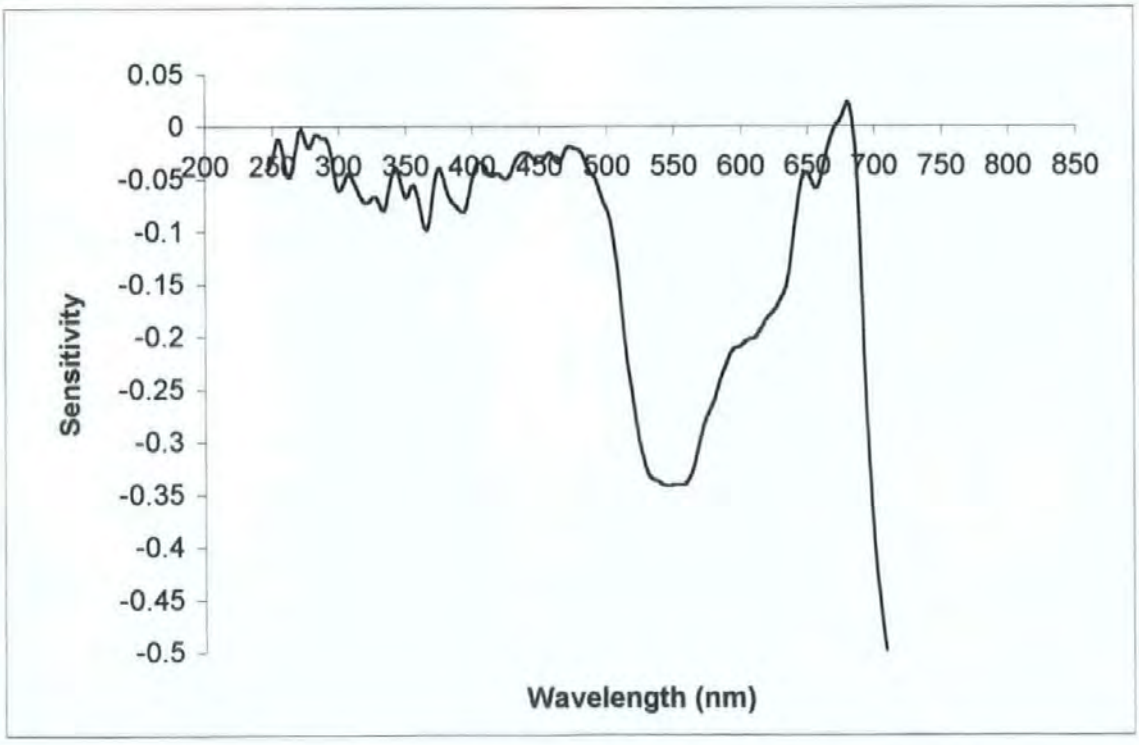

Figure 56: Sensitivity of *E.intestinalis in vivo* reflectance to Irgarol 1051 exposure for (250 ug/l) 7 days

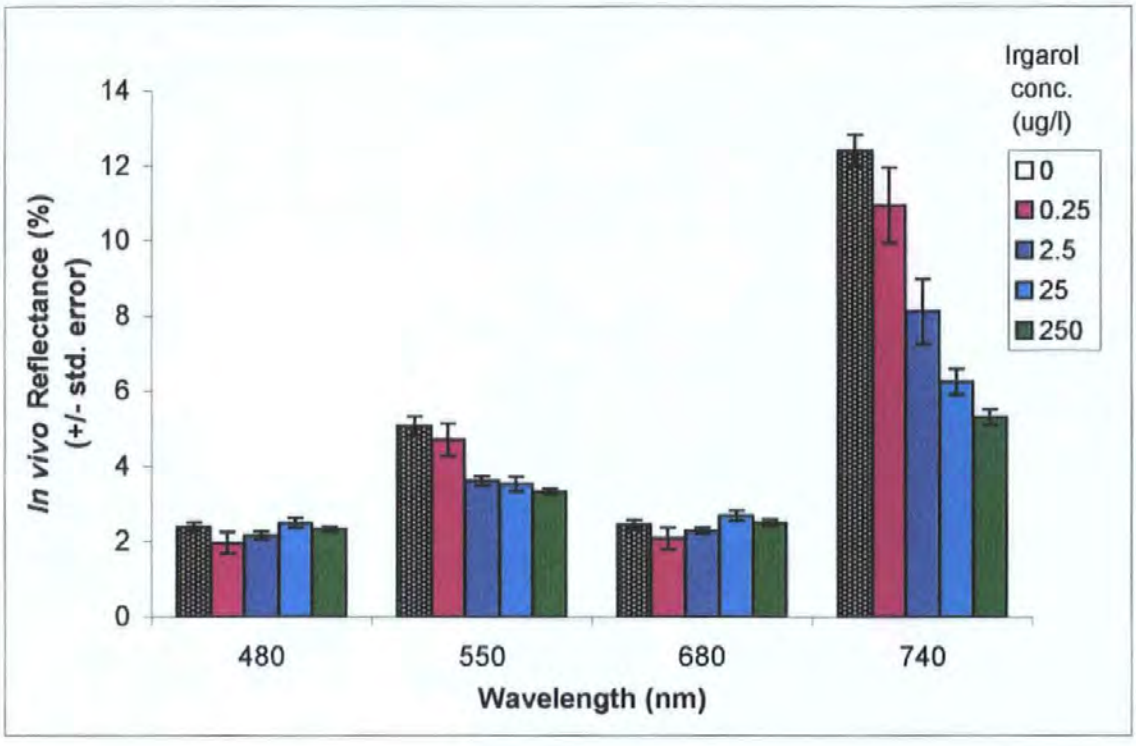

Figure 57: ***In vivo*** **reflectance at specific wavelengths (nm) of** ***E. intestinalis*** **exposed to Irgarol 1051 for 7 days**

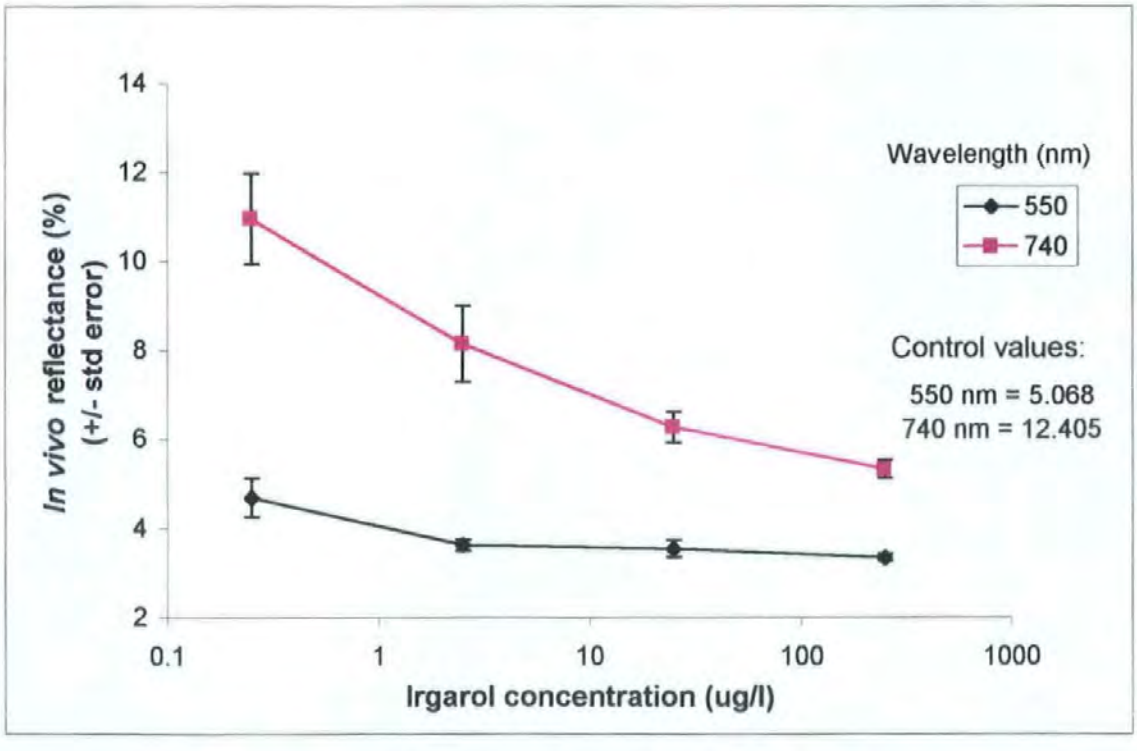

Figure 58: Effect of irgarol 1051 on the ***in vivo*** **reflectance (%) of** ***E. intestinalis*** **at 550 and 740 nm after 7 days exposure**

LOEC = 25 and 2.5 µg/l respectively) with an increase in Irgarol 1051 concentration. Dose response curves (Figure 58) were obtained by plotting the *in vivo* reflectance at these wavelengths against the Irgarol 1051 concentration. From this, it appears that *in vivo* reflectance at 740 nm is the most sensitive wavelength when *E.intestinalis* is treated with Irgarol 1051. This indicates that the Irgarol 1051 could be having a considerable effect on the structure of the algae.

Reflectance ratios are often used to detect whether a plant is stressed. Carter, 1994, devised various reflectance ratios for different plant species and stress agents. Certain ratios were found to be affected by several stress agents but some ratios e.g. 695/420, 605/760, 695/760 and 710/760 were found to be affected by all the stress agents. These ratios were applied to the *in vivo* reflectance data for *E. intestinalis* when treated with Irgarol 1051 but it was found that these ratios were not as affected as other ratios devised by using the suitable wavelengths from the sensitivity results (Figure 56). Figure 59 illustrates the effect Irgarol 1051 has on certain *in vivo* reflectance ratios after 7 days exposure. The 740/680 ratio, devised from the *E. intestinalis* results, is plotted with the 695/420 ratio devised by Carter, 1994.

In vivo spectrophotometry has potential in monitoring pollutant damage by providing appropriate quantitative parameters. The region of the spectrum from 500 – 750nm is characterised by absorption by chlorophylls a and b, carotenes and xanthophylls. From the results it is apparent that there are certain wavelengths, both for reflectance and transmittance factor, which appear to be affected most by the addition of Irgarol 1051. For example, it appears that the 582/440 nm transmittance factor ratio and the 740/680 reflectance ratio show a clear dose response. If these ratios or wavelengths are specific to treatment with Irgarol 1051 or herbicides with this mode of action it may be possible to identify the specific stresses that the algae has been exposed to. It would, therefore, be

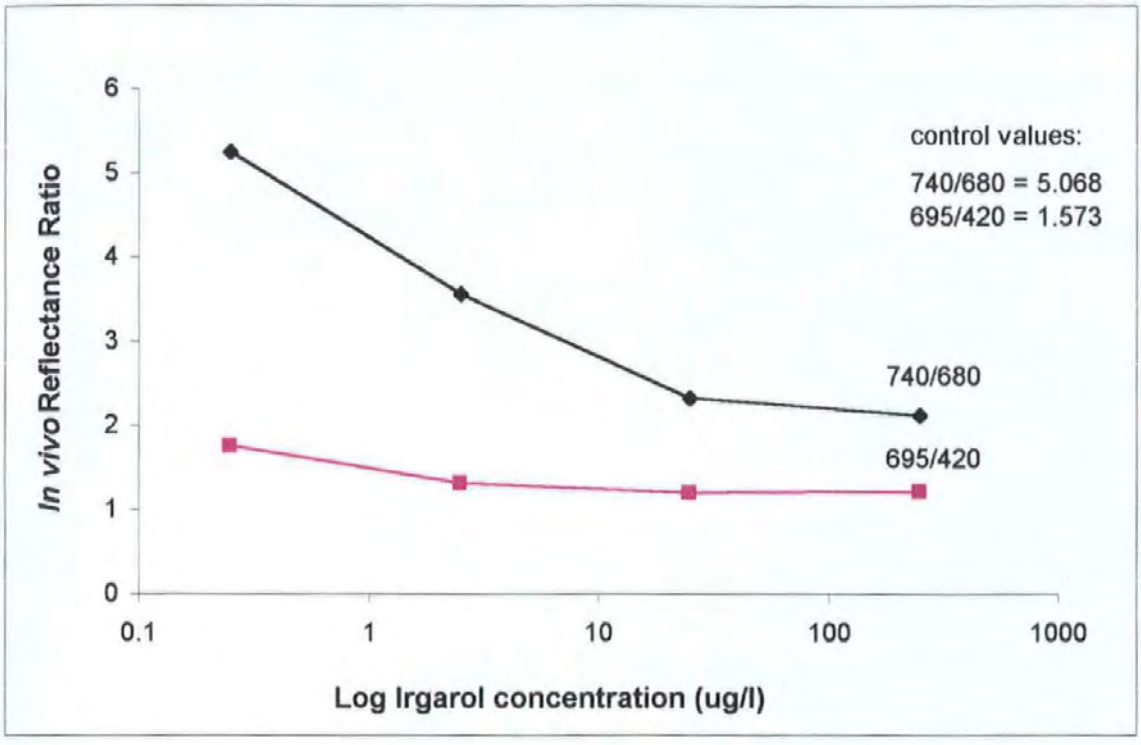

Figure 59: Effect of Irgarol 1051 on the 740/680 and 695/420 *in vivo* reflectance ratios of *E. intestinalis* after 7 days exposure

more effective to use these specific ratios rather than the ratios devised by Carter, 1994, which are affected by all the stress agents that were tested. However, care would have to be taken in interpreting results because, as with the fluorescence, other stresses could be responsible for certain responses. If certain reflectance ratios were specific to certain stress agents, this would be extremely useful in environmental studies. Reflectance ratios may even be more useful than transmittance factor ratios as they may have the potential in being used in remote sensing.

From the results of the experiments involving exposure of *Enteromorpha intestinalis* to Irgarol 1051 it can be seen that Irgarol 1051 does have an effect. These effects are considerable and include changes in the *in-vivo* spectral properties of the algae and also changes in growth and fluorescence. It was also observed that there was a dose response relationship between the irgarol concentration applied to the algae and inhibition of sporulation in the thallus of the algae although this needs further confirmation.

5.2 ATRAZINE

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is not directly used in the marine environment but runoff and leaching are two ways it could potentially reach this system. It has a solubility of 30 mg/l at 20 °C in freshwater and is persistent in the marine environment (Riedel-de Haën, 1994). Atrazine is a triazine herbicide that is used primarily on higher plants but there have been reports of it reaching the marine environment and it is now a common contaminant of surface waters of agricultural regions (Hersch and Crumpton, 1987). The potential of it reaching aquatic systems has now increased as it is sometimes used to control aquatic weeds, for example the water hyacinth in tropical and sub-tropical waters (Rana and Srinivas, 1992). Levels ranging from 120 – 5800 ng/l have

been reported in coastal waters of Germany and biological testing of these levels on marine phytoplankton resulted in a decrease in photosynthesis and a reduction in chlorophyll levels (Bester *et al* 1995).

The aim of this study was to investigate the use of *in vivo* spectral properties of *E.intestinalis* to monitor effects of atrazine exposure using growth, fluorescence (mainly F_v/F_m) and fertility to assess the health of the algae.

5.2.1 EXPERIMENTAL DESIGN

Collection of algae

E.intestinalis was collected from Wembury Bay, identified and acclimated as described in the Methods section.

Preparation of solutions

For the first experiment, atrazine stock solutions were made by dissolving atrazine in ethanol as described in the Methods chapter. Treatment solutions were then made by adding 100 µl of each stock solution to Instant Ocean containing nutrients (50 mg/l $NaNO_3$ and 10 mg/l Na_2HPO_4) to give concentrations of 2, 10, 50, 250 and 1250 µg/l.

Higher concentrations of atrazine were used in the second experiment. The treatment solutions were then made by adding 200 µl of each stock solution to Instant Ocean containing nutrients (50 ml/l $NaNO_3$ and 10 mg/l Na_2HPO_4) to give concentrations of 0.22, 2.2, 22, 220 and 2200 µg/l (equivalent to 0.001, 0.01, 0.1, 1 and 10 µM). 200 µl was used because the concentrations of atrazine were higher than in the first experiment and it was important that the atrazine dissolved in the highest concentration stock. A carrier control containing 200 µl ethanol was also prepared and was the control that was used in subsequent data analysis.

Experimental setup

Intercalary sections (2.5 cm long) of *E.intestinalis* were cut from healthy fronds and placed in tri-divided petri dishes each containing 50 ml of the relevant atrazine solution (as described in the Methods section).

Parameters measured

Growth was measured on day 7 for experiment 1 and day 4 for experiment 2, and was recorded as Relative Growth Rate. Fertility was recorded as the percentage of sections that sporulated during the experiment with sections that did sporulate subsequently being removed as discussed earlier.

Fluorescence measurements (F_v/F_m) were recorded before exposure (0 hours) and at 24, 48 96 and 168 hours for experiment 1 and at 0, 48 and 96 hours for experiment 2.

The *in vivo* spectral properties of the algae were measured at the same times as fluorescence using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with the Labsphere RSA-UC-40 integrating sphere.

5.2.2 RESULTS AND DISCUSSION

Growth

Figure 60 shows the effect of atrazine exposure on the growth of *E.intestinalis*. Growth is hardly affected until levels of 250 µg/l atrazine are reached with the alga exposed to 1250 µg/l atrazine for 7 days exhibiting a relative growth rate of only 5 %. However, this was not sufficient to be able to calculate an accurate EC_{50} and so the second experiment involving a wider range of concentrations was set up. From the growth results of the second experiment (figure 63) it was evident that growth was significantly ($P < 0.05$) affected by atrazine, with a resulting LOEC of 50 µg/l. The algae exposed to the highest concentration (2200 µg/l) showed no growth and, consequently, an EC_{50} for growth after 96 hours of 143 µg/l was calculated from the dose response curve (figure 64). As with the

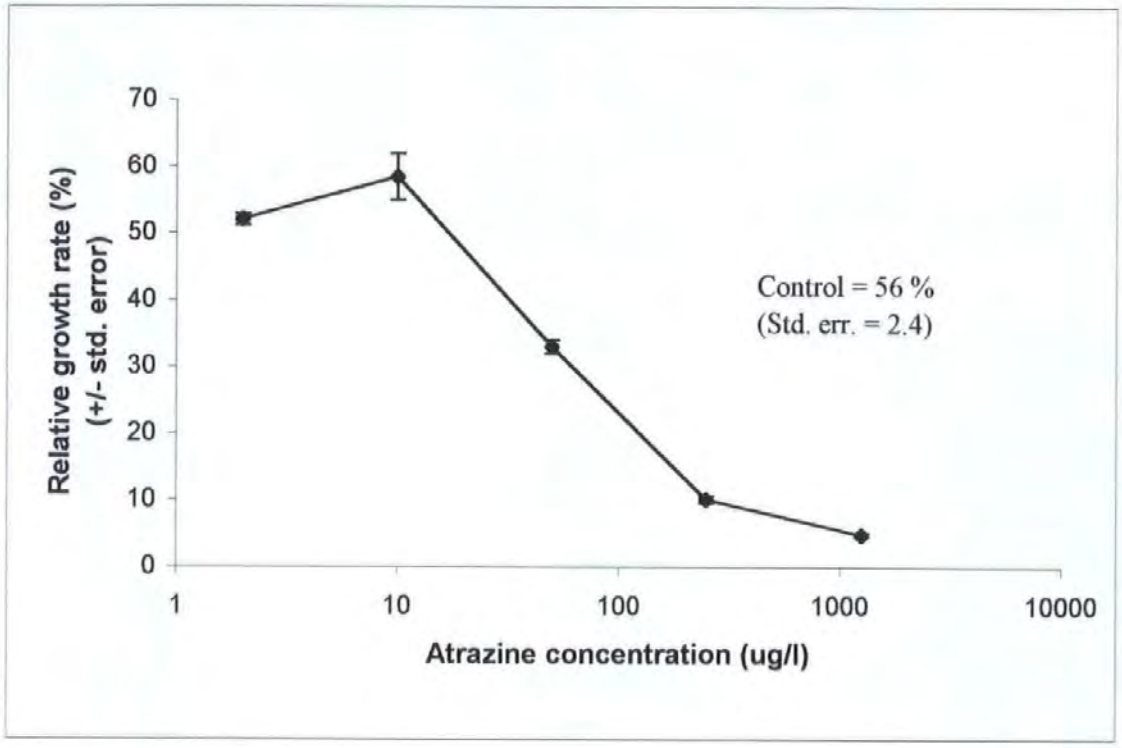

Figure 60: **Relative growth rate of *E.intestinalis* exposed to atrazine (ug/l) for 7 days**

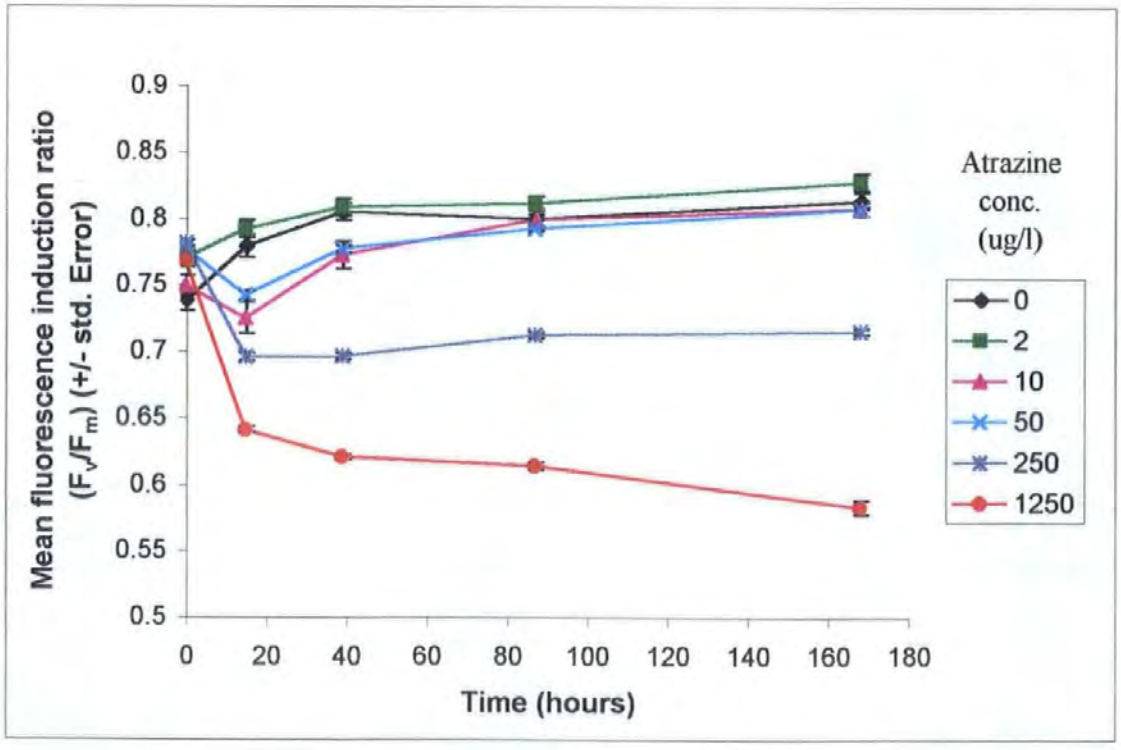

Figure 61: **Effect of atrazine (ug/l) on fluorescence (F_v/F_m) of *E.intestinalis***

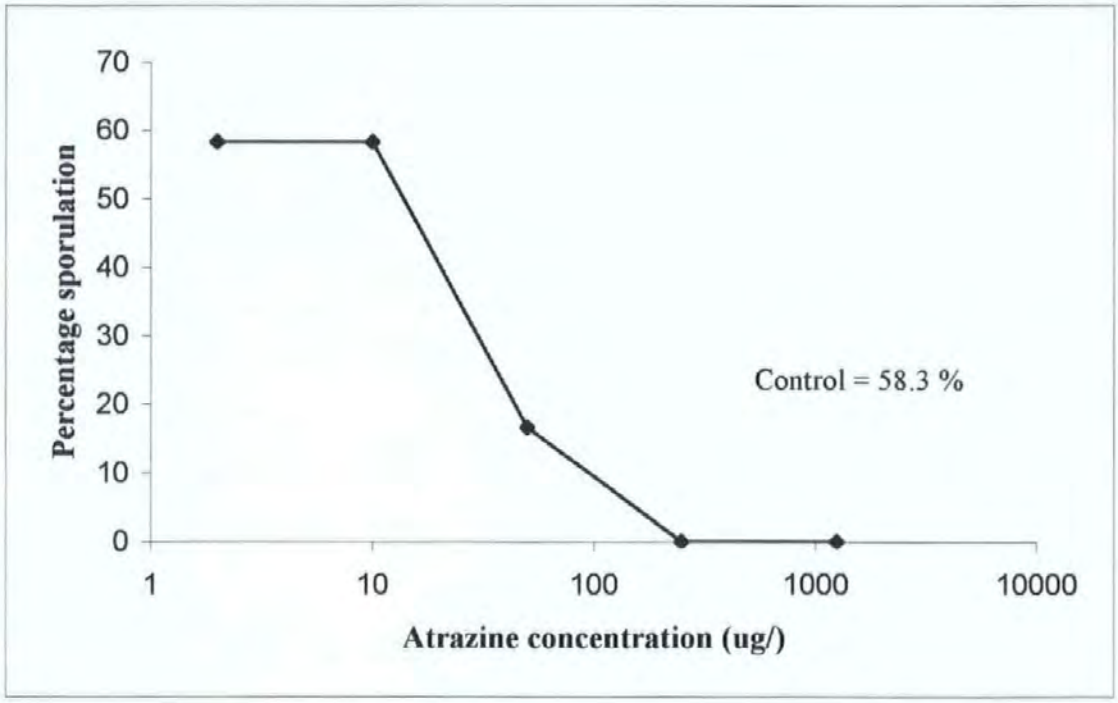

Figure 62: **Effect of atrazine (ug/l) on the sporulation of** *E.intestinalis*

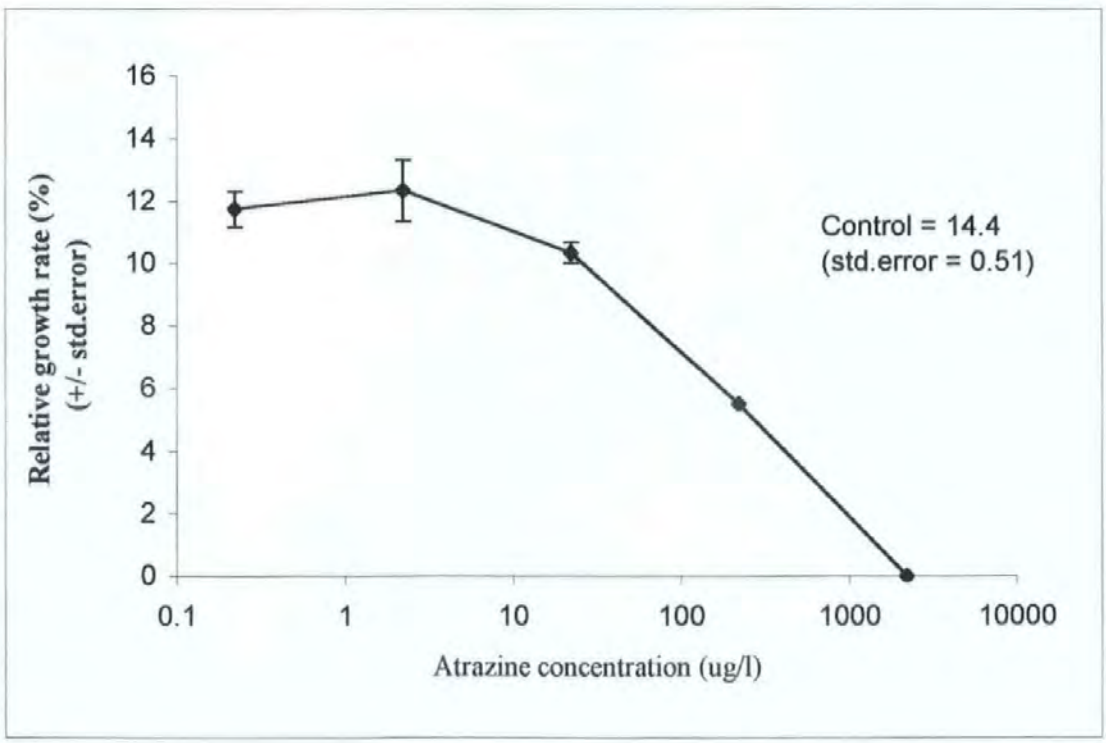

Figure 63: **Effect of atrazine on relative growth rate of *E.intestinalis* after 4 days exposure**

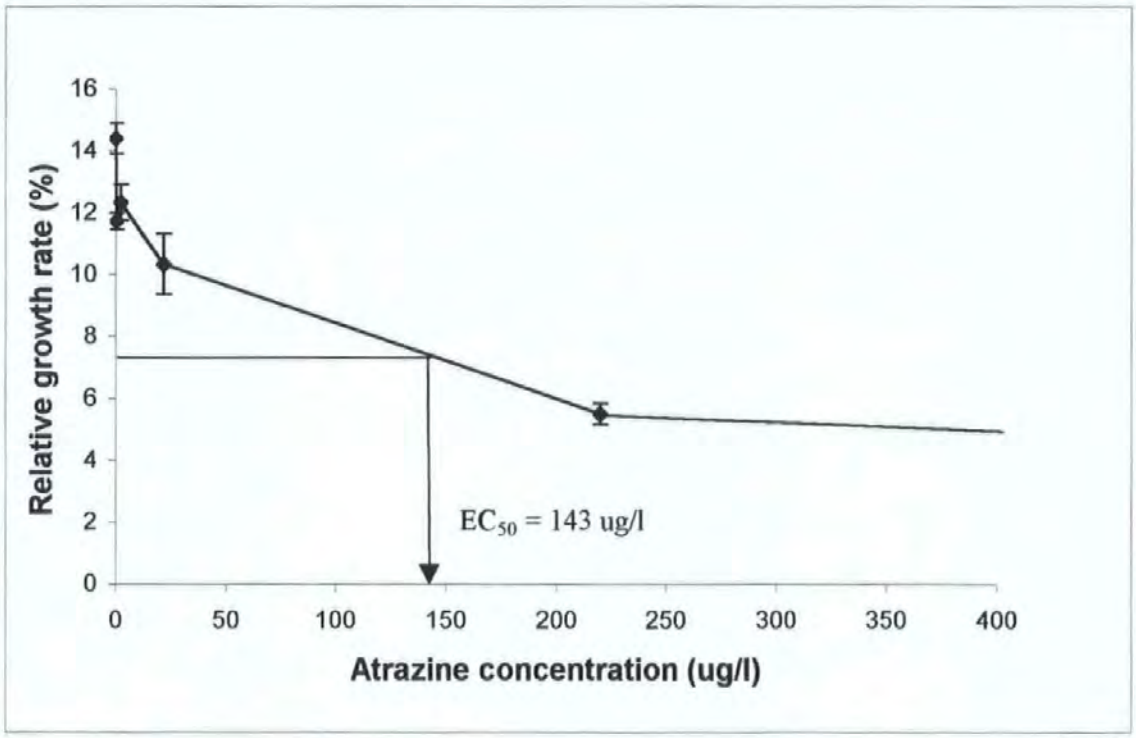

Figure 64: **EC_{50} for growth of *E.intestinalis* when exposed to atrazine (ug/l) for 4 days**

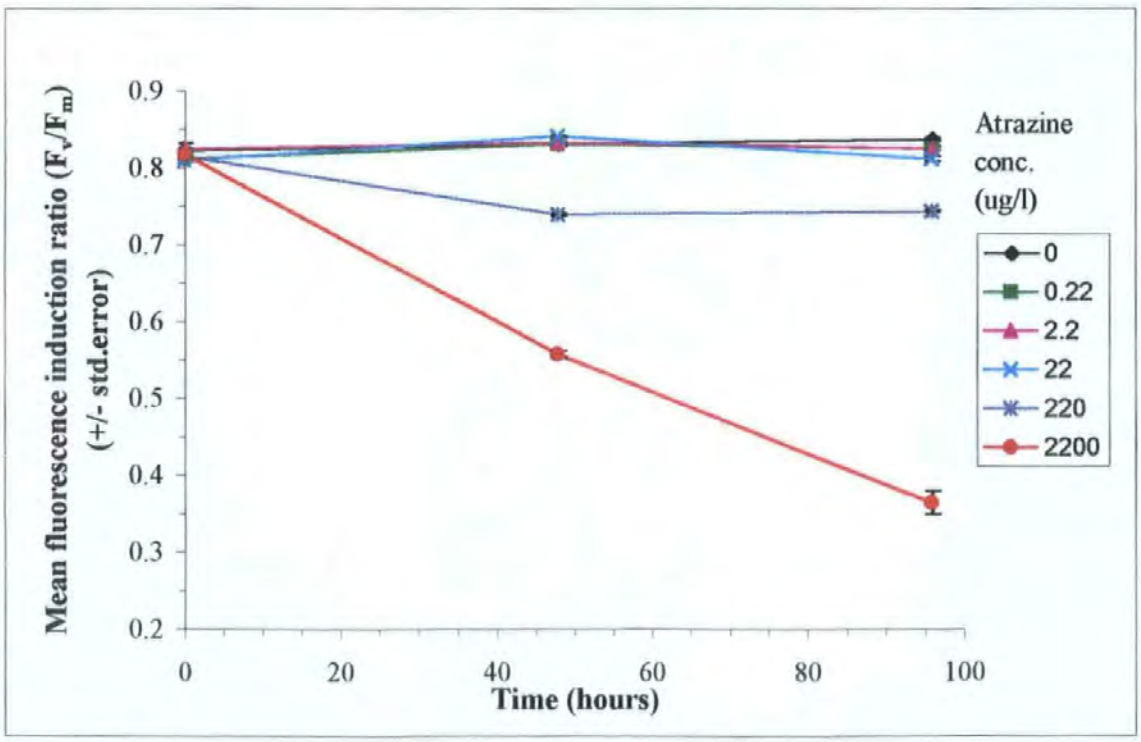

Figure 65: **Effect of atrazine (ug/l) on fluorescence induction ratio (F_v/F_m) of *E.intestinalis***

Irgarol 1051 experiments, it was evident that the growth of the algae exposed to low concentrations (0.22 and 2.2 µg/l) was greater than that of the control sections which supports the possibility certain triazine herbicides may stimulate growth at subtoxic concentrations (Ashton and Crafts 1981, Fedtke, 1982). Fertility was also affected by exposure to atrazine with 250 µg/l and above exhibiting no sporulation, contrasting with a control level of 58.3% (Figure 62)

Fluorescence

The effect of atrazine on the fluorescence (F_v/F_m) of *Enteromorpha intestinalis* is illustrated in figures 61 and 64. As mentioned previously, fluorescence induction ratios, F_v/F_m, are a sensitive indicator of specific damage to the photosynthetic system II of the algae and, results indicate that PS II activity is significantly ($p < 0.05$) affected by exposure to atrazine, as expected for a triazine herbicide, within 18 hours for concentrations of 250 µg/l and above and had an LOEC of 250 µg/l. However, there was not a dramatic decrease in F_v/F_m until concentrations as high as 2200 µg/l were used, when it is evident that the PS II activity of the algae was being severely inhibited. This is an extremely high concentration, far greater than found ordinarily in natural situations, but was still not high enough to calculate an EC_{50} as exposure after 96 hours resulted in a F_v/F_m value of 0.36. This is in contrast to Irgarol 1051, which had a much lower EC_{50} of 16.8 µg/l. However, figure 66 shows the Area above the fluorescence curve was significantly affected by exposure to atrazine, with concentrations of 2200 µg/l resulting in a low reading of 132 in contrast with a control value of 6018, resulting in an EC50 of 650 µg/l (figure 67). This indicates that electron transfer from the reaction centres to the quinone pool has been blocked (Bolhar-Nordenkampf, 1993) as the parameter of Area is proportional to the pool size of the electron acceptors Q_A on the reducing side of Photosystem II (Hansatech manual, 1995).

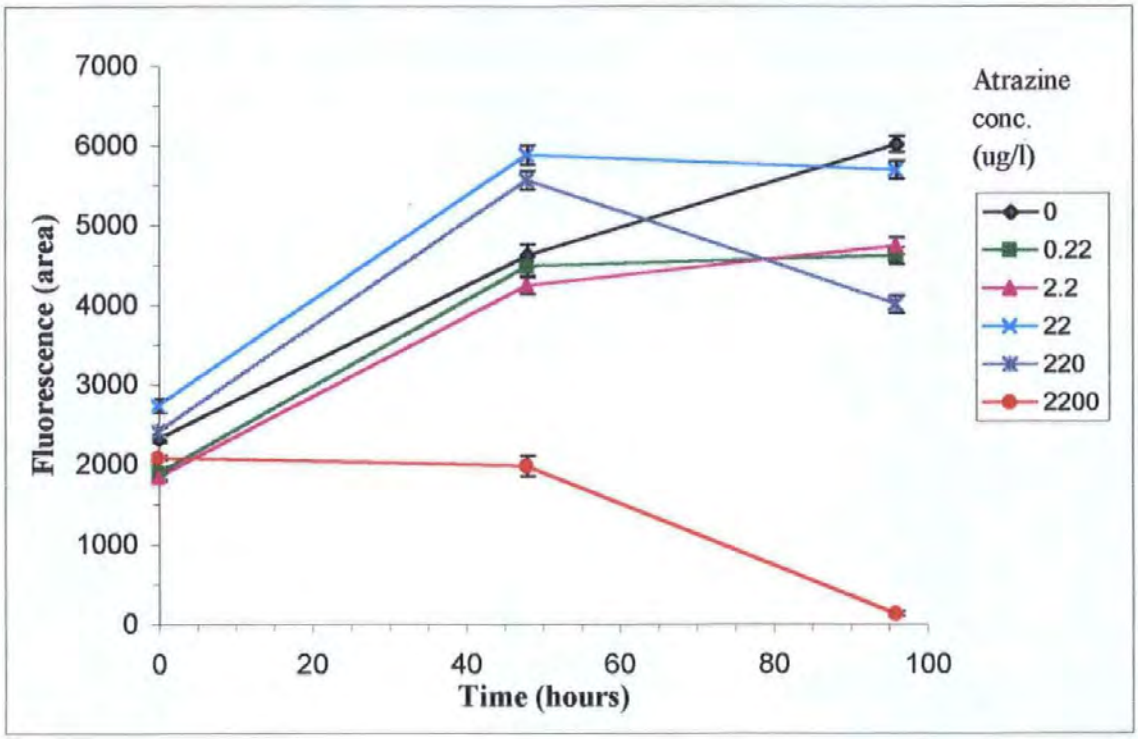

Figure 66: **Effect of atrazine (ug/l) on fluorescence (area above fluorescence curve) of *E.intestinalis***

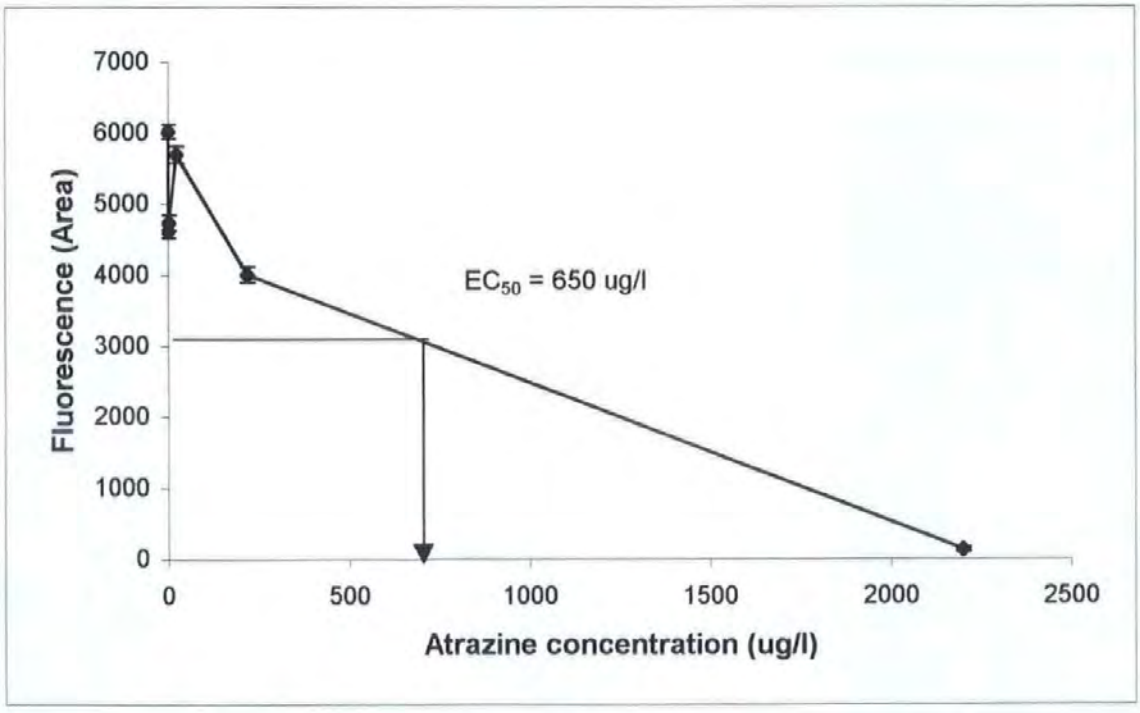

Figure 67: **Fluorescence (Area above fluorescence curve) dose response of *E.intestinalis* with exposure to atrazine (ug/l) for 96 hours**

In vivo transmittance factor

Figure 68, the effect of 7 days exposure of 1250 µg/l atrazine on transmittance factor, shows that, although there are some differences from the control, these are not as obvious as the experiments involving Irgarol 1051. There is a slight increase in transmittance factor between the wavelengths of 510 – 700 nm but there is little evidence to indicate any effect on pigments. If pigments were affected, it would be expected that transmission would be dramatically increased at the major wavelengths that chlorophyll a (440 and 680 nm) and b (470 and 650 nm) absorb light. When a plot of sensitivity (as discussed earlier) was produced there did not seem to be any particular wavelengths that were affected by atrazine exposure (figure 69) which is in contrast to the Irgarol 1051 exposure (figure 50) but supports the idea that pigments are not being directly affected in this timescale. When the difference between the treated and control transmittance factor and was plotted (figure 70), a different response was observed than with Irgarol 1051, with the regions from 320 – 700 nm being increased with atrazine exposure. This indicates that although it is having some effect, atrazine may be having a different structural effect on the algae than Irgarol 1051. The difference between Irgarol 1051 and atrazine may have something to do with Irgarol 1051 exerting more of a non-specific narcotic effect as well as a specific photosynthesis inhibitor effect. It was not possible to use the *in vivo* transmittance factor sensitivity results to plot a dose response curve for atrazine. This is in contrast to Irgarol 1051 but is interesting in that it indicates that although atrazine and Irgarol 1051 are both triazine herbicides, they exhibited different effects and so the use of *in vivo* spectral properties may possibly be used to discriminate between the herbicides.

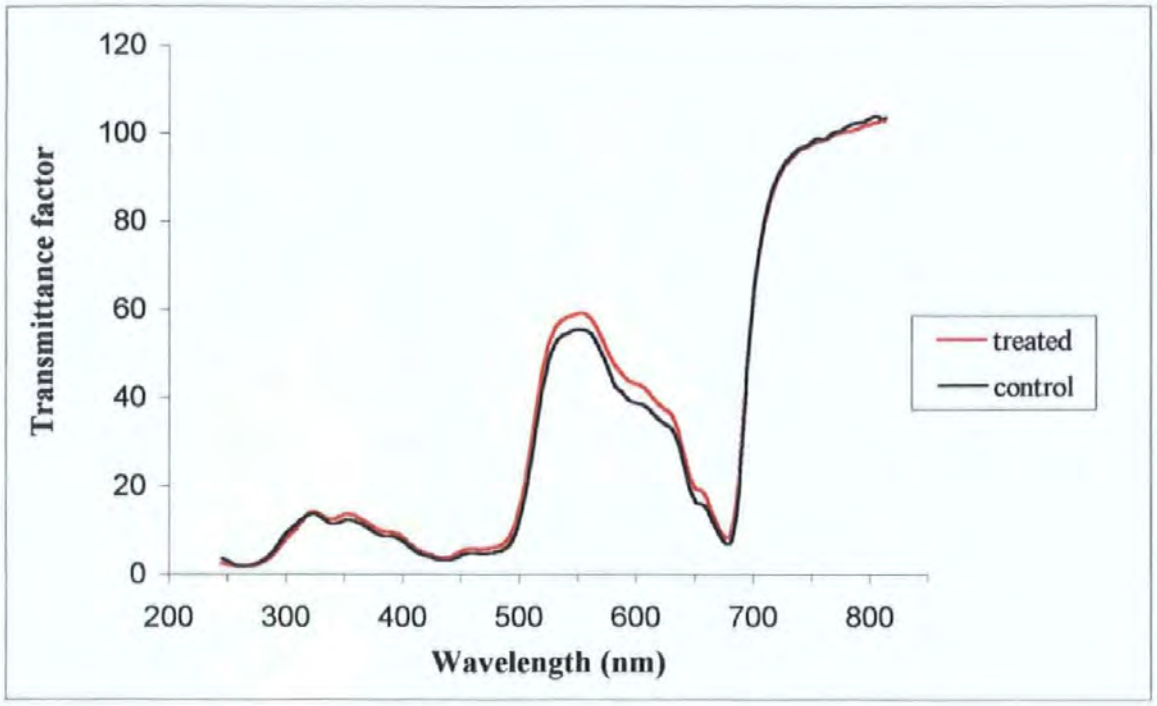

Figure 68: **Effect of atrazine (1250 ug/l) on *in vivo* transmittance factor of *E.intestinalis* after 7 days exposure**

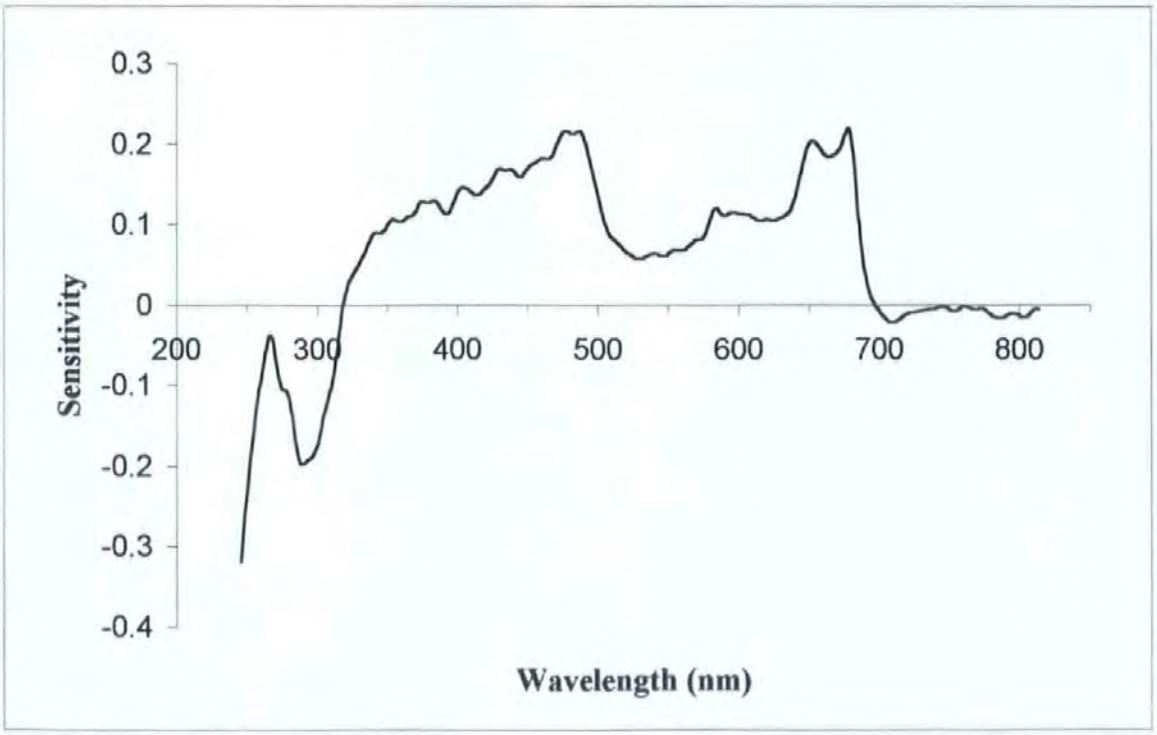

Figure 69: **Sensitivity of *in vivo* transmittance factor of *E.intestinalis to* atrazine (1250 ug/l) exposure for 7 days**

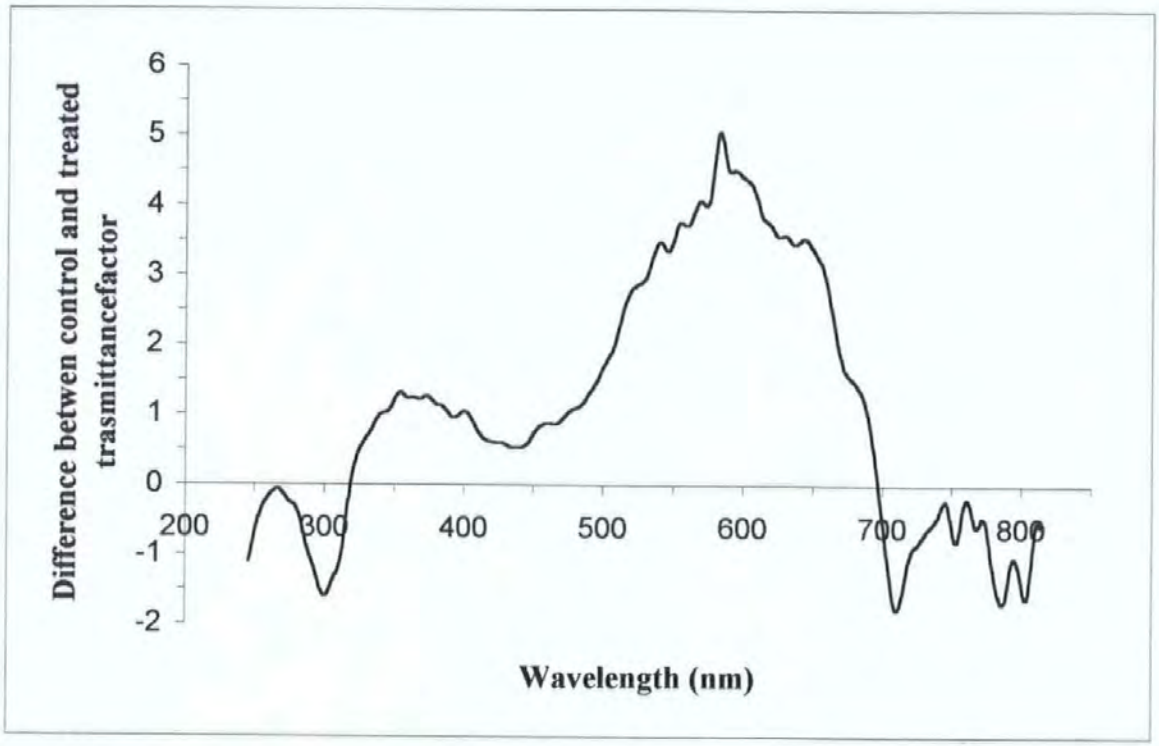

Figure70: **Difference between the control *in vivo* transmittance and *in vivo* transmittance factor of E.intestinalis when treated with 1250 ug/l atrazine for 7 days**

In vivo reflectance

Figure 71 shows the effect of treatment with 1250 µg/l atrazine on the *in vivo* reflectance of the algae after 7 days. The response was similar to that exhibited by algae exposed to Irgarol 1051 as a decrease in reflectance was observed especially in the regions of 500 – 600 nm and above 700 nm. The results again support that changes are unlikely to be due to chlorophyll directly because, as with the Irgarol 1051, there is very little effect at 680 and 440 nm. The region that appears to be dramatically lowered is above 700 nm, indicating that the changes are likely to be structural. The sensitivity graph plotted (figure 72) is extremely similar to that obtained for the sensitivity *in vivo* reflectance when *E.intestinalis* was exposed to Irgarol 1051. This could have significant advantages in that the use of in vivo spectral properties may be indicative of the pollutant the alga has been exposed to. For instance, it may be possible that a certain *in vivo* reflectance indicates a particular class of pollutant *i.*e. Herbicides that have a structural effect on the algae, but that an additional *in vivo* parameter *i.e.* transmittance factor may be able to distinguish between the pollutants. This would obviously be extremely useful as an addition to the current range of biomonitors used in ecotoxicology. The fact that the results for in vivo reflectance are similar both for atrazine and Irgarol 1051 indicates that this could be have potential for remote sensing, where they would be the most pertinent, as suitable wavelengths or ratios could be selected.

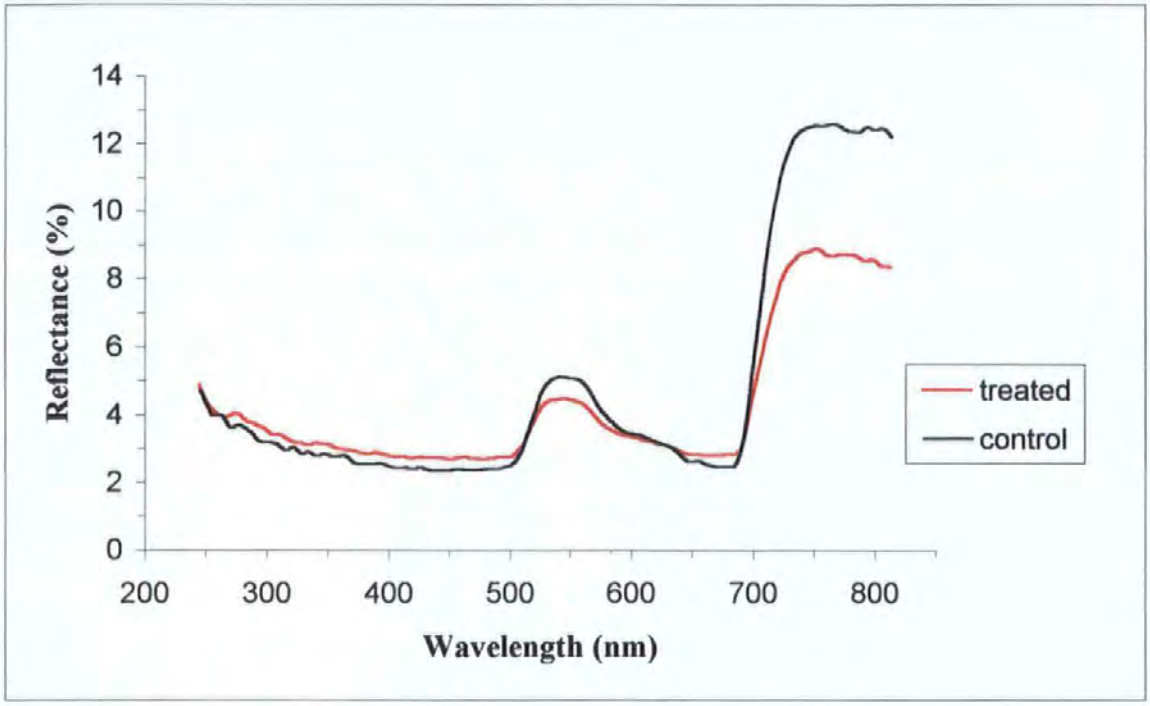

Figure 71: **Effect of atrazine (1250 ug/l) on*in vivo* reflectance of *E.intestinalis* after 7 days exposure**

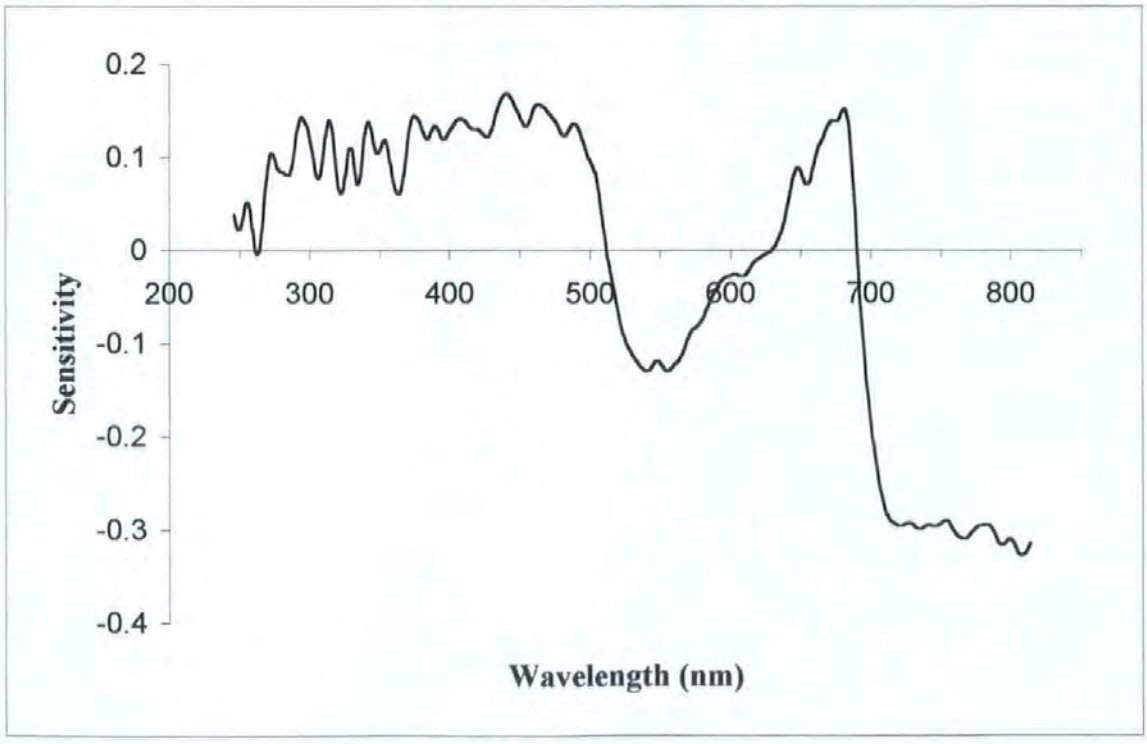

Figure 72: **Sensitivity of *in vivo* reflectance of *E.intestinalis* when exposed to atrazine (1250 ug/l) for 7 days**

5.3 IRGAROL 1051 AND COPPER COMBINED

Irgarol 1051 and copper exhibit different effects on *E.intestinalis* as illustrated in the previous experiments. However, as mentioned previously they are often used together in antifouling paints to prevent the colonisation of boat hull surfaces by algae. it was therefore decided to investigate the effects of exposing the algae to both pollutants simultaneously to investigate possible interactive effects.

5.3.1 EXPERIMENTAL DESIGN

Collection and acclimatisation of algae

E.intestinalis was collected from Wembury Bay, Southwest England, identified and acclimatised as described in the Methods section.

Preparation of solutions

The Irgarol 1051 concentration for all treatment solutions remained constant, at 7 µg/l with concentration based on the EC_{50} for growth obtained in the previous experiments using Irgarol 1051. As with the standard Irgarol 1051 experiments, a carrier control containing 100 µl ethanol was also required in addition to a control containing only Instant Ocean and nutrients (50 mg/l $NaNO_3$ and 10 mg/l Na_2HPO_4) and was used for all comparisons with treated algae.

Copper stocks were made using copper chloride in MilliQ Ultrapure water. This was added to the different treatments to give concentrations of 0, 25, 50, 100, 200, 500 µg/l copper. All the glassware used was acid washed before the experiment to ensure there was no metal contamination and it must be remembered that concentrations were nominal.

The different combined copper and Irgarol treatments used, in addition to controls, were, therefore, as follows:

Sol. A	Sol. B	Sol. C	Sol. D	Sol. E	Sol. F	
7	7	7	7	7	7	µg Irgarol/l
0	25	50	100	200	500	µg copper/l

Experimental setup

Intercalary sections (2.5 cm long) of *E. intestinalis* were cut from healthy fronds and placed in tri-divided petri dishes, each containing 50 ml of the relevant treatment solution (as described in the Methods section).

Parameters measured

Growth was measured on day 7 and was recorded as Relative Growth Rate (RGR). Fertility, a secondary observation, was recorded as the percentage of sections that sporulated during the experiment. As discussed earlier, sections that sporulated were removed from the experiment completely.

Fluorescence measurements (F_v/F_m) were recorded at 0, 38, 91 and 168 hours.

The in vivo spectral properties were taken at 0 hours and 168 hours (7 days) using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with the Labsphere RSA-UC-40 integrating sphere.

5.3.2 RESULTS AND DISCUSSION

Growth

Figure 73 shows the effect of the combined Irgarol 1051 and copper solutions on the growth of the algae. It is evident from this that Irgarol 1051 is having the most damaging effect as the growth of treatment A (7 µg/l Irgarol 1051 with no copper) is as seriously

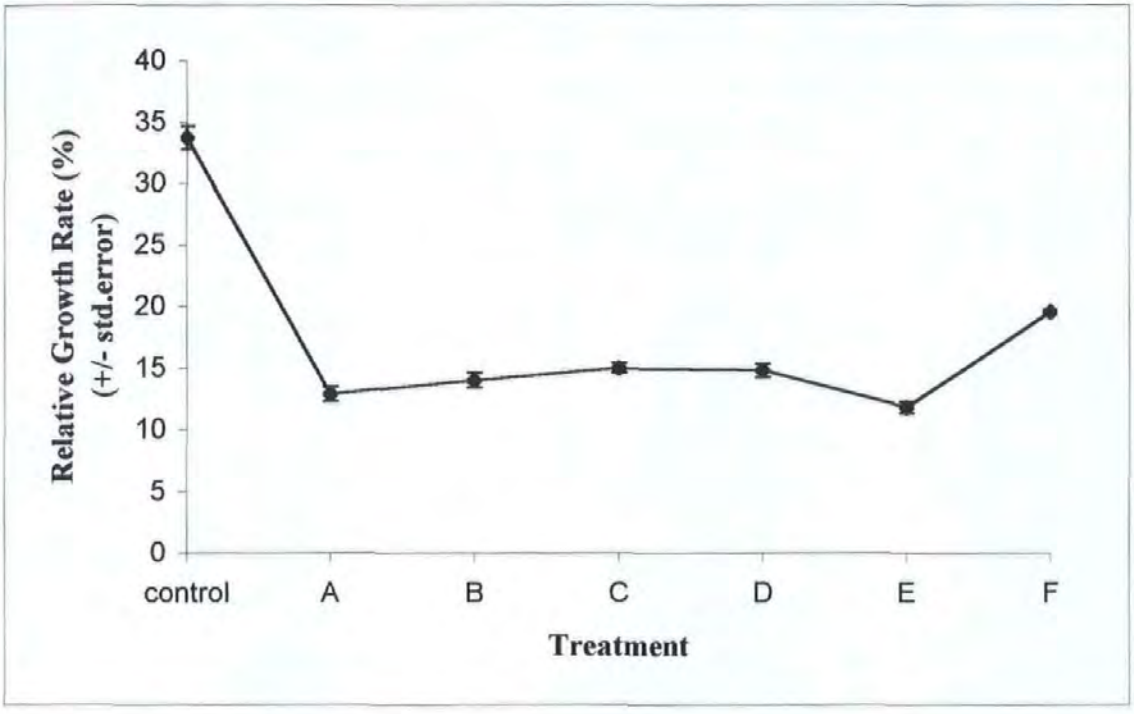

Figure 73: **Effect of copper and Irgarol 1051 combined on relative growth rate of *E.intestinalis* after 7 days exposure**

	Treatment						
	Control	**A**	**B**	**C**	**D**	**E**	**F**
Copper (ug/l)	0	0	25	50	100	200	500
Irgarol (ug/l)	0	7	7	7	7	7	7

Table 7: **Concentration of copper (ug/l) and Irgarol 1051 (ug/l) in the combined copper and Irgarol 1051 treatments**

affected as the solutions containing the Irgarol 1051 with the higher levels of copper. However, treatment F (7 µg/l Irgarol 1051 with 500 µg/l copper) still exhibited considerable growth which contrasts with the original copper experiment where treatment with 500 µg/l copper resulted in zero growth.

Fluorescence

Figure 74 illustrates the effect of the combined Irgarol 1051 with copper solutions on the fluorescence induction ratio (F_V/F_m) of the algae. As with the growth, there is an obviously significant effect ($p < 0.05$) for all treatments, which appears to be primarily due to the Irgarol 1051 exposure. This effect was noticeable after 38 hours and indicates, as discussed earlier, that some inhibition of PS II is occurring, indicating Irgarol 1051 is therefore having a serious detrimental effect on PS II but the algae is able to survive. However, it is interesting that the copper also had an effect on the fluorescence induction ratio (F_V/F_m) as the values drop as the copper concentration increases indicating a possible additive effect. The algae is, however, able to carry on photosynthesising even with the additive effect of copper and so must be tolerating the copper by either excluding its uptake, binding or detoxifying the copper ions.

Fertility

As with previous experiments, the control exhibited the highest levels of sporulation with treatments C, D and F resulting in no sporulation at all. This indicates that it is the high levels of copper that is preventing sporulation, as treatments A and B (zero and 25 µg/l copper respectively together with 7 µg/l Irgarol 1051) still exhibited a relatively high proportion of sections that sporulated (25 % compared with a control value of 42 %).

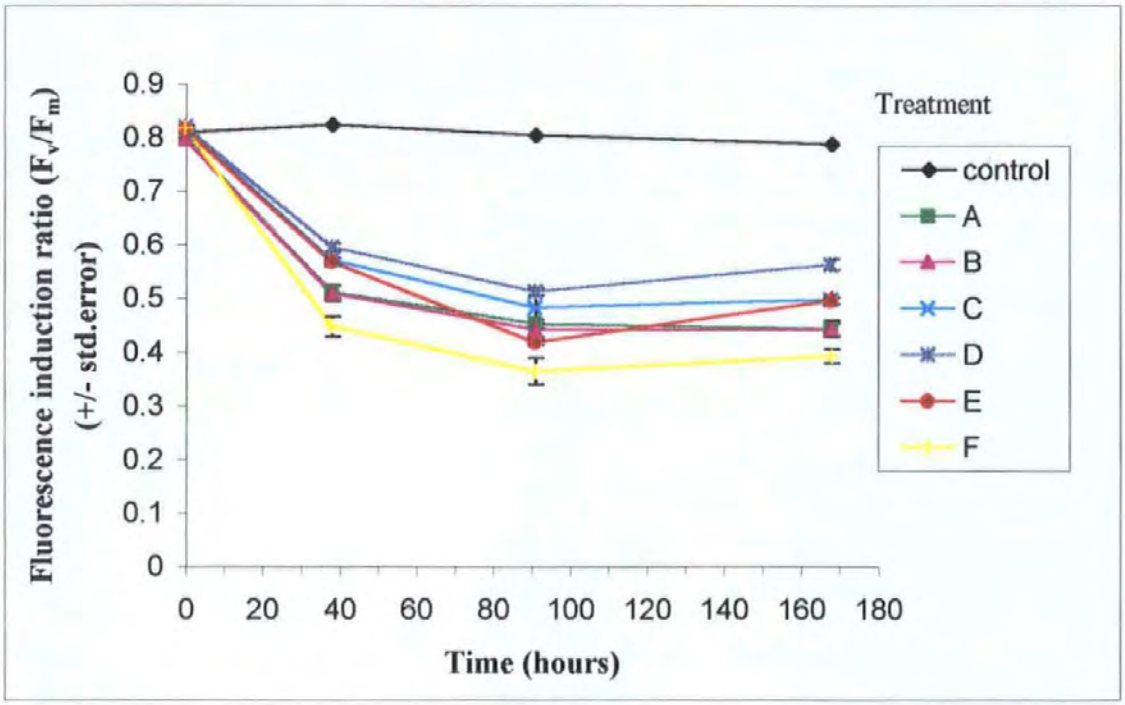

Figure 74: **Effect of combined copper and Irgarol 1051 on fluorescence induction ratio (F_v/F_m) of *E.intestinalis***

In vivo transmittance factor

It is evident from figure 75 that the treatments had an effect on the *in vivo* transmittance factor, with some treatments exerting a different effect than others. Although there is some increase in the *in vivo* transmittance factor across the majority of the spectra for all treatments, the treatment that appears to be having the most obvious effect is 'F' containing 7 µg/l Irgarol 1051 and 500 µg/l copper. When a graph illustrating the difference between treated and control sections was plotted, Figure 76, this treatment, although containing both Irgarol 1051 and copper, is very similar to the in vivo transmittance results obtained from the experiments involving copper alone. This was confirmed by the production of a sensitivity plot (Figure 77), which shows that the most sensitive wavelengths in the regions of chlorophyll absorption (440 nm and 680 nm). This indicates that the high concentrations of copper are breaking down or affecting chlorophyll turnover in some way. These results are extremely promising as they indicate that it may be possible to identify specific pollutant signatures even from algae that has been exposed to a variety of pollutants.

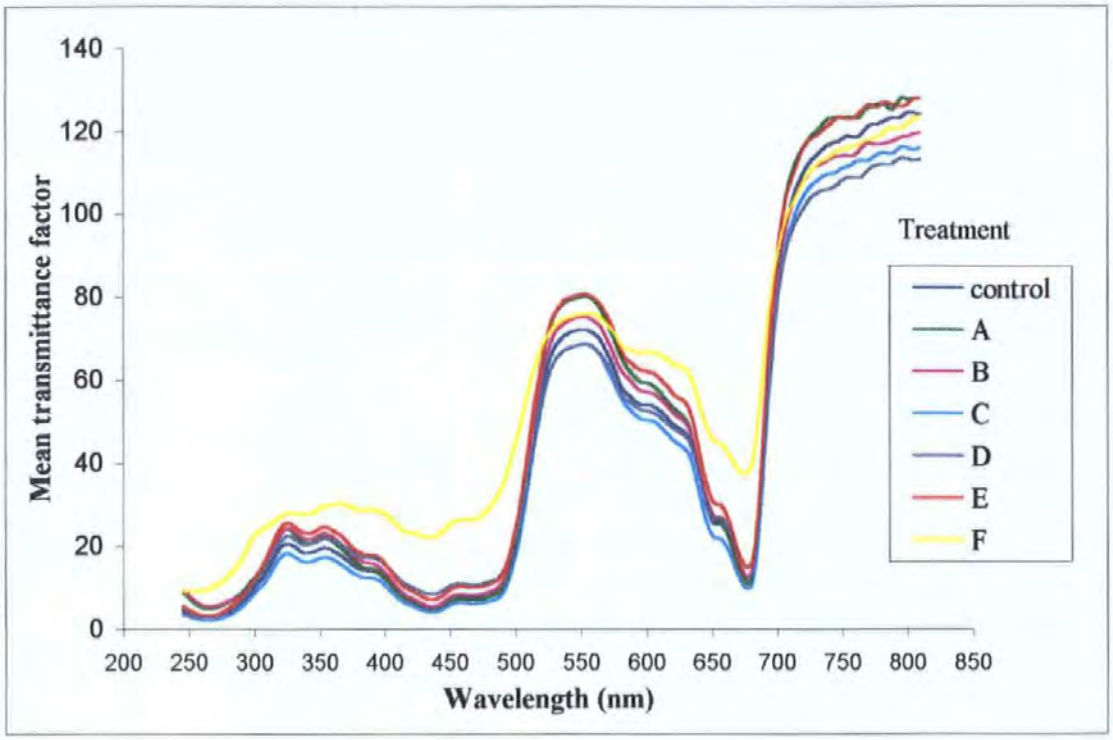

Figure 75: **Effect of Irgarol 1051 and copper combined on *in vivo* transmittance factor of *E.intestinalis* after 7 days exposure**

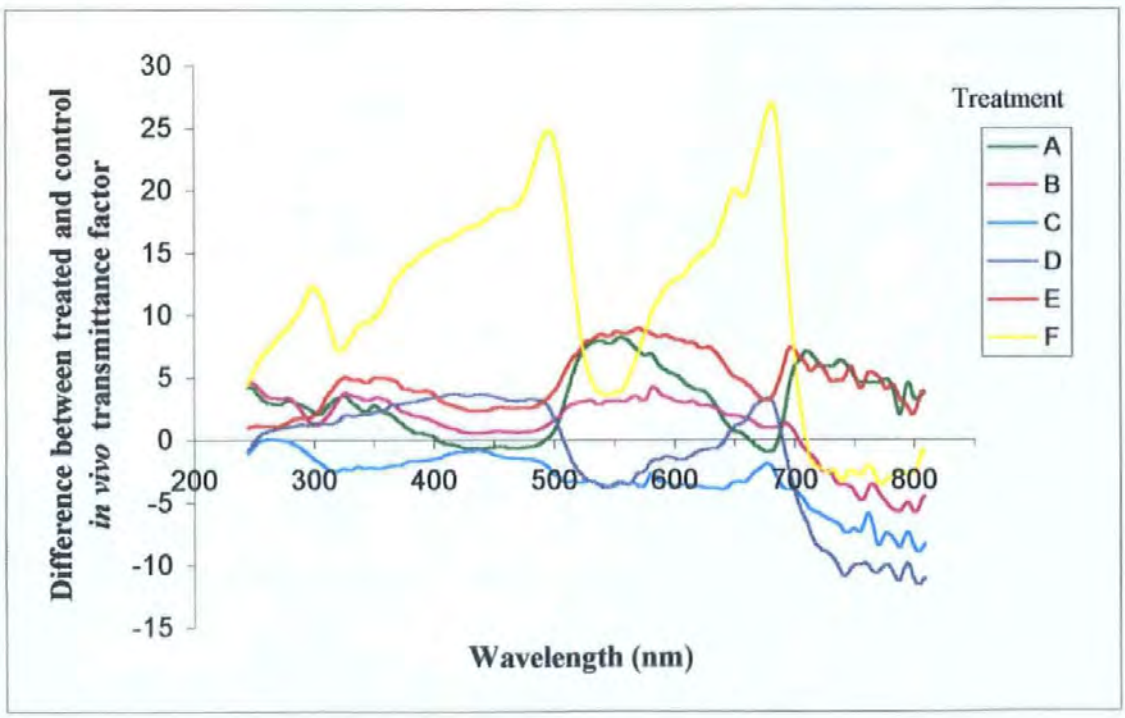

Figure 76: **Difference between treated and control *in vivo* transmittance factor of *E.intestinalis* after 7 days exposure**

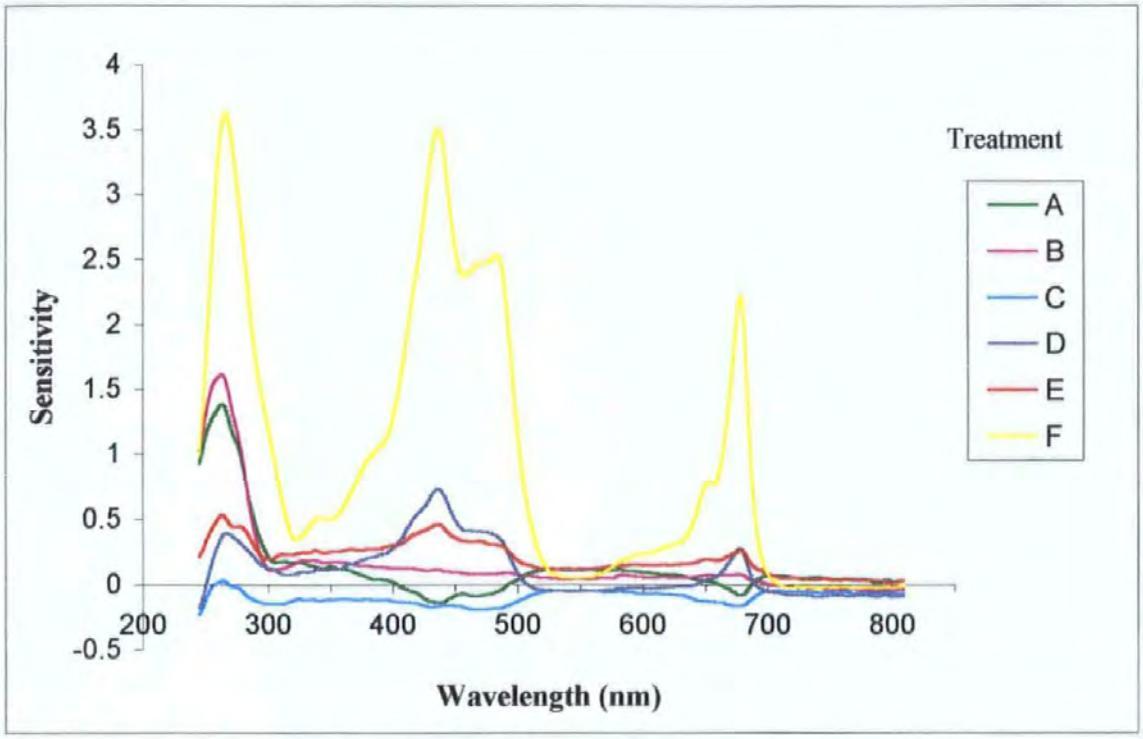

Figure 77: **Sensitivity of *in vivo* transmittance factor of *E.intestinalis* to combined copper and Irgarol 1051 exposure for 7 days**

***In vivo* reflectance**

Figure 78 illustrates the effect of the combined Irgarol 1051 and copper treatments on the *in vivo* reflectance of *E.intestinalis* and it can clearly be seen that there is an effect on *in vivo* reflectance for all treatments. The figure showing the difference, figure 79, between the treated and control values indicate that the signature appears to be most like that which Irgarol 1051 exhibited during the individual experiments, with a general lowering of in vivo reflectance especially at 550 nm and above 700 nm. This could, as discussed previously, indicate some sort of structural effects. However, when a plot of sensitivity was calculated, figure 80, it appears that copper is also exerting an effect on the *in vivo* reflectance at the higher concentrations. Although treatments E and F (200 µg/l and 500 µg/l copper respectively, together with 7 µg/l Irgarol 1051) appear to exhibit the same wavelengths of sensitivity as Irgarol 1051 exposure, the sensitivity spectra have been raised which corresponds with copper exposure.

This combination experiment involving Irgarol 1051 and copper indicates that the use of the *in vivo* spectral properties has great potential as it is able to 'detect' the structural changes exerted by Irgarol 1051 and the pigment effects caused by copper exposure. This would obviously be a huge advantage in a biomonitoring situation as not only could exposure to some form of pollutant be ascertained, it may also be possible to identify the actual pollutant or class of pollutant by using a combination of different *in vivo* spectral property calculations.

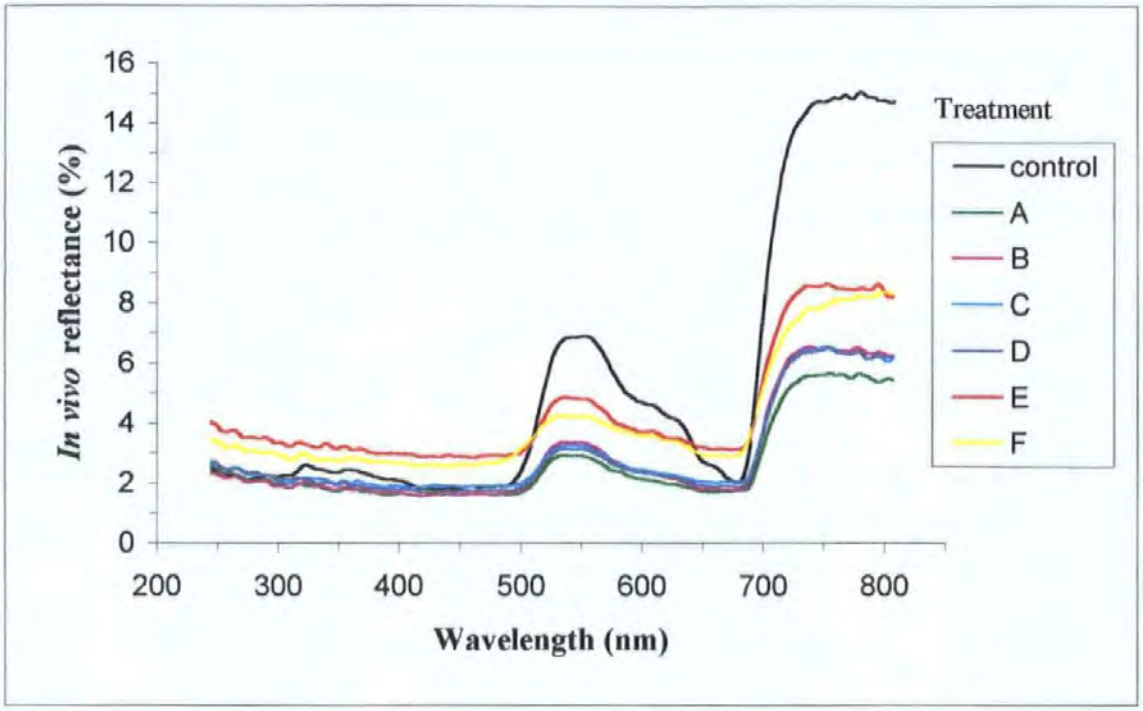

Figure 78: Effect of irgarol 1051 and copper combined on *in vivo* reflectance of *E.intestinalis* after 7 days

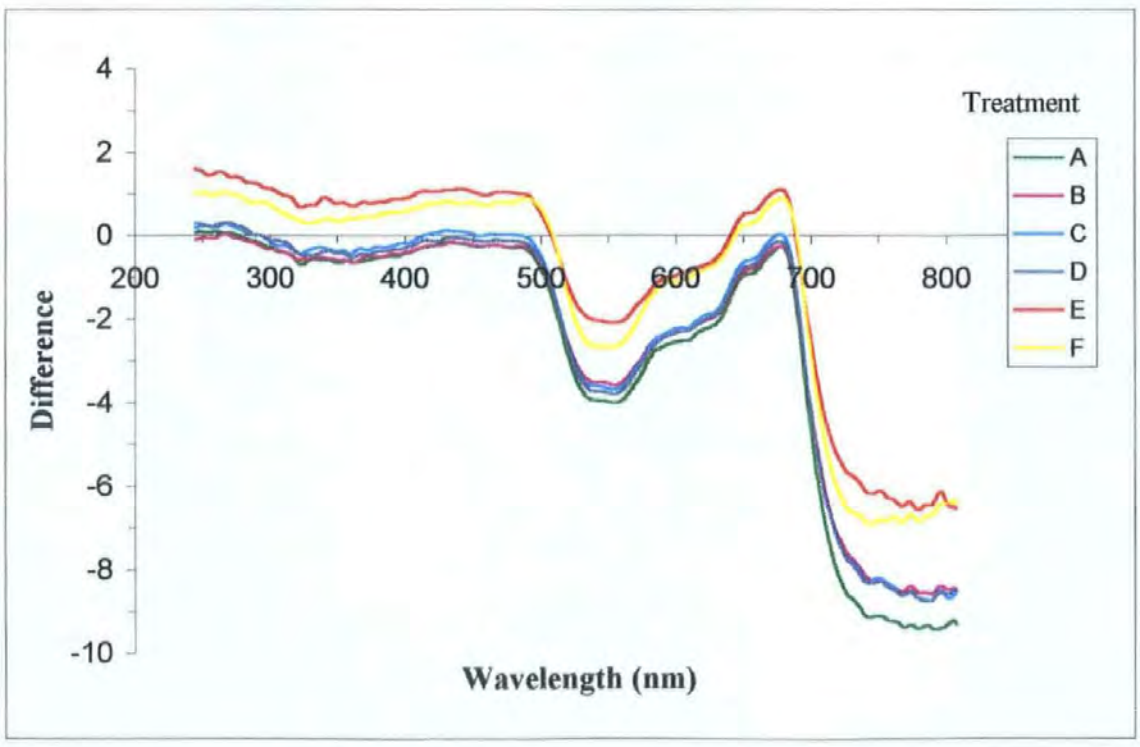

Figure 79: Difference between treated and control *in vivo* reflectance of *E.intestinalis* after 7 days exposure to combined copper and Irgarol 1051

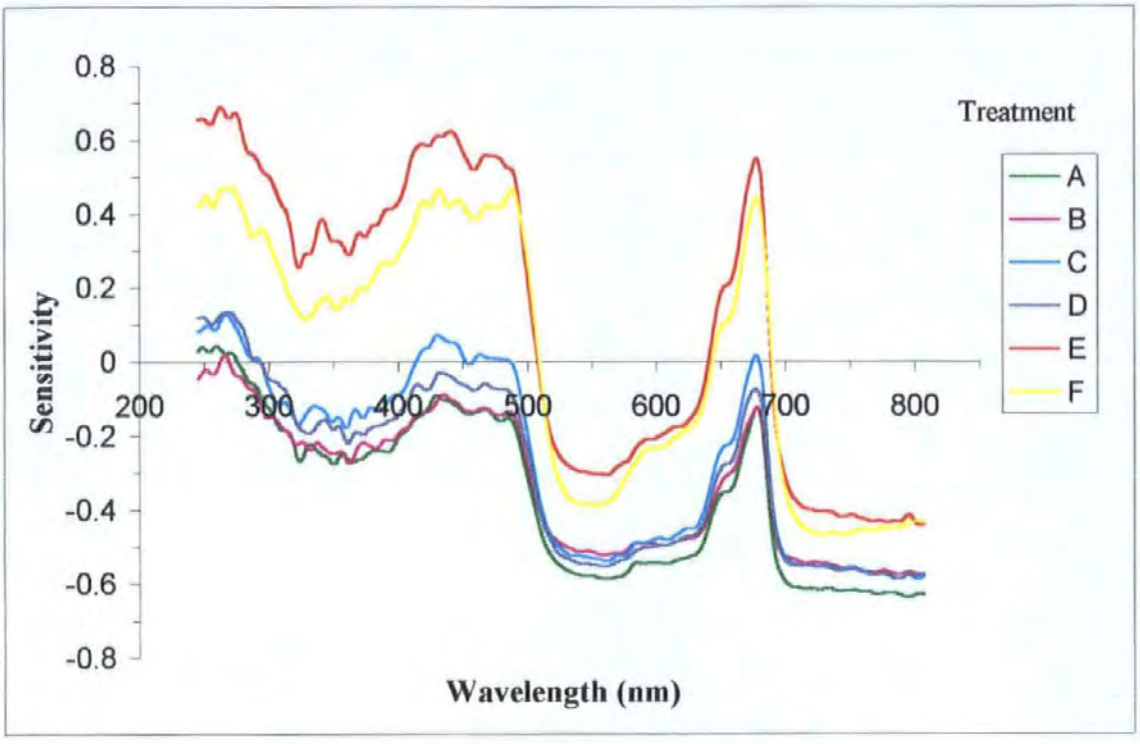

Figure 80: Sensitivity of *in vivo* reflectance of *E.intestinalis* exposed to combined copper and irgarol 1051 for 7 days

6. ALCOHOLS

Although macroalgae have been used frequently to monitor trace metals they have been rarely used to study the abundance of trace organic contaminants in aquatic systems. It is possible that this could be due to their low lipid contents which may reduce their bioaccumulation capacity of hydrophobic compounds and their potential to metabolise organic pollutants (Phillips, 1994). However, there can be high levels of hydrocarbons dissolved in seawater and levels of a few micrograms per litre offshore and higher values nearshore have been reported (Law, 1981). Polynuclear aromatic hydrocarbons have now been monitored for several years in eastern Mediterranean seaweeds (Yilmaz *et al*, 1998) and there is increasing concern about hydrocarbon pollutants. The organic toxicants used in this study were a homologous series of alcohols ranging from C1 to C9 which are considered to be representative of the many hydrophobic industrial chemicals that are important environmentally and are non-polar narcotic toxicants (Donkin, 1994).

The use of a homologous series of organic contaminants enables Quantitative Structure Activity Relationships (QSARs) to be used. These can predict the toxicological behaviour of a chemical depending on its structural properties and physiochemical properties. Different compounds in the group, depending on their environmental and toxicological behaviour, can be shown to change in a systematic and predictable way in relation to these features, and mathematical relationships can often be fitted (Donkin, 1994). These Structure Activity Relationships are now frequently used in the study of organic chemicals in the environment. As well as the structure of the chemical, which allow them to be grouped, it is also important to know its behaviour. This information can then be used to show that the environment and toxicological behaviour of a chemical changes in a systematic and predictable way in relation to these properties. This often results in the

possibility of fitting precise mathematical relationships to determine toxicity, otherwise known as QSARs.

The QSARs plotted in this study were based on carbon number, structure, and octanol/water partition coefficient (Log K_{OW}), behaviour of the molecule. The octanol/water partition coefficient is now universally accepted as a standard method (Dearden, 1985) as hydrophobicity is obviously one of the most important molecular characteristics in determining the behaviour of organic chemicals in the marine environment.

6.1 EXPERIMENTAL DESIGN

Collection and acclimatisation of the algae

E.intestinalis was collected from Wembury Bay, washed and sorted and acclimated in the laboratory as described in the Methods section.

Preparation of solutions

A homologous series of alcohols were set up from C1 (methanol) to C9 (nonanol). The alcohols ranged from 95 – 99.8 % purity and were added to Instant Ocean containing nutrients to result in the following concentrations:

Methanol (BDH) - 0, 50, 100, 150 g/l

Ethanol (Rathburn) - 0, 10, 25, 50, 75, 100 g/l

Propan-1-ol (BDH) - 0, 5, 10, 15, 25, 50 g/l

Butan-1-ol (SIGMA) - 0, 2, 5, 9, 14, 20 g/

Pentan-1-ol (BDH) - 0, 0.66, 1.32, 3.29, 5.26, 7.89 g/l

Hexan-1-ol (BDH) - 0, 0.4, 0.8, 1.2, 1.6, 2.0 g/l

Heptan-1-ol (BDH) - 0, 0.1, 0.2, 0.3, 0.45, 0.6 g/l

Octan-1-ol (SIGMA) - 0, 5, 25, 50, 100, 200 mg/l

Nonanol - 0, 5, 25, 50, 100, 200 mg/l

The alcohol concentrations were chosen after several trial runs and consideration of solubility of each alcohol was also taken into account. The solubility of each alcohol in freshwater at 25 °C (Schultz *et al.* 1990) was halved to estimate the solubility at 33 ppt salinity and a temperature of 15 °C and corresponded with maximum concentrations used by Schild 1996. The alcohol was added to the Instant Ocean containing nutrients in a vortex and the solutions were stirred for a minimum of 4 hours to ensure the alcohol dissolved. When large quantities of the lower carbon alcohols, especially methanol, were added, corrections were made to the salinity of the Instant Ocean.

Experimental setup

Intercalary sections (25 mm long) of *E.intestinalis* were cut from healthy fronds and placed in 400 ml screwtop jars, each containing 300ml treatment solution as described in the Methods section.

Parameters measured

Fluorescence measurements for the complete alcohol series were recorded before exposure (0 hours) and at 96 hours.

The *in vivo* spectral properties of the algae exposed to the alcohols C1 (methanol) to C8 (octan-1o-l) were measured before exposure (0 hours) and at 96 hours using the Pye Unicam SP8-100 UV/Vis spectrophotometer with diffuse reflectance accessory 790824.

6.2 RESULTS AND DISCUSSION

Fluorescence

In addition to the *in vivo* spectral properties, fluorescence measurements were also recorded to assess the health status of the algae. These results had a similar pattern for each of the alcohols and these can be seen in figures 81-86, plots of the fluorescence

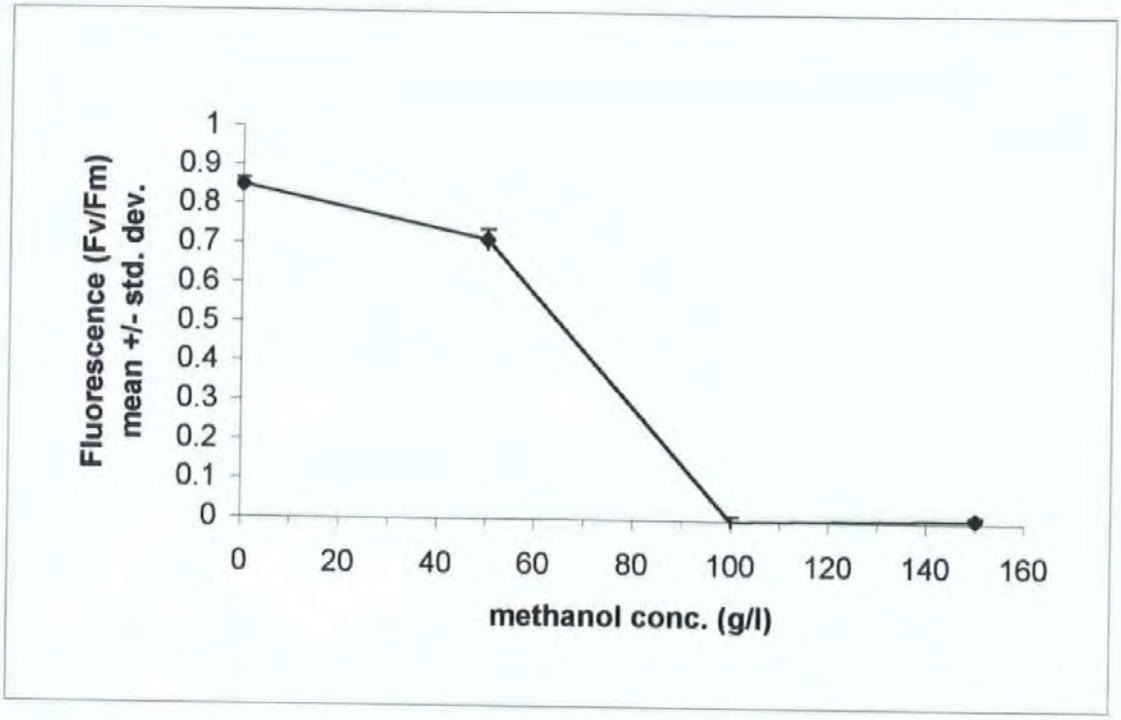

Figure 81: **Effect of methanol (g/l) on fluorescence induction ratio (Fv/Fm) of *E.intestinalis* after 96 hours exposure**

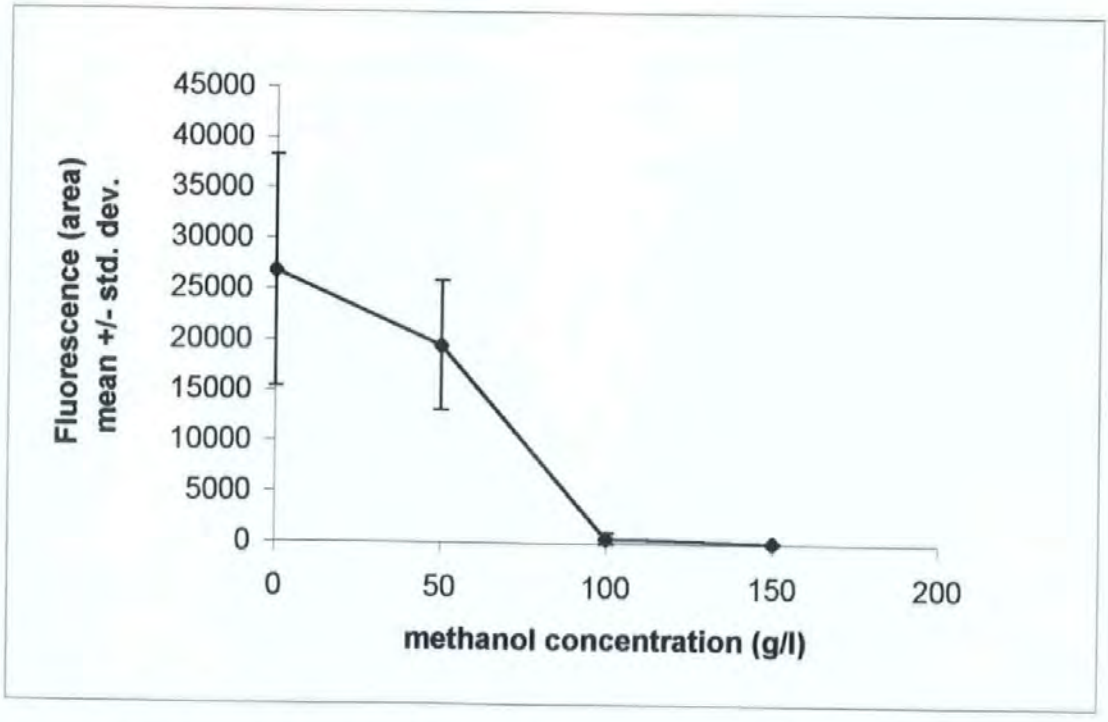

Figure 82: **Effect of methanol (g/l) on fluorescence (area above fluorescence curve) of *E.intestinalis* after 96 hours exposure**

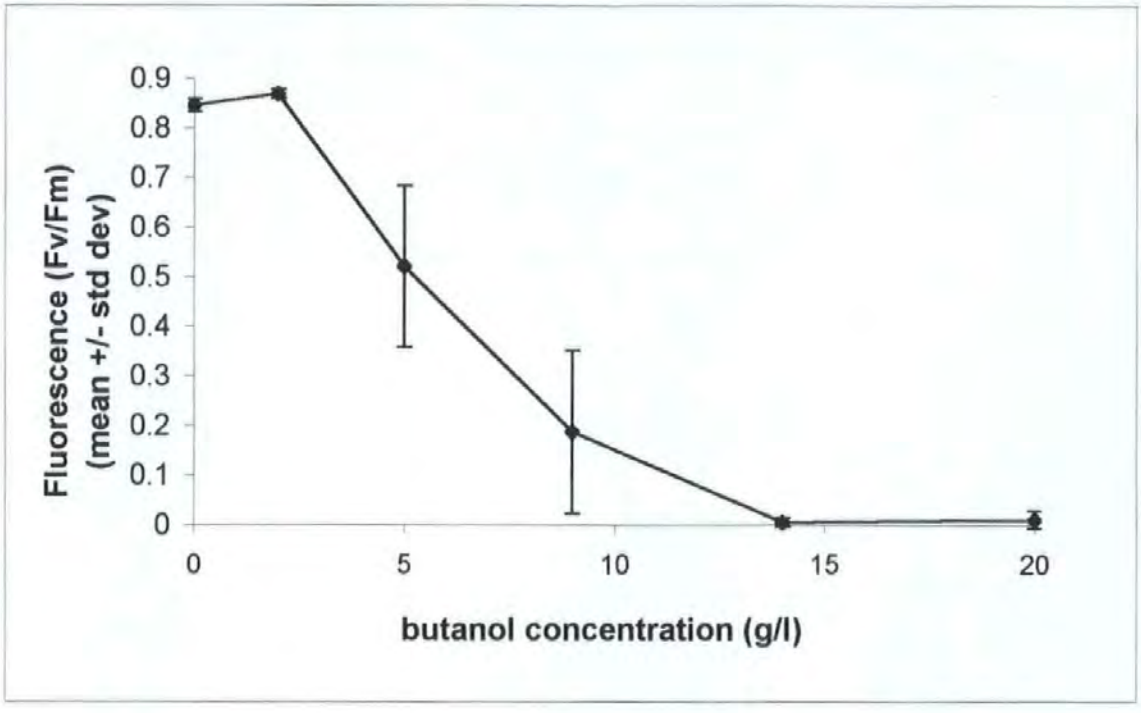

Figure 83: Effect of butanol (g/l) on fluorescence induction ratio (Fv/Fm) of *E.intestinalis* after 96 hours exposure

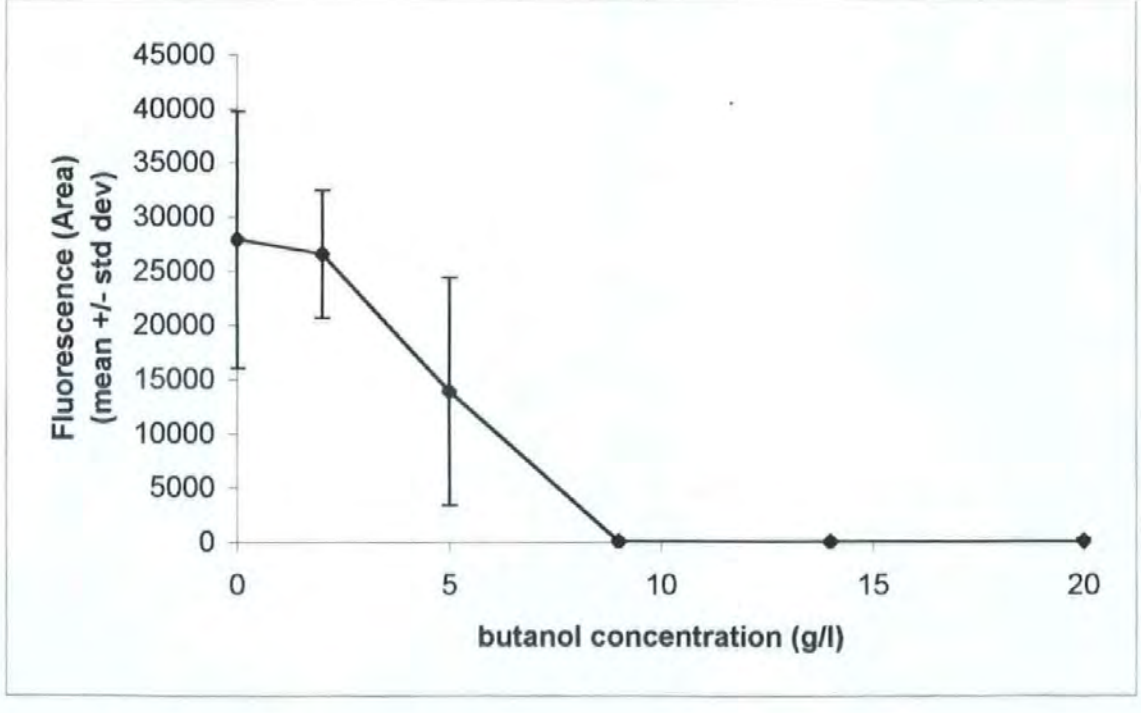

Figure 84: Effect of butanol (g/l) on fluorescence (area above fluorescence curve) of *E.intestinalis* after 96 hours exposure

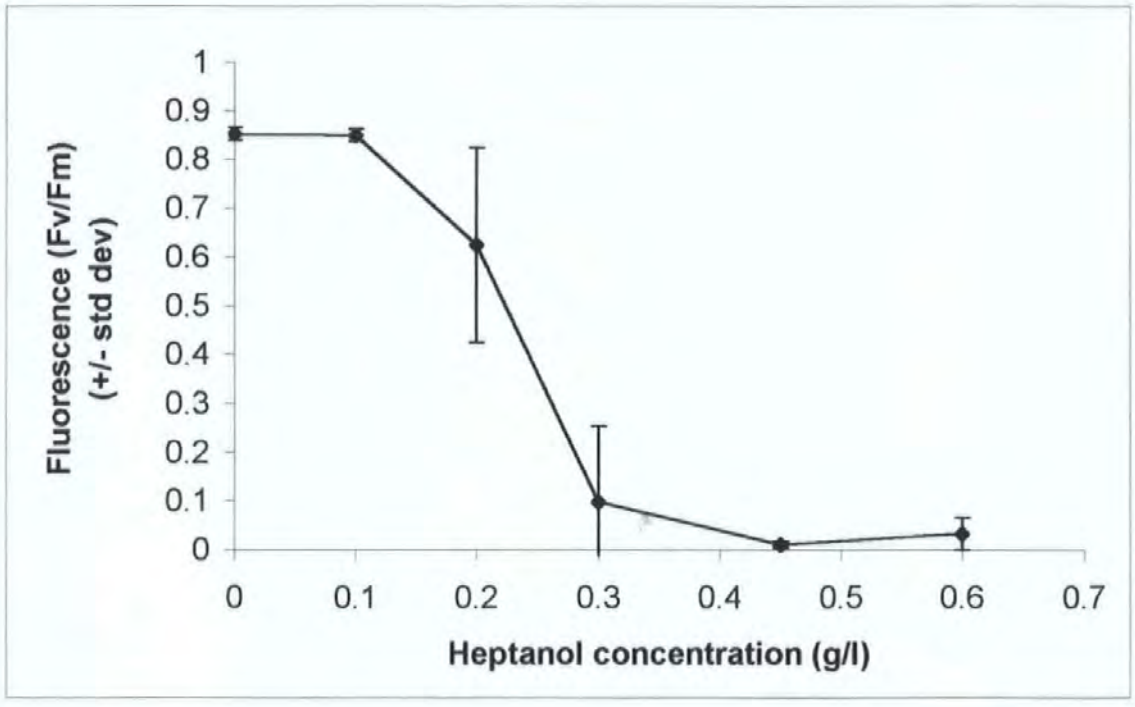

Figure 85: **Effect of heptanol (g/l) on fluorescence induction ratio (Fv/Fm) of *E.intestinalis* after 96 hours exposure**

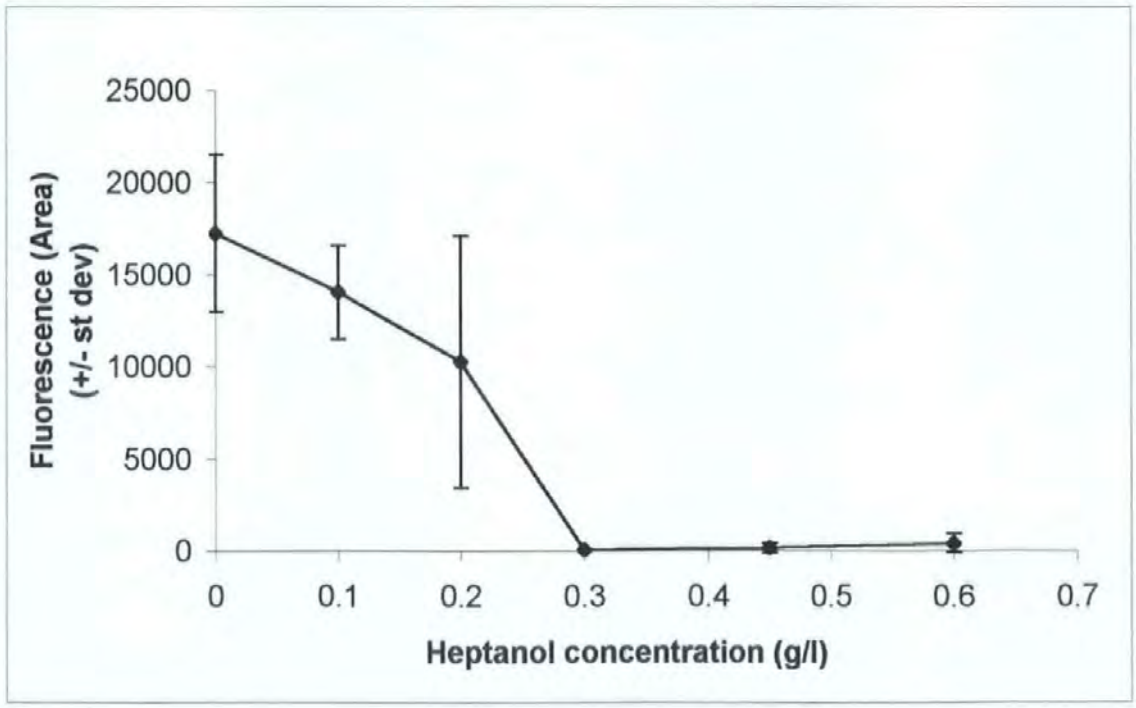

Figure 86: **Effect of heptanol (g/l) on fluorescence (area above fluorescence curve) of *E.intestinalis* after 96 hours exposure**

induction ratio (F_v/F_m) and the size of the area above the fluorescence curve between F_0 and F_m. For both fluorescence measurements, there is sudden drop with concentration, which ensures that minimal errors will be encountered when calculating EC_{50} values. The results were highly significant for all alcohols ($P < 0.05$) with resulting LOECs, using a 95 % confidence limit, as follows: methanol (50 g/l), butanol (5 g/l), and octanol (25 µg/l).

In vivo spectral properties

The effects of the alcohols on the *in vivo* spectral properties of *E.intestinalis* exhibited a similar pattern across the complete range of alcohols used. This is demonstrated in figures 87 - 104 which were selected to show that the same effect occurs as the carbon number of the alcohol increases. This suggests that the series of alcohols used all have the same mode of action on *E.intestinalis*.

In vivo transmittance

The *in vivo* transmittance results (figures 87, 93 and 99) all show a gradual increase in the transmittance of the *E.intestinalis* as the alcohol concentration increases, resulting in spectra that have a very high transmittance across the entire wavelength range at the highest alcohol concentrations. The spectra produced in response to alcohol exposure exhibit an overall increase across the wavelengths for *in vivo* transmittance but they also show sensitivity at wavelengths associated with pigments, in particular chlorophylls. This indicates that there is a combination of effects occurring, both on structure of the algae and pigment content, which is a result that would be expected from a non-specific hydrocarbon.

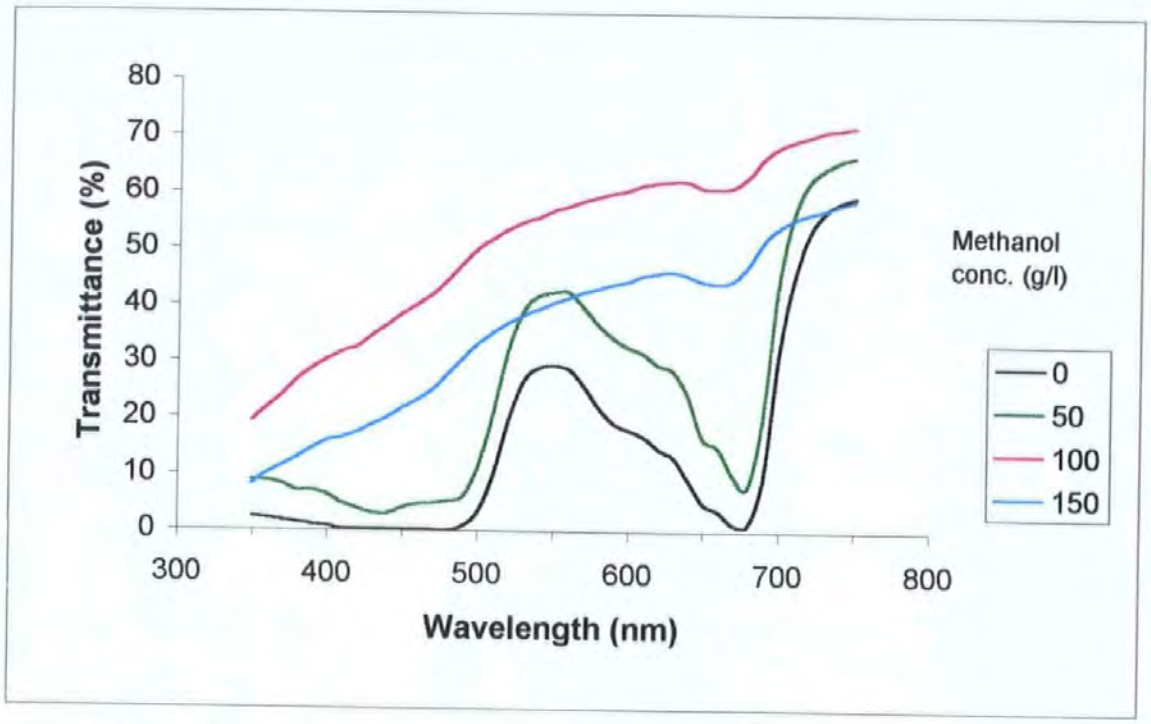

Figure 87: **Effect of methanol (g/l) on *in vivo* transmittance of *E.intestinalis* after 96 hours exposure**

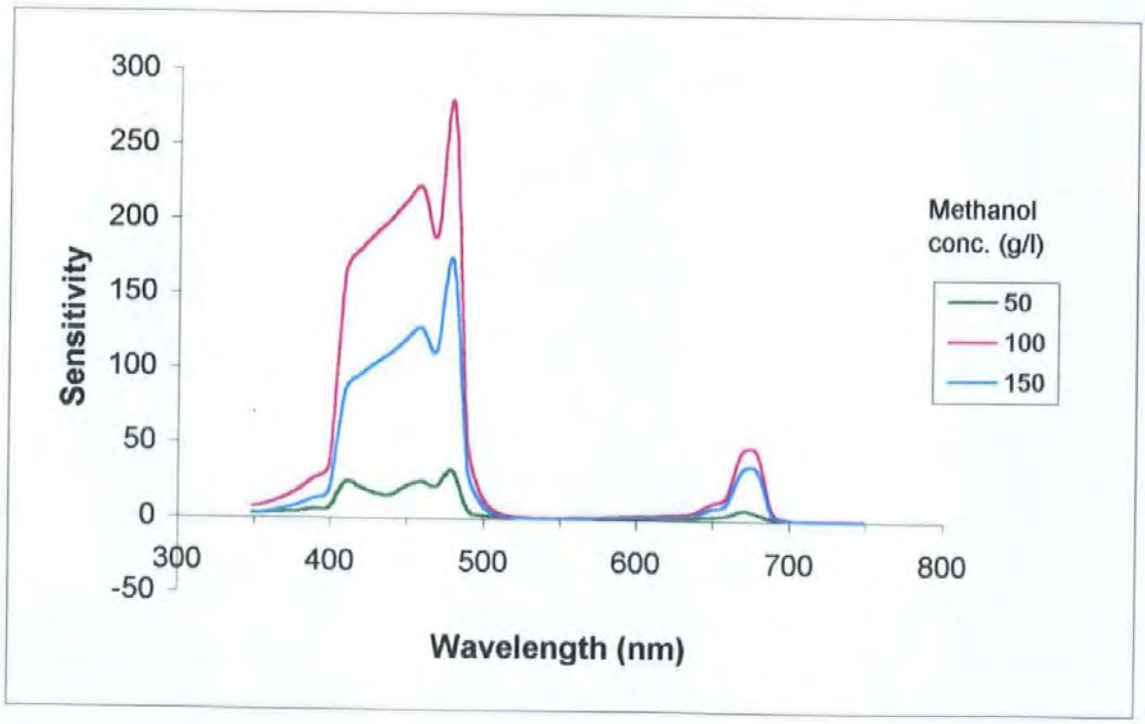

Figure 88: **Sensitivity of *in vivo* transmittance of *E.intestinalis* to methanol (g/l) exposure for 96 hours**

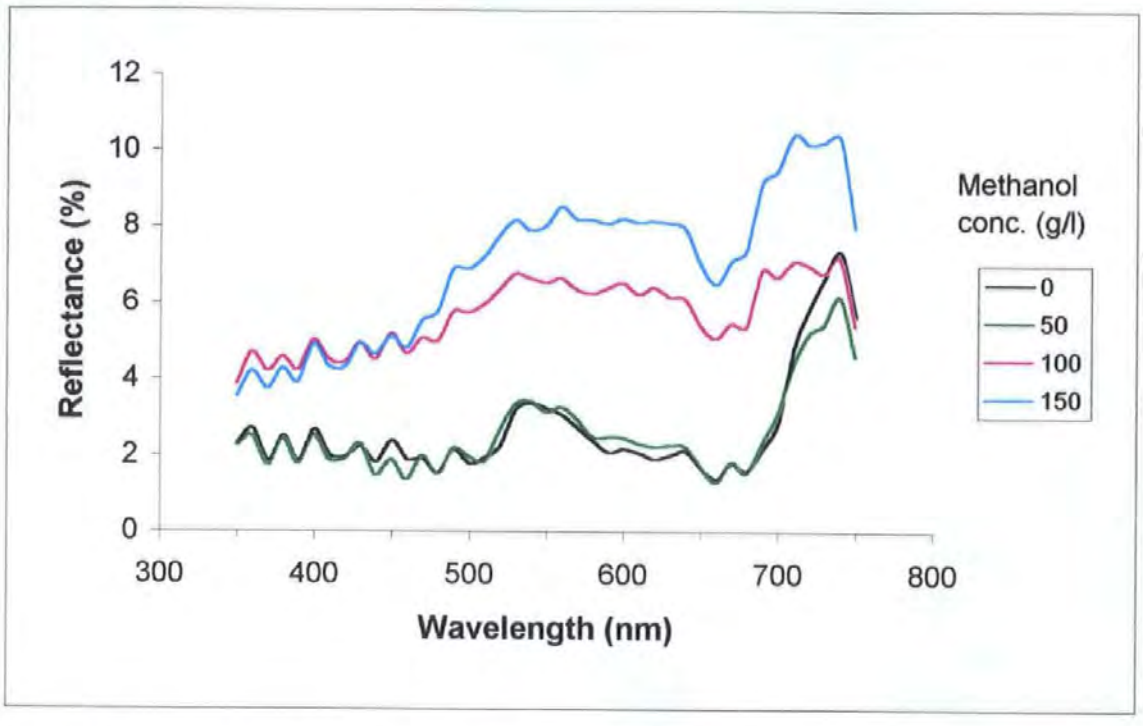

Figure 89: **Effect of methanol (g/l) on *in vivo* reflectance of *E.intestinalis* after 96 hours exposure**

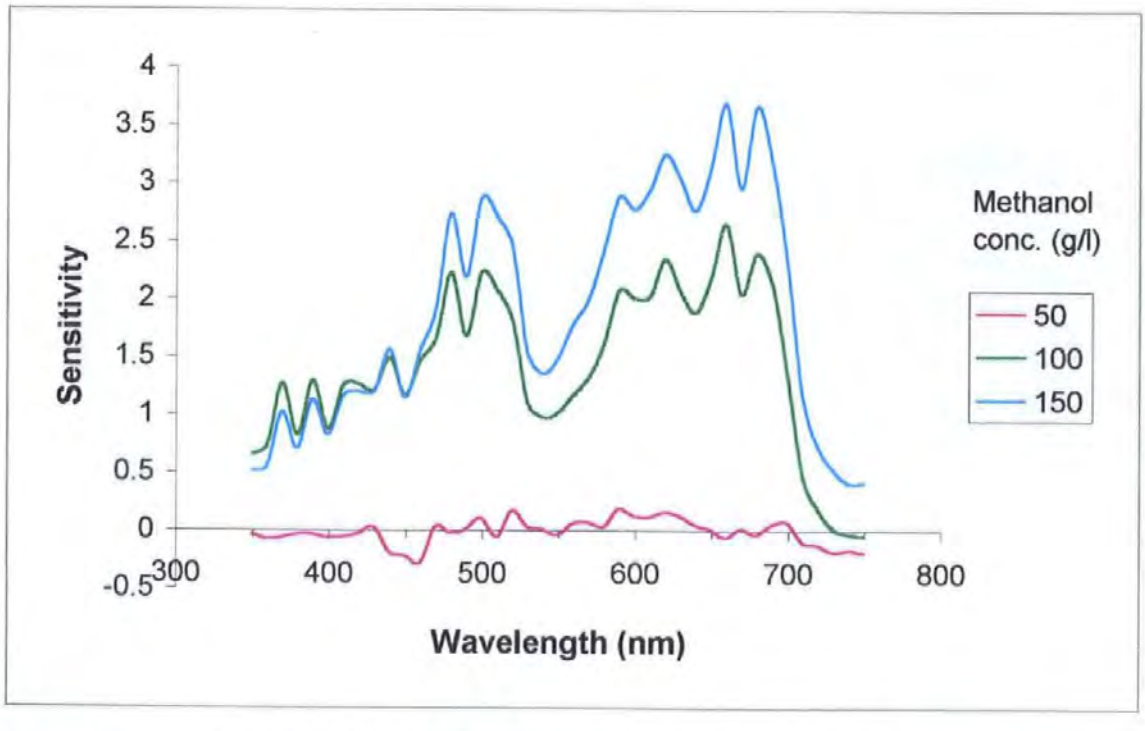

Figure 90: **Sensitivity of *in vivo* reflectance of *E.intestinalis* to methanol (g/l) exposure for 96 hours**

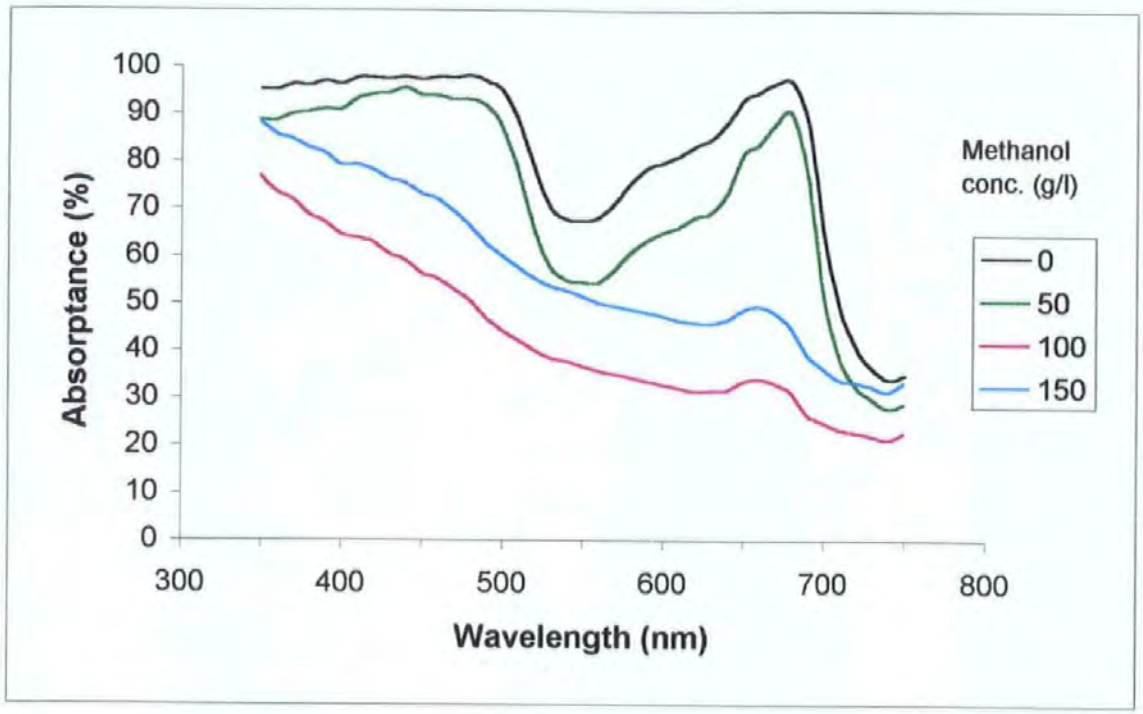

Figure 91: **Effect of methanol (g/l) on *in vivo* absorptance of *E.intestinalis* after 96 hours exposure**

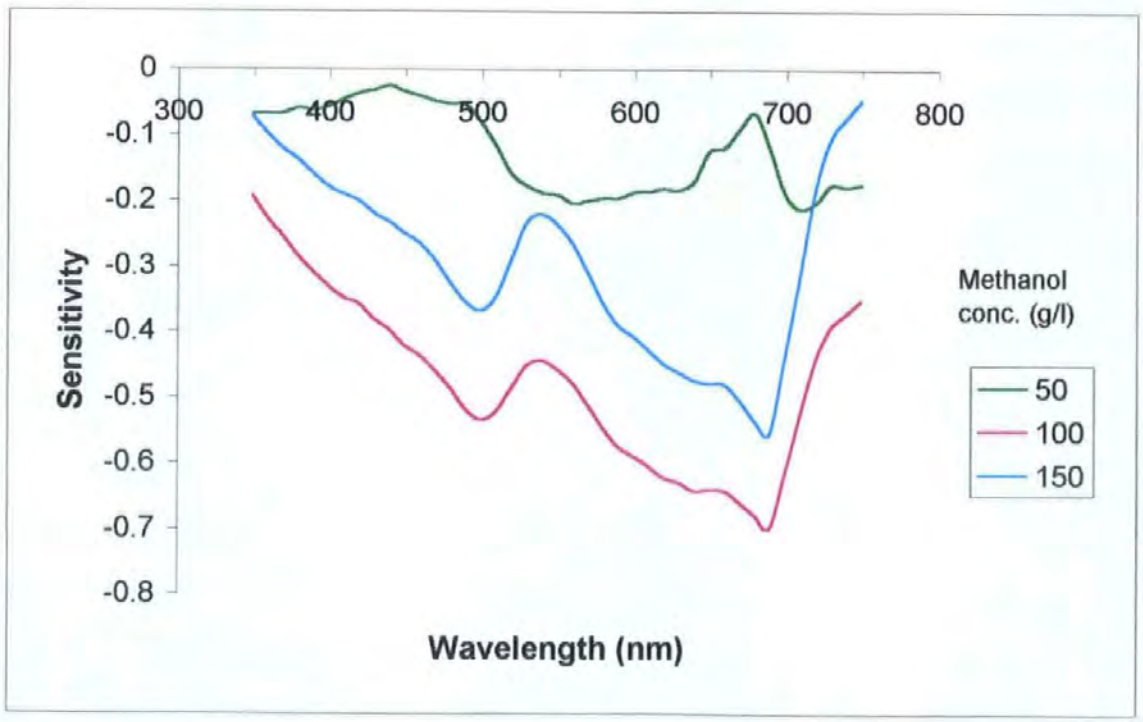

Figure 92: **Sensitivity of *in vivo* absorptance of *E.intestinalis* to methanol (g/l) exposure for 96 hours**

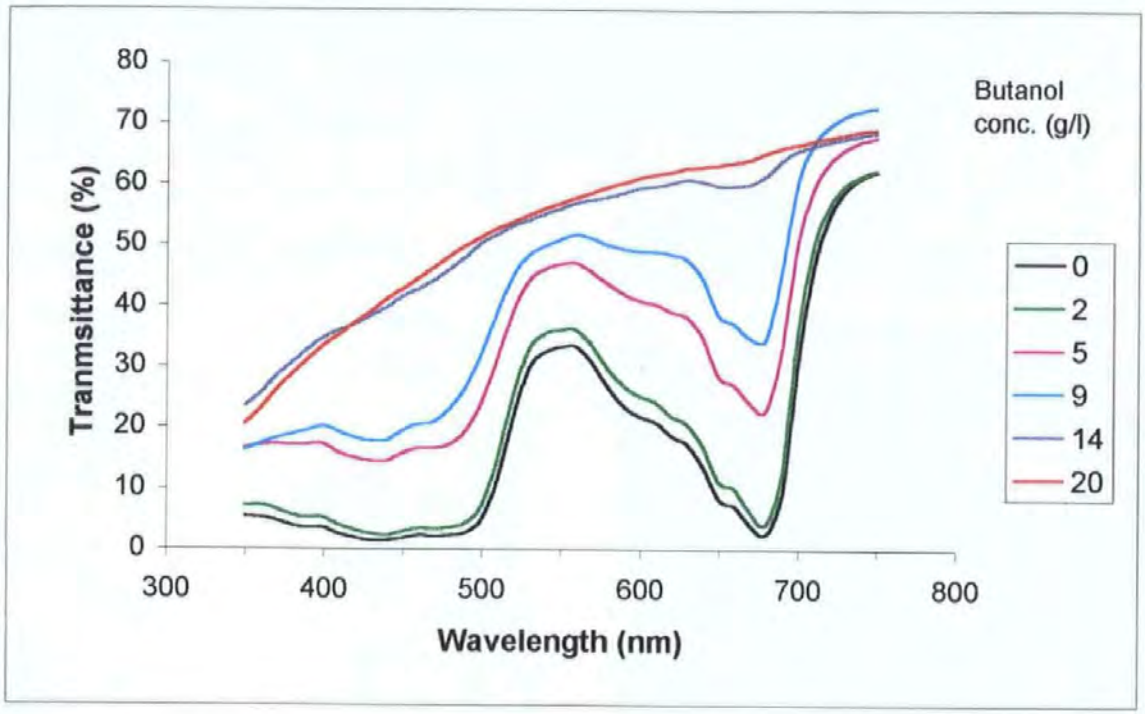

Figure 93: **Effect of butanol (g/l) on *in vivo* transmittance of *E.intestinalis* after 96 hours exposure**

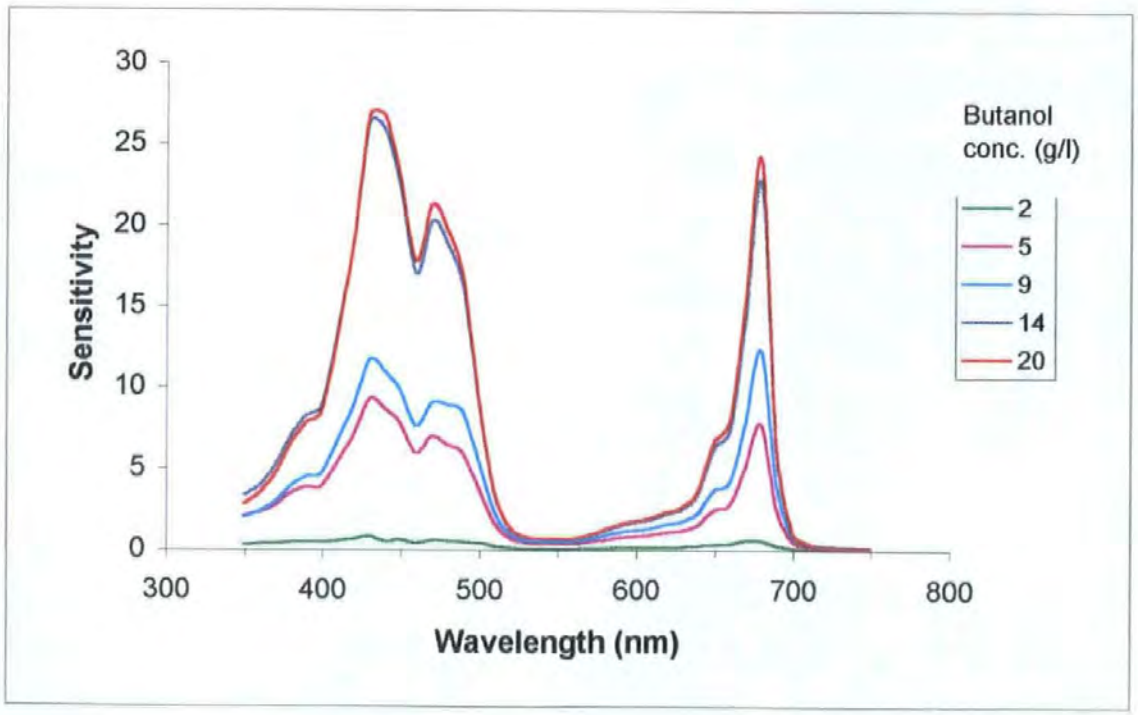

Figure 94: **Sensitivity of *in vivo* transmittance of *E.intestinalis* to butanol (g/l) exposure for 96 hours**

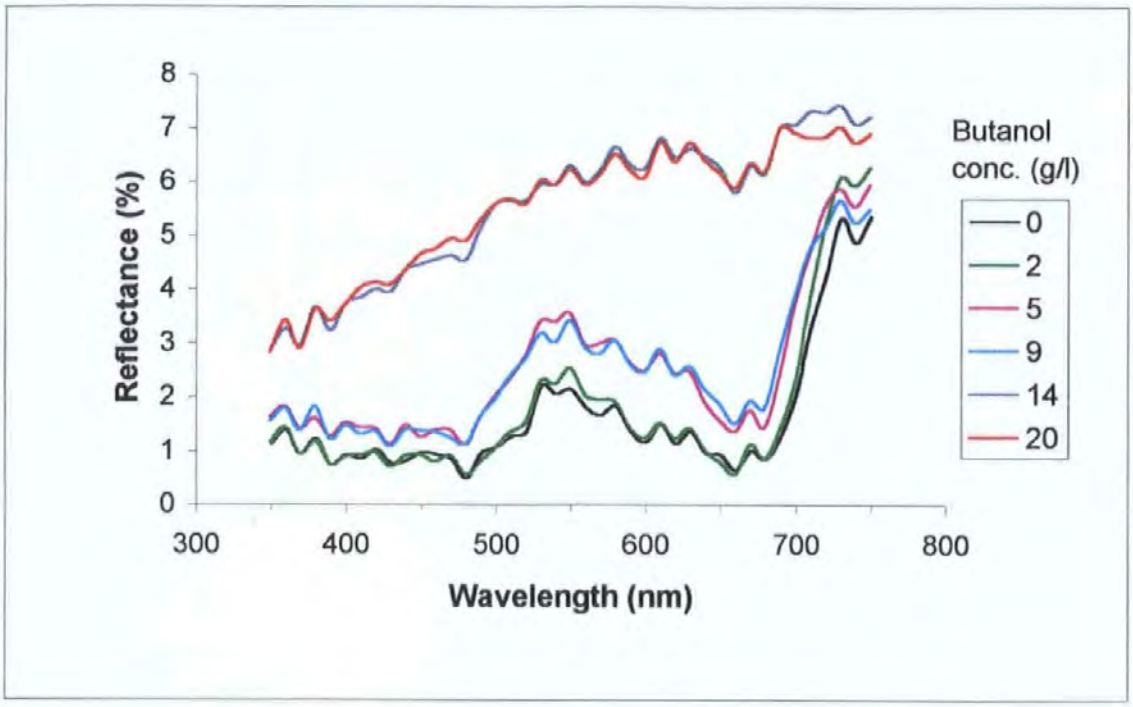

Figure 95: **Effect of butanol (g/l) on *in vivo* reflectance of *E.intestinalis* after 96 hours exposure**

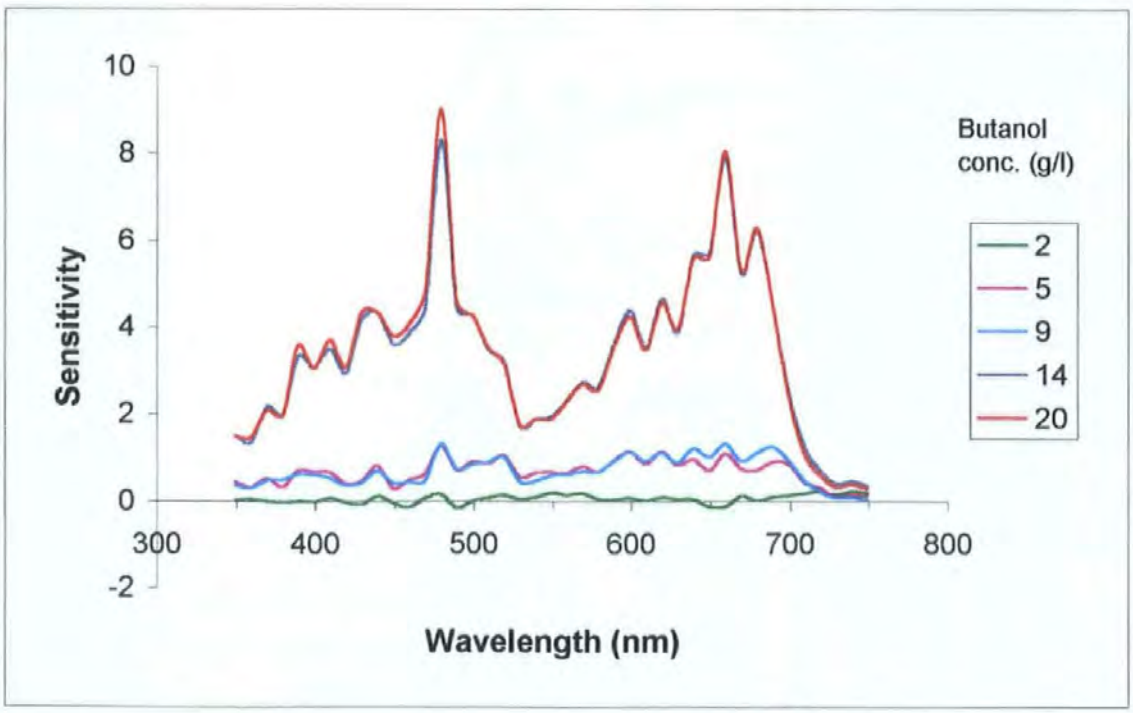

Figure 96: **Sensitivity of *in vivo* reflectance of *E.intestinalis* to butanol (g/l) exposure for 96 hours**

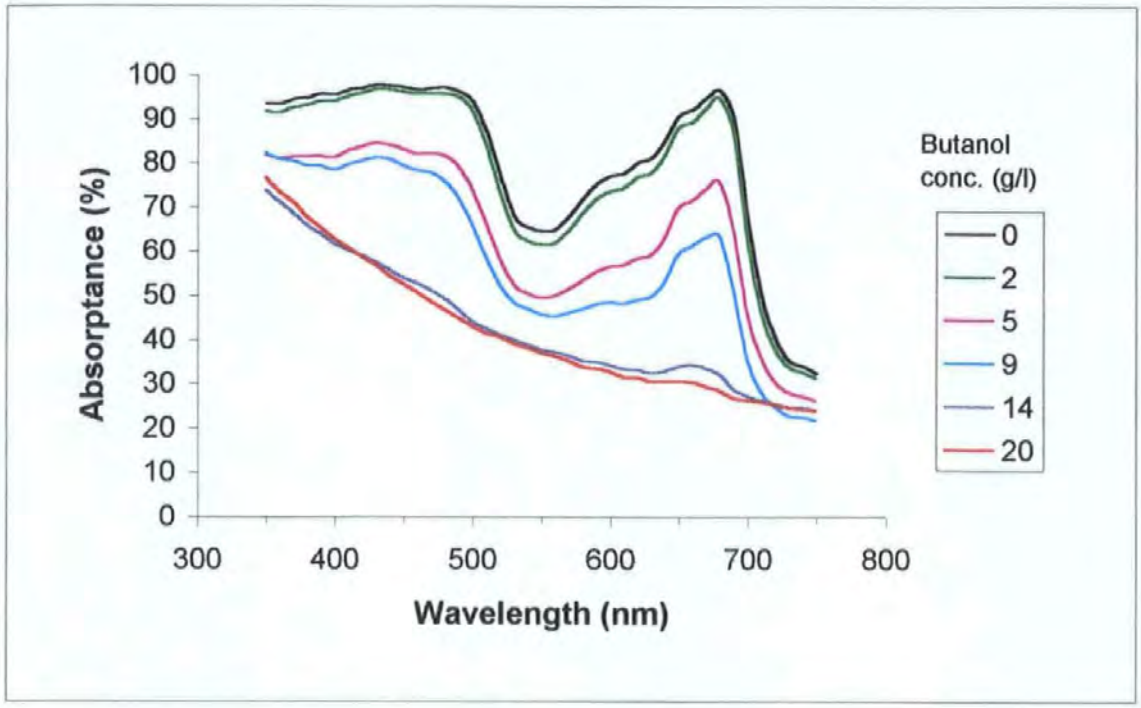

Figure 97: **Effect of butanol (g/l) on *in vivo* absorptance of *E.intestinalis* after 96 hours exposure**

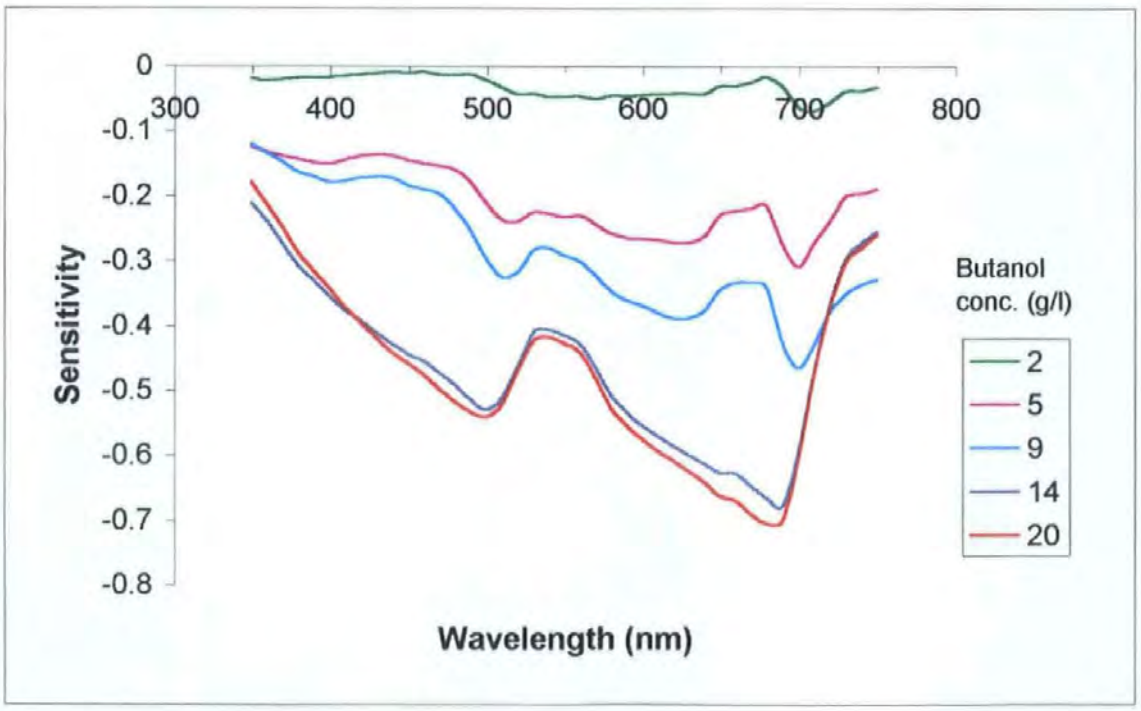

Figure 98: **Sensitivity of *in vivo* absorptance of *E.intestinalis* to butanol (g/l) exposure for 96 hours**

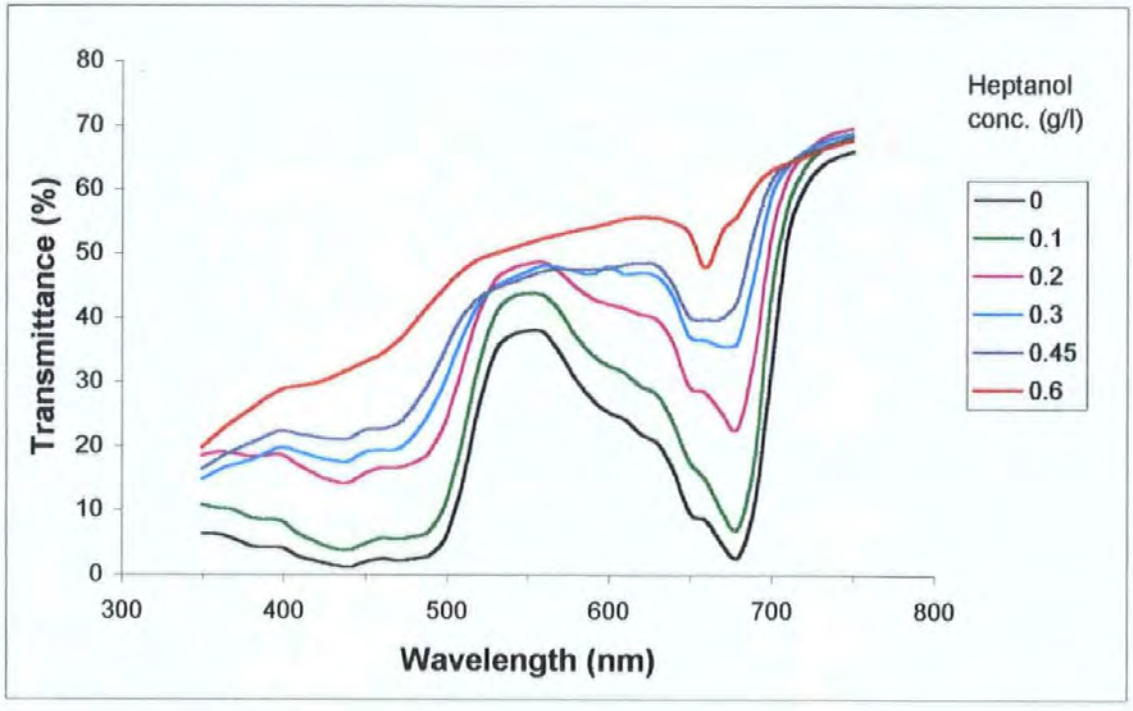

Figure 99: **Effect of heptanol (g/l) on *in vivo* transmittance of *E.intestinalis* after 96 hours exposure**

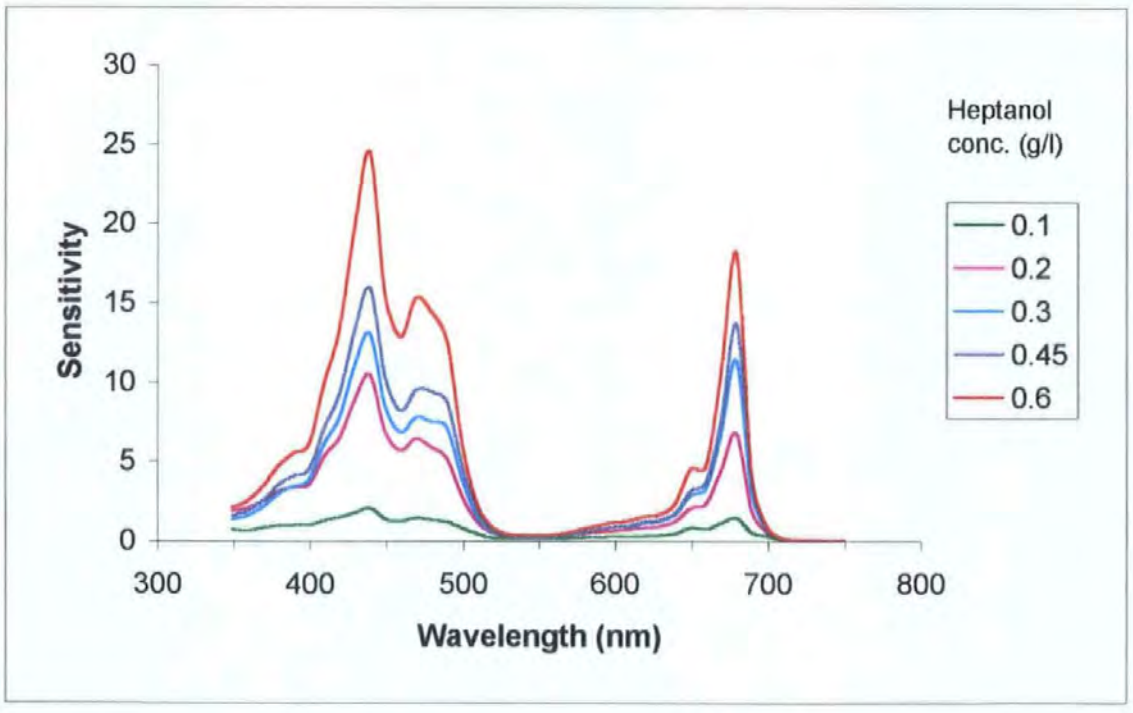

Figure 100: **Sensitivity of *in vivo* transmittance of *E.intestinalis* to heptanol (g/l) exposure for 96 hours**

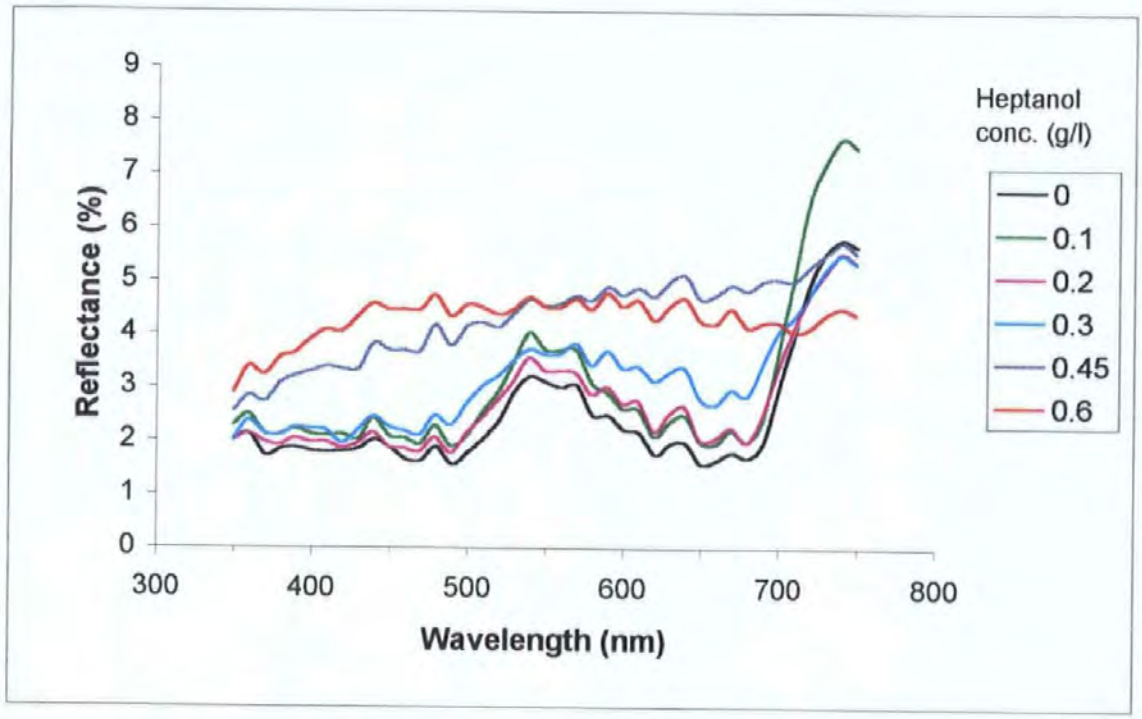

Figure 101: **Effect of heptanol (g/l) on *in vivo* reflectance of *E.intestinalis* after 96 hours exposure**

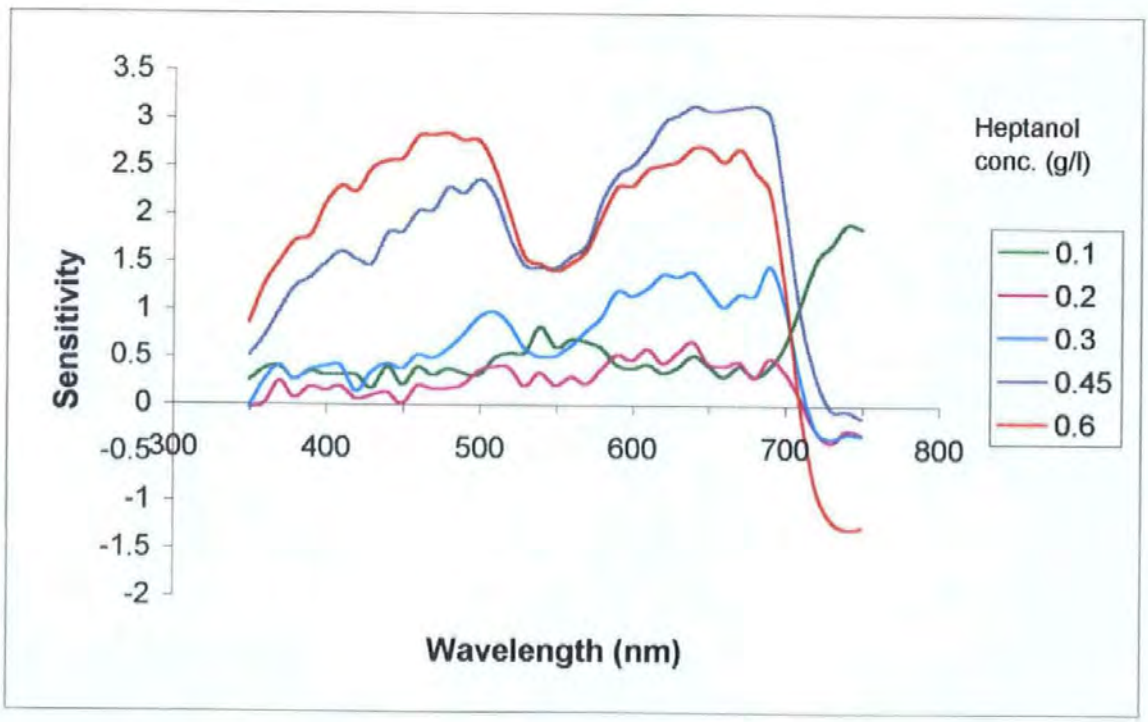

Figure 102: **Sensitivity of *in vivo* reflectance of *E.intestinalis* to heptanol (g/l) exposure for 96 hours**

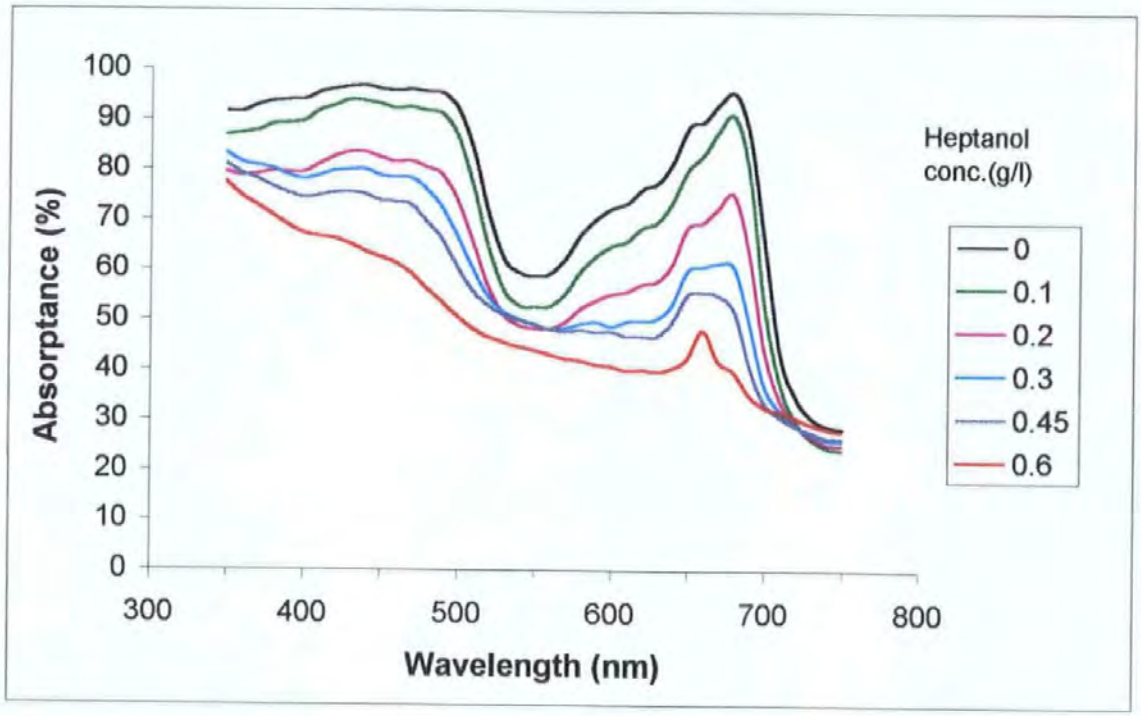

Figure 103: **Effect of heptanol (g/l) on *in vivo* absorptance of *E.intestinalis* after 96 hours exposure**

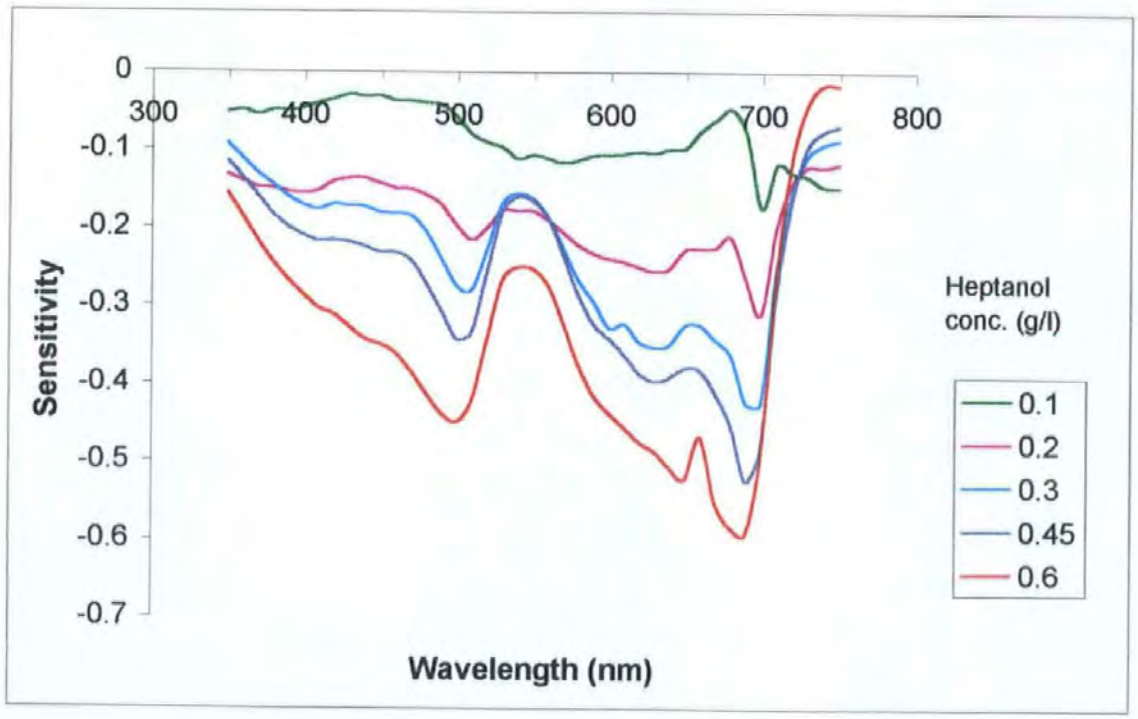

Figure 104: **Sensitivity of *in vivo* absorptance of *E.intestinalis* to heptanol (g/l) exposure for 96 hours**

***In vivo* reflectance**

The *in vivo* reflectance of *E.intestinalis* (Figures 89, 95 and 101) also increases as the alcohol concentration increases for each alcohol but, as with the in vivo transmittance, specific wavelengths relating to chlorophylls are also affected. It is possible that the thylakoid membranes may be affected by the alcohols, which would account for the changes in structure and pigment content encountered.

***In vivo* absorptance**

The use of the Pye Unicam SP8-100 UV/Vis spectrophotometer with diffuse reflectance accessory 790824 enables the absorptance to be calculated as described in Chapter 2 and the effect of methanol, butan-1-ol and heptan-1-ol on the *in vivo* absorptance of E.intestinalis are illustrated in figures 91, 97 and 103. This demonstrates the relationship between *in vivo* reflectance, transmittance and absorptance and it can be seen that an increase in the *in vivo* reflectance and transmittance results in a corresponding decrease in *in vivo* absorptance.

The *in vivo* spectral properties of *E.intestinalis* exposed to the series of homologous alcohols used indicate that chlorophyll is being affected as there are very definite peaks in at 680 nm and 440 nm in the sensitivity spectra which would correspond to chlorophyll a. At the highest concentrations of each alcohol the transmission increased and the absorptance decreased dramatically across the entire spectrum. This could indicate that the alcohol is affecting structure of the frond as well as pigments at these higher concentrations. This corresponds with Schild *et al*, 1995, when it was found that high concentrations of alcohols resulted in breakdown in the frond in neutral red retention studies.

The *in vivo* reflectance is obviously the most relevant for extrapolating the laboratory studies to the field for use in remote sensing but it was decided to use some of the in vivo transmittance and absorptance results to devise ratios and to investigate dose response relationships. This was decided because the noise level of the scans was not so high relative to the signal and several very obvious peaks in the sensitivity spectra were apparent and so they exhibited potential for detecting stress by the alcohol solutions.

Use of results to produce QSAR's

Because the series of homologous alcohols used in these experiments are similar compounds and the results obtained indicate that these alcohols have similar modes of action, there is a possibility of constructing a quantitative structure activity relationship (QSAR). QSAR's are a method of estimating the toxic effects of a compound by using both the physical and structural construction of a compound (Landis and Yu, 1995). Schild *et al*, 1995, used an ion leakage technique to validate the QSAR approach to quantify the response of *Enteromorpha intestinalis* to organic pollutants. It was therefore decided to construct QSARs for results taken from the *in vivo* spectral properties of the algae and to compare them with QSARs constructed using fluorescence measurements (May *et al*, 1998) and published (Schild *et al*, 1996) QSARs involving neutral red uptake and ion leakage in *E.intestinalis*.

The results of the effect of the alcohols on the *in vivo* absorptance indicated that there was a significant decrease in absorptance at 680 nm with increasing alcohol concentration (figures 91, 97 and 103). This was true for all alcohols from C = 1 to C = 8. EC_{50} values were therefore calculated from the linear regression equations for the alcohols and represented the concentration at which 50 % decrease of the absorptance at 680 nm from the control occurred (figures 105 - 112).

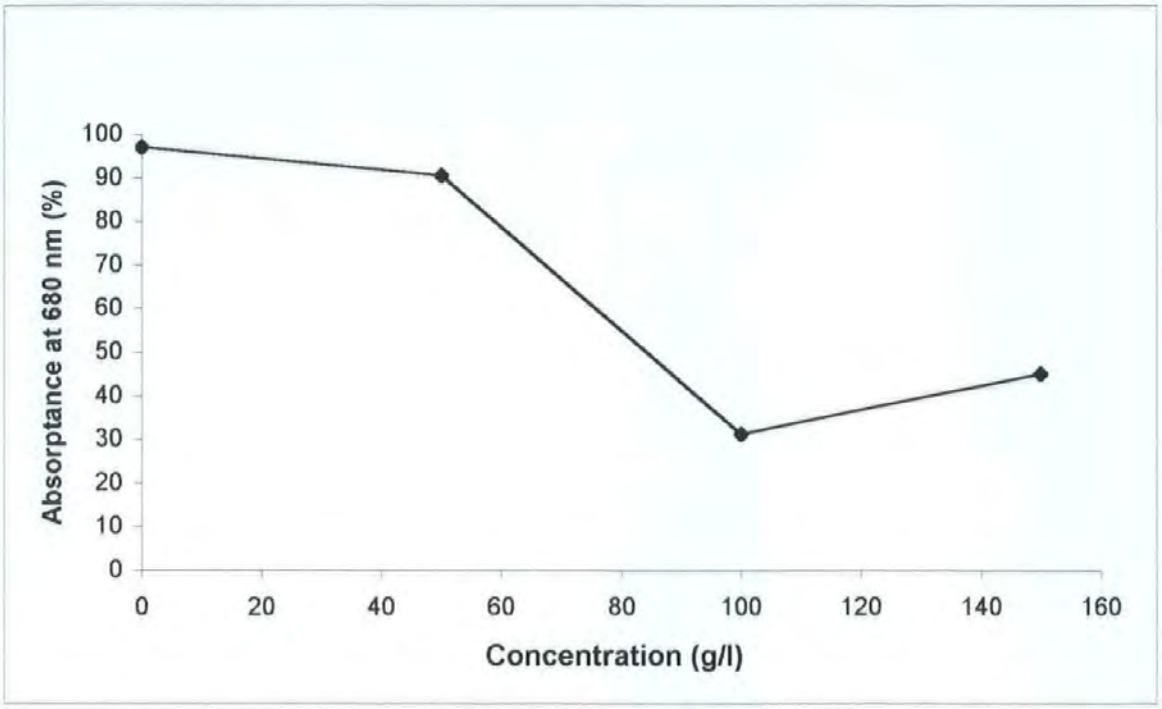

Figure 105: **Effect of methanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

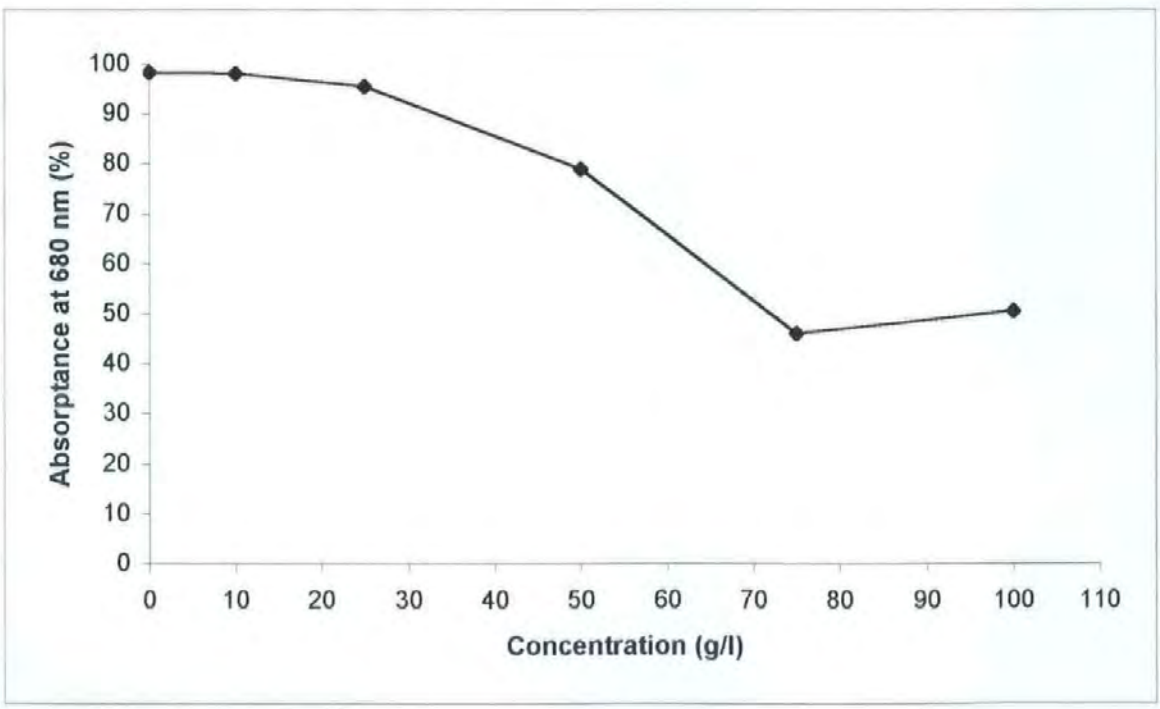

Figure 106: **Effect of ethanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

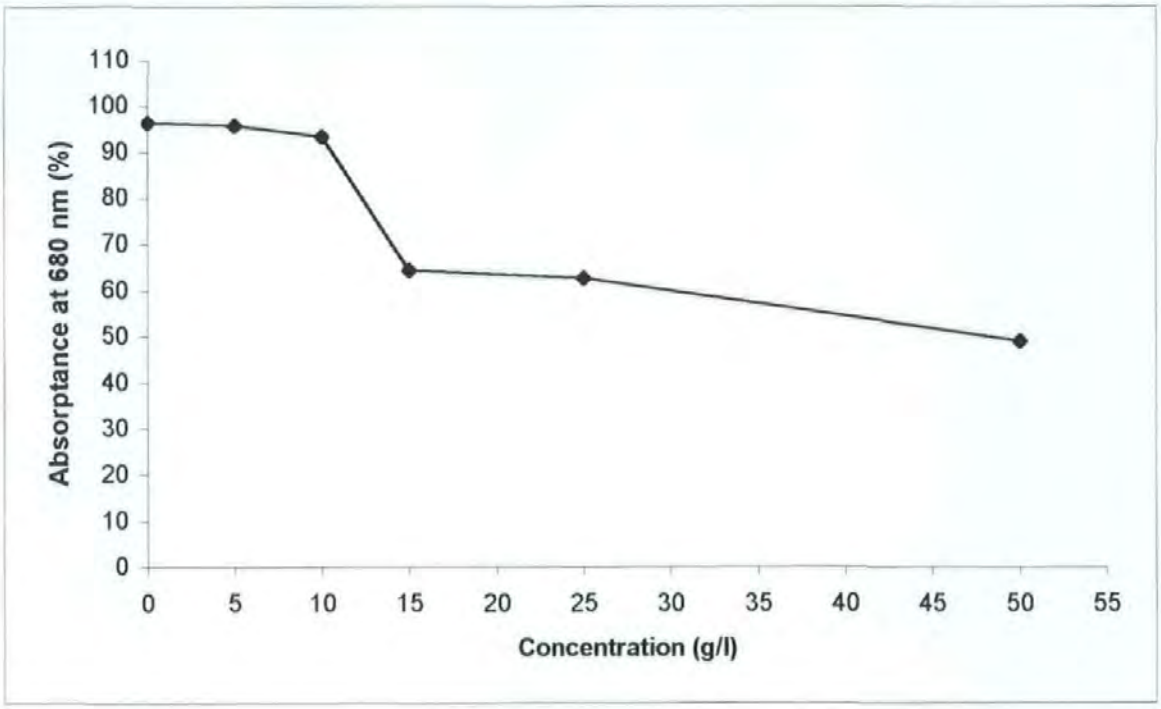

Figure 107: **Effect of propanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

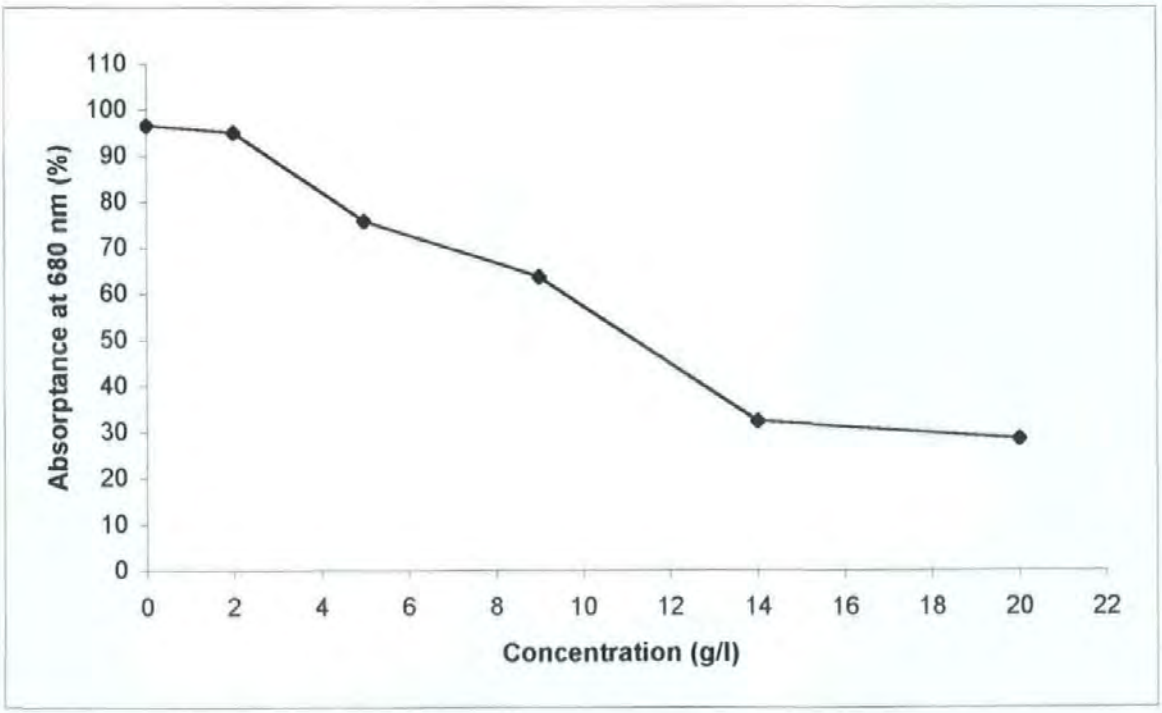

Figure 108: **Effect of butanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

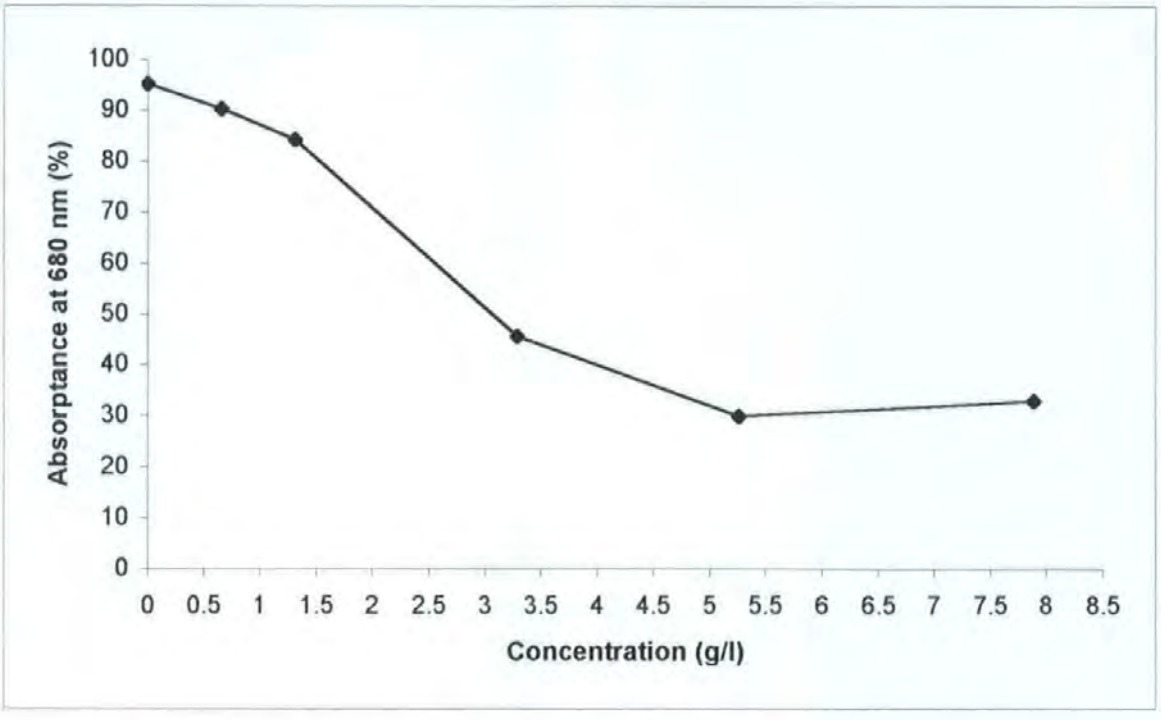

Figure 109: **Effect of pentanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

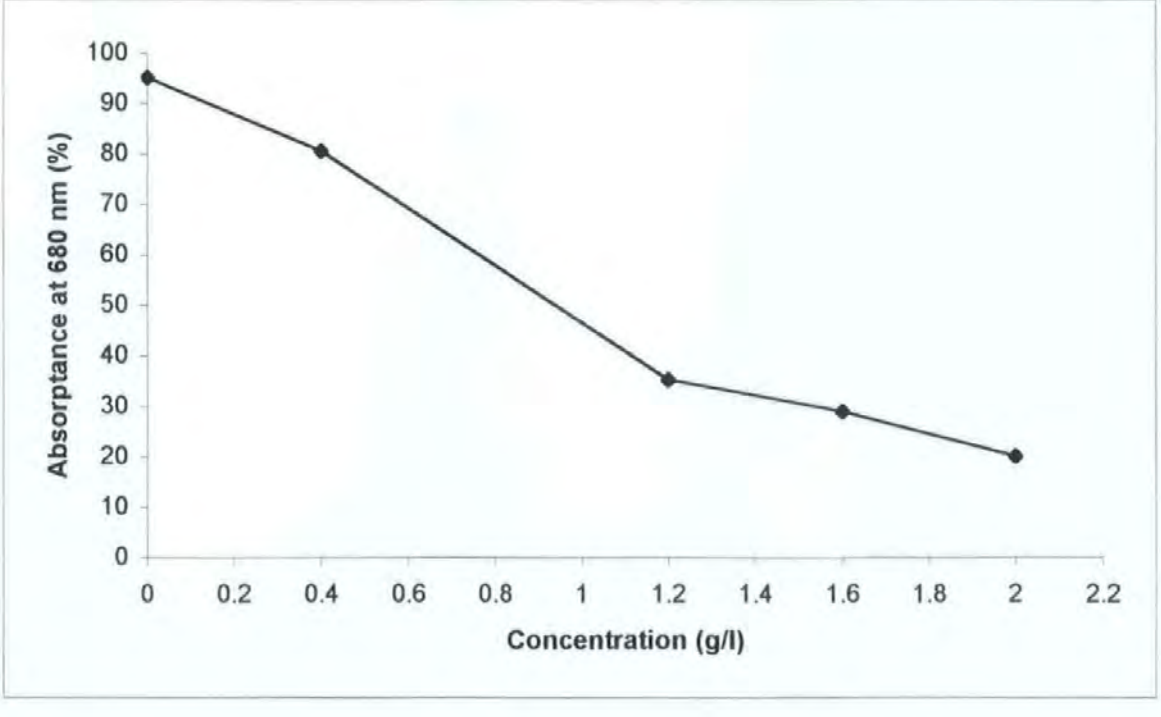

Figure 110: **Effect of hexanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

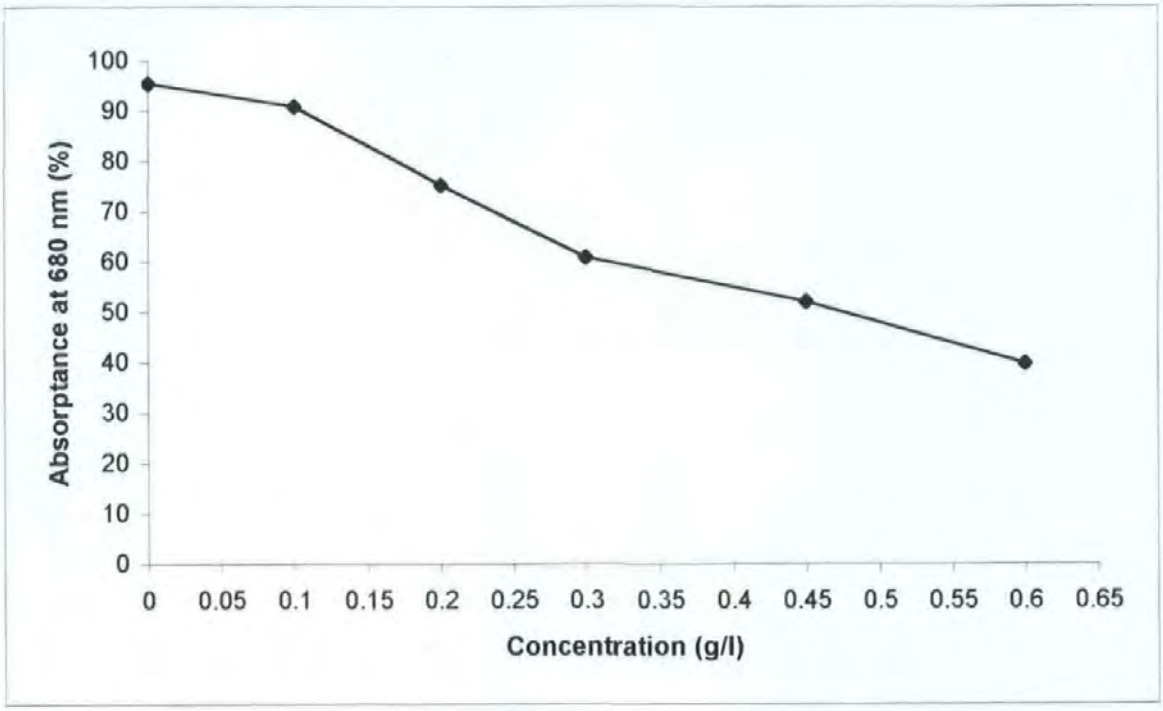

Figure 111: **Effect of heptanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

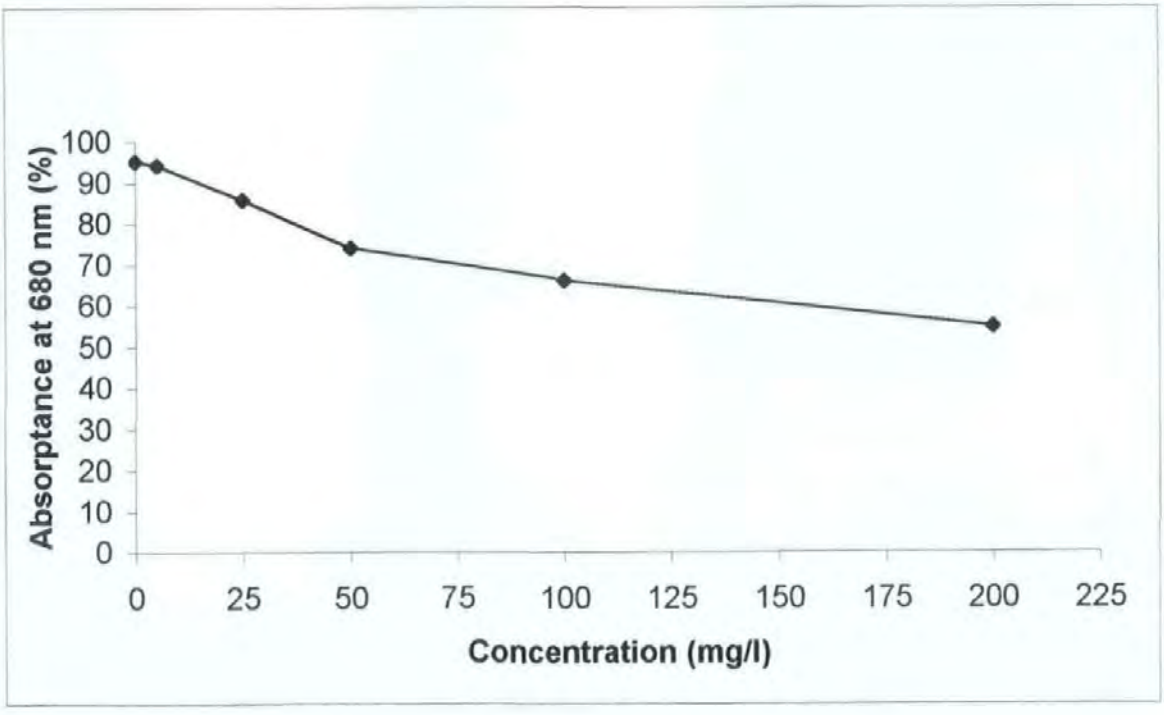

Figure 112: **Effect of octanol (g/l) exposure for 96 hours on *in vivo* absorptance at 680 nm in *E.intestinalis***

The resultant EC_{50}s were then plotted against both the carbon number of each alcohol and also the octanol/water partition coefficient (figures 113 and 114). Both QSARs were linear and the equations of the regression lines were:

Carbon number:

Log EC_{50} = -0.4165 carbon number + 2.5199, $n = 6$, $R^2 = 0.9804$

Log K_{ow} :

Log EC_{50} = -0.7695 Log K_{ow} + 1.4919, $n = 6$, $R^2 = 0.9682$

When this was compared with QSARs involving ion leakage of *E.intestinalis* (Schild *et al.* 1996) a close correlation was observed. The QSAR involving Log K_{ow} also compared favourably with a QSAR plotted using the EC_{50} values taken from the fluorescence data (F_v/F_m) (figure 115). The fact that spectral analysis (% absorptance at 680 nm), fluorescence (F_v/F_m) and ion leakage all produced QSARs with equations that were very close in slope, intercept and R^2 values may indicate that all the parameters are reflecting damage to membranes caused by this series of non-specific narcotics. The QSARs for the spectral analysis (% absorptance at 680 nm) and fluorescence (F_v/F_m) were extremely similar and this probably correlates with the damage being to the thylakoid membrane within the chloroplast. This indicates that the use of the *in vivo* spectral properties is useful as a technique in investigating the effect of alcohols on the algae. It also has the advantage in that many different parameters can be used from the *in vivo* reflectance, transmittance or absorptance and so more information about the alcohol effect can be obtained. This is, of course, in addition to the technique being non-invasive and the possible extrapolation to remote sensing applications.

The QSARs produced for both carbon number and log K_{ow} against toxic effect, both *in vivo* absorptance at 680 nm and fluorescence, were linear and had very high correlation coefficients, $R^2 = 0.962$ and 0.9153 respectively, and are characteristic for non-specific

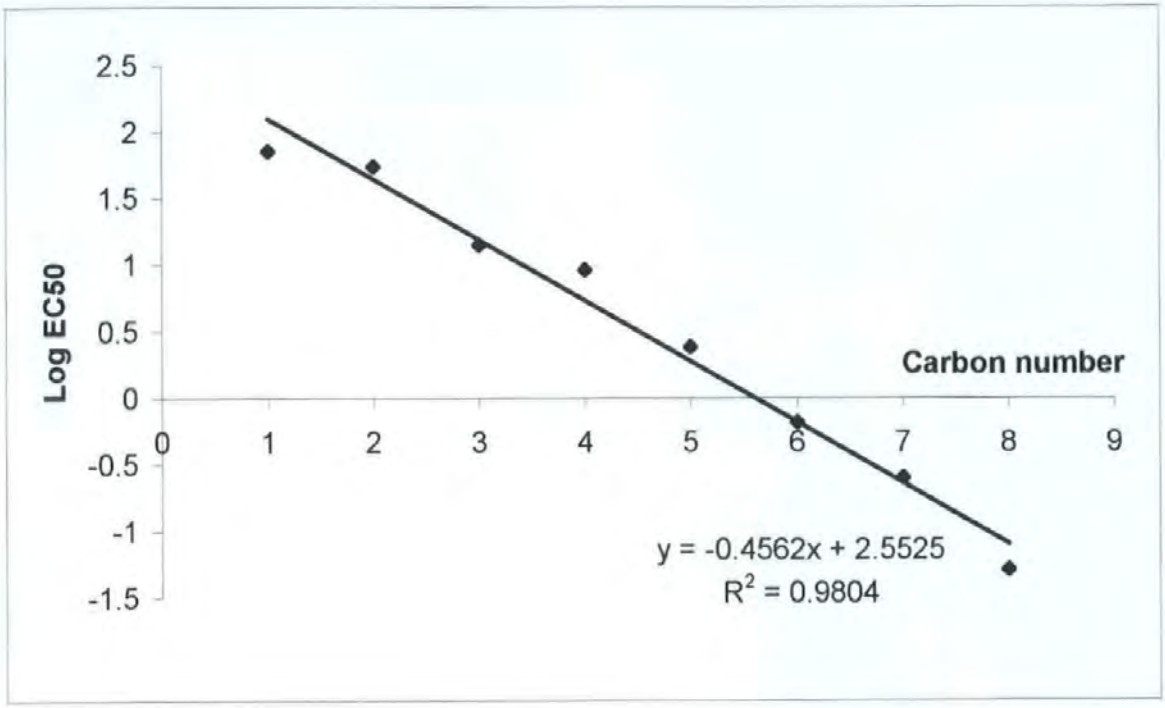

Figure 113: **QSAR of *in vivo* absorptance at 680 nm using carbon number**

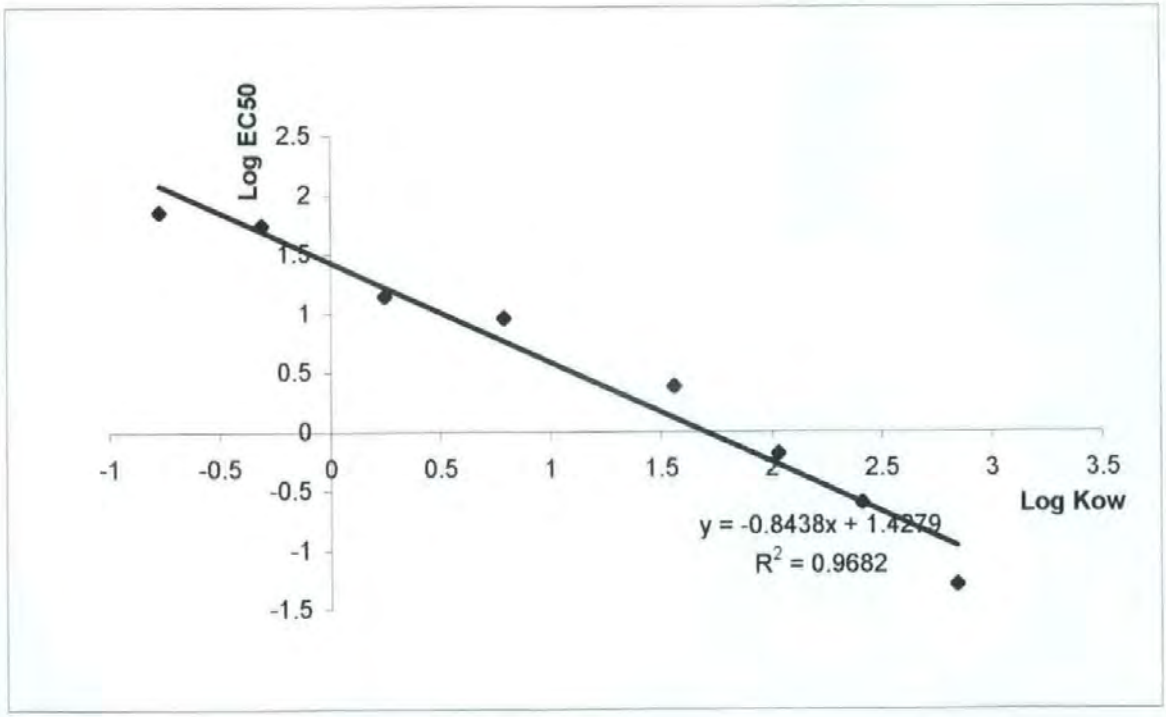

Figure 114: **QSAR of *in vivo* absorptance at 680 nm using Log K_{OW}**

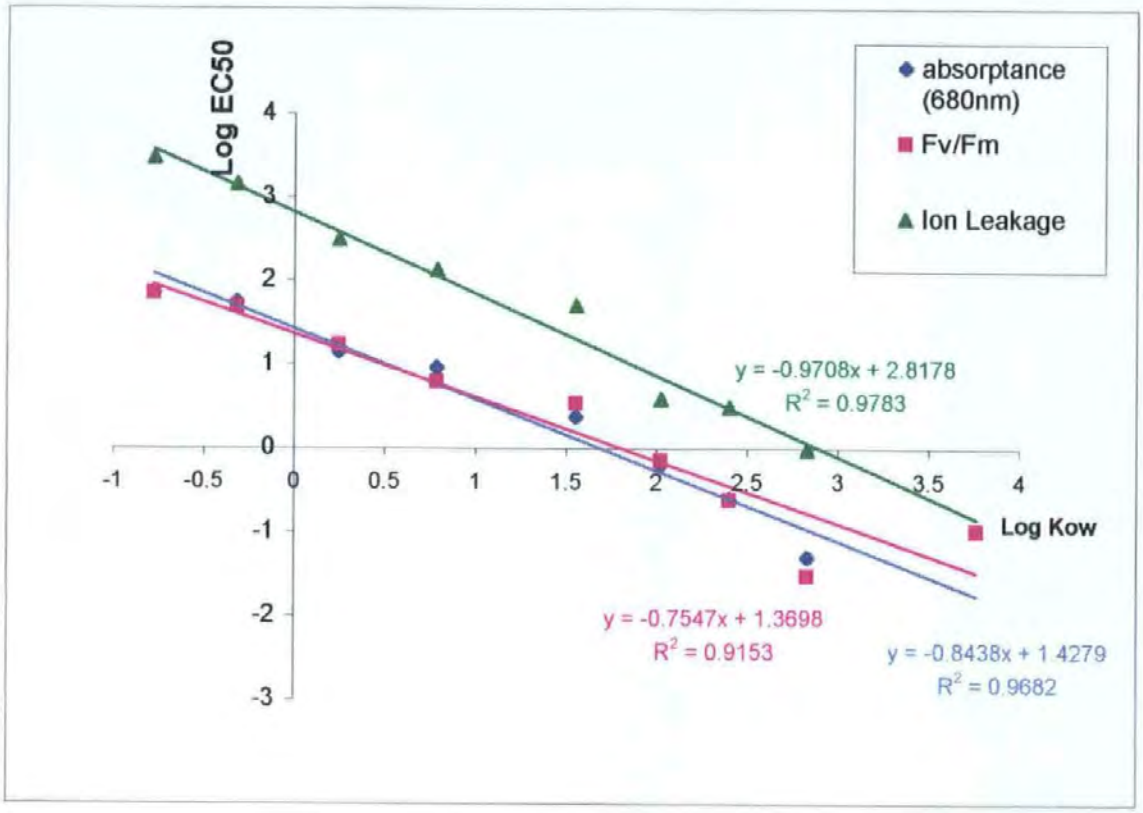

Figure 115: **QSARs using fluorescence, *in vivo* absorptance at 680 nm and ion leakage**

narcotic alcohols (Hansch *et al*, 1989). In addition to being similar to a QASR produced using ion leakage and *E.intestinalis* as mentioned above, they also had a similar pattern to that produced using fish cells with the same pollutants (Dierickx and Van De Vyver, 1991) indicating that the method is suitable for use in wider applications. QSARs are now used extensively in ecotoxicological studies as, if they result from narcosis, they can be highly correlated between different organisms which enables the toxicity of a compound to a particular species to be predicted using a QSAR derived from using another species (Zaroogian et al, 1985).

Results are presented for alcohols ranging from C=1 to C=8. The QSARs demonstrate that as the size of the compound increases a resulting increase in toxicity is observed. However, toxicity of a homologous series of compounds only increases until they are a particular size, one reason in aquatic systems being that the solubility may decrease below the concentration required to produce a toxic effect (Donkin *et al*, 1991). Schild *et al*, 1995, used the method of plotting aqueous solubility and toxicity against carbon number to find where the lines intersected to ascertain the 'cut-off' point and determined that alcohols with a carbon number greater than 8 would not be toxic in marine systems. They tested decanol (n=10) and found this to be true. However, during this study nonanol (C=9) was used to produce some preliminary results and it was found that toxic effects did occur, using fluorescence as a parameter, and so it is likely that the 'cut-off' point in marine systems is actually alcohols with a carbon number greater than 9.

By examining the effect of a homologous series of organic pollutants on the in vivo spectral properties it is evident that the method was sensitive and accurate enough to produce a quality QSAR with a slope approaching 1. This is important as the use of QSARs and related approaches can be used to 'predict the physical and chemical properties of organic contaminants, their bioaccumulation, biodegradation and toxicity'

(Donkin, 1994. A QSAR provides a baseline and for non-specific narcosis this can then be used to assess the toxicity of other chemicals. If a chemical is plotted with a QSAR and it is an outlier, it can be determined from this whether it has enhanced toxicity or is less toxic. If it has enhanced toxicity, this implies it must be acting by an additional mechanism (Lipnick, 1991; cited by Donkin, 1994), whereas if it appears to be less toxic than predicted by baseline narcosis, it may be because its physiochemical properties lie outside the range required to produce this response (Donkin, 1991). Most chemicals in the environment are found as complex mixtures (Hardy *et al*, 1987) and it has been found that if the toxicity of a mixture of compounds act by the same mechanism, illustrated by fitting a QSAR, the effect is concentration additive (Widdows and Donkin, 1991). This would obviously be an important consideration when determining contaminant effects in the marine environment.

7. APPLICATION OF *IN VIVO* SPECTROPHOTOMETRY TO SAMPLES COLLECTED FROM DIFFERENT FIELD SITES

The laboratory results obtained in this study certainly offer potential for the use of *in vivo* spectral properties as a biomonitor. However, in addition to laboratory studies, it is also important to assess whether the technique can be extrapolated for use in field studies. If laboratory studies can be used to identify specific spectral signatures, due to pollutant exposure, then it may be possible that these signatures appear in data collected using material taken from natural situations. This has the advantage of not only being able to detect but also the possibility of distinguishing between particular pollutants.

7.1 EXPERIMENTAL DESIGN

Collection of material

E.intestinalis was collected from each sample site and was taken back to the laboratory, washed, identified and put in holding tanks as described in Chapter 3. All algae were collected at low tide and, where possible, from similar types of shore location.

Choice of sites

The sites were chosen because of the potential or recorded pollution at each site and were selected along the South-West coastline. As mentioned previously, the control site used for all experiments in this study was Wembury Bay (OS SX517484) because this site is considered to be relatively free from pollution and is consequently a marine conservation area (part of the Marine Nature Reserve, DW Trust). A study by Scarlett *et al* (1997) found that levels of Irgarol 1051 at Wembury Bay were less than 1 ng dm^{-3} at high water in 1995. This was the lowest level recorded for all the sites tested around the Plymouth Sound area, which is located at the mouth of the river Plym and the river Tamar. *E.intestinalis* was also collected from Mothecombe (OS SX610473) and Thurlestone (OS

SX660435) which are relatively close to Wembury Bay along the same part of the coastline and could also be considered to be 'clean' sites as they are not subject to a history of pollution.

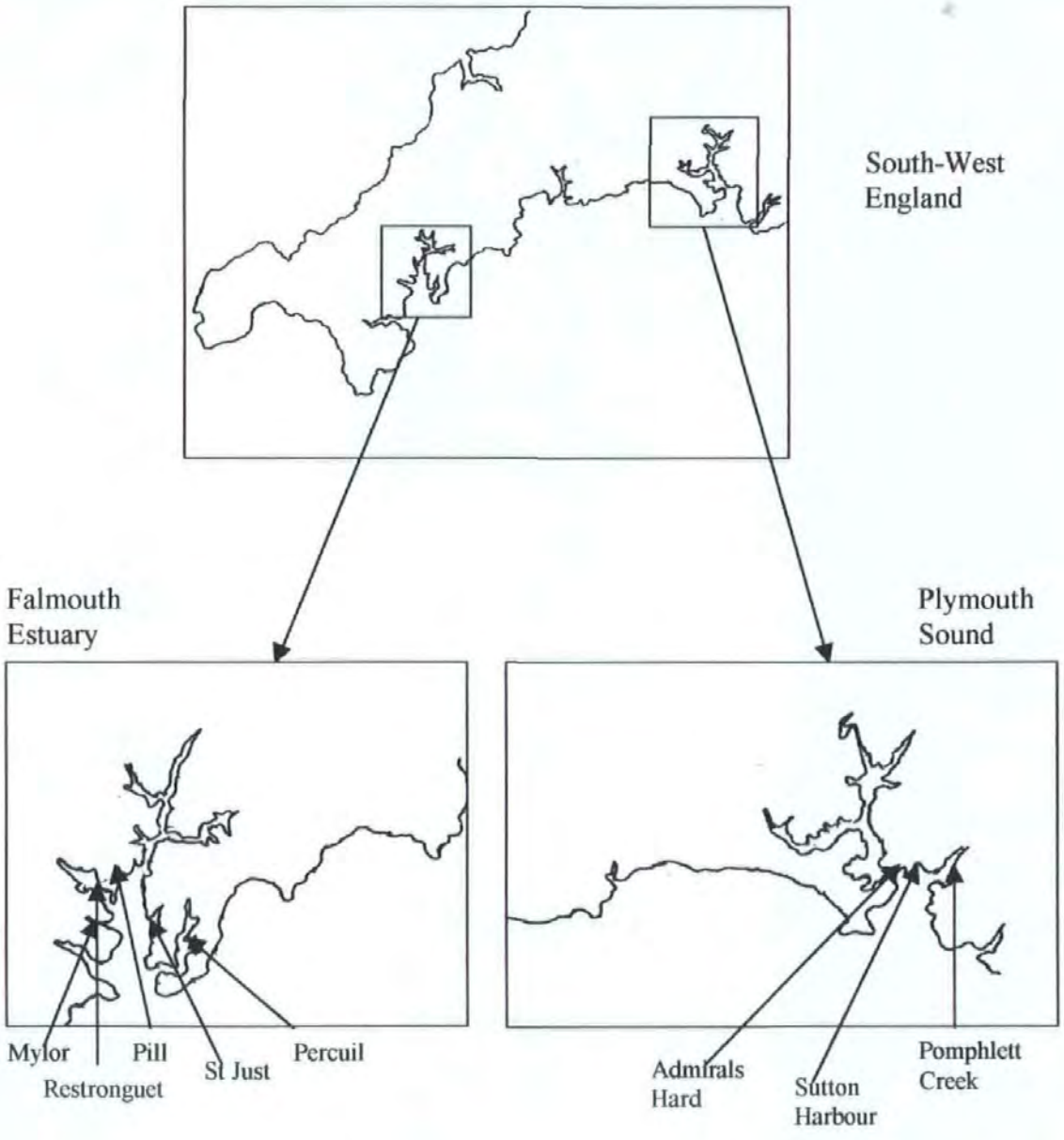

Figure 116: *Location* of potentially polluted field sites

Plate 6: Wembury Bay

Plate 7: Mothecombe

Plate 8: Thurlestone

Several sampling sites were chosen from the Fal estuary in Cornwall because of the history of the Fal being polluted by heavy metals, especially copper and zinc (Bryan and Gibbs 1983). The sites chosen were the five main creeks in the Fal estuary and these are known to exhibit a gradient of metal pollution with the highest concentration in Restronguet to the lowest in Percuil (Bryan and Gibbs 1983, Somerfield *et al* 1994). *E.intestinalis* was collected from

- Restronguet (OS SW385810)
- Mylor (OS SW356809)
- Pill (OS SW385827)
- St. Just (OS SW361848)
- Percuil (OS SW343857)

In addition, three Plymouth sites were chosen due to their location to potential sources of pollutants - Sutton Harbour (OS SX486543), Admirals Hard and Pomphlett Creek. Sutton Harbour is an enclosed harbour that is extremely close to a commercial fishing fleet (less than 100 m away), a marina with approximately 200 boats and 'Queen Anne's Battery' which is another large marina containing approximately 300 boats. In addition, Sutton Harbour maintains a minimum water level of 3 m above chart datum by the use of lock gates that are closed for approximately 12 hours a day (Scarlett *et al* 1997). It would be expected that levels of Irgarol 1051 would be higher in water where there is a high level of boating activity and this was confirmed by Scarlett *et al* 1997 where the maximum levels were found to be as high as 127 ng dm^{-3}. Admirals Hard is also in Plymouth Sound and is actually located at the mouth of the river Tamar which is used by large naval vessels. Pomphlett Creek is located at the mouth of the river Plym which is used to a lesser extent then the other 2 sites by large commercial vessels but it does contain many small craft moorings. Pomphlett Creek is a small boatyard but it is also extremely close to a sewage works.

Macroalgae have been used in studies as biomonitors for a number of reasons as discussed in Chapter 1. However, it must be remembered when using macroalgae as a potential monitor of pollution at a site that there are many factors to be considered. Algae represent static monitors of pollution and information can be ascertained from the presence or absence of particular species, studies involving the use of different parameters *e.g.* growth of fluorescence and analysis of the composition of the algae. Factors that can affect results include the part of the algae used, season, thallus thickness, type of algae, position on the shore and other pollutants present in the environment. *E.intestinalis* has been used as a monitor of trace metal pollution (Say *et al* 1990) because it is widespread, has a thin thallus and its use avoids the potential problem of the dilution effect encountered in *Fucus*

sps. due to apical growth. However, studies have shown (Barreiro *et al,* 1993) that it is important to standardise the time, place of collection and the part of the algae sampled.

There is frequently intra-site variation between levels of pollutants encountered and this needs to be considered when selecting the place of algal collection. Some species of brown macroalgae, *e.g. Ascophyllum nodosum,* can provide information of the history of pollution at a site because they remain in the same position for many years. *Enteromorpha intestinalis,* by contrast, is a seasonal macroalgae and can, therefore, only provide information on pollution at that site that season. This needs to be taken into consideration as pollution at a site can vary from season to season. Samples of *E.intestinalis* for this study were taken from similar places on the shoreline for each site. However, it must be remembered that they each only represent a sample of one place at each site and, therefore, have been exposed only to the pollutants present at that position of the site.

Metal analysis

Metal analysis of the *E.intestinalis* samples to determine tissue levels of copper and zinc was carried out as described in Chapter 3.

Parameters measured

Intercalary sections, ten sections from each site, 2.5 cm long of *Enteromorpha intestinalis* were cut from healthy frond and were used for the fluorescence and spectral measurements.

The *in vivo* spectral properties of the samples of *E.intestinalis* were measured using the ATI Unicam UV4 UV-Vis spectrophotometer equipped with a Labsphere RSA-UC-40 integrating sphere to assess whether their use could determine pollution effects between sites. These were used in conjunction with metal content of the algae. In addition, fluorescence (F_v/F_m and area above the fluorescence curve between F_0 and F_{max}) measurements were taken to assess algal health status.

7.2 RESULTS AND DISCUSSION

Fluorescence

The measurement of the fluorescence parameters to assess the health status of the *E.intestinalis* produced some interesting results (Figures 117 and 118). The mean fluorescence induction ratio (F_v/F_m) (figure 117) clearly shows that the values obtained for the Wembury *E.intestinalis* (control for all experiments) were lower than many of the samples collected from the other sites. The sites that exhibited the highest levels were Pill, Mylor, St. Just, Sutton Harbour and Admirals Hard. It is possible that this is because the 24 hour acclimation period in Instant Ocean with nutrients could have allowed the algae to enter a recovery phase as this is often exhibited by *E.intestinalis*. The Restronguet *E.intestinalis* does not appear to have this 'elevated' level of F_v/F_m values. This could be because Restronguet is polluted to such an extent that some of the organisms, including *E.intestinalis*, could become resistant to the pollution. The consequences of this could be that the algae either does not enter a period of recovery when the pollutant is removed or that removal of the pollutant may even have a detrimental effect on the algae. However, the F_v/F_m results for all the sites were in the 'normal' range for healthy algae.

An alternative possibility is that the high fluorescence, F_v/F_m, results may be caused by exposure to pollutants. The typical 'healthy' F_v/F_m value is generally in the region of 0.8 and figure 117 illustrates that the values obtained from some of the sites are much higher than this, in some cases up to 0.87. If F_v/F_m increases, see figure 1 in Introduction, it must due to F_v increasing or F_m decreasing. As $F_v = F_m - F_0$, if F_m decreases, this must be accompanied by F_0 decreasing if F_v is going to increase. It could, therefore, be that the observed F_v/F_m might be due to pollutant exposure, which has resulted in the lowering of typical F_m and F_0 values, resulting in an elevated F_v/F_m.

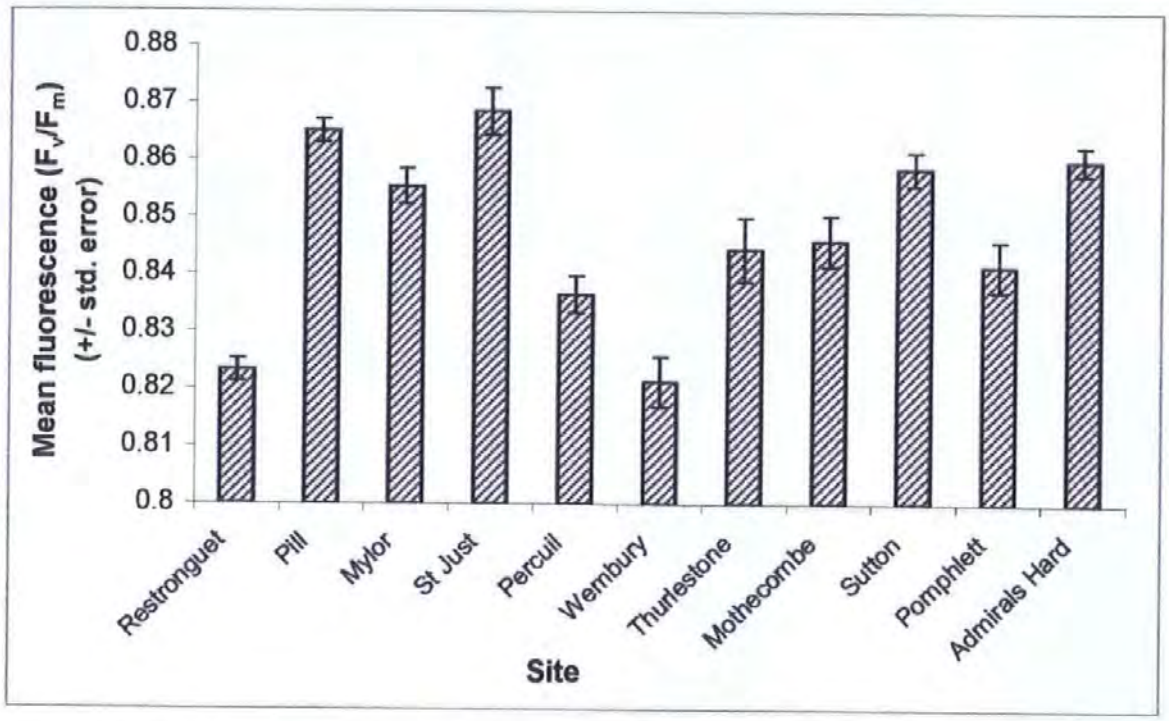

Figure 117: Mean Fluoresence (F_v/F_m) of *E.intestinalis* collected from different sites after 48 hours acclimation

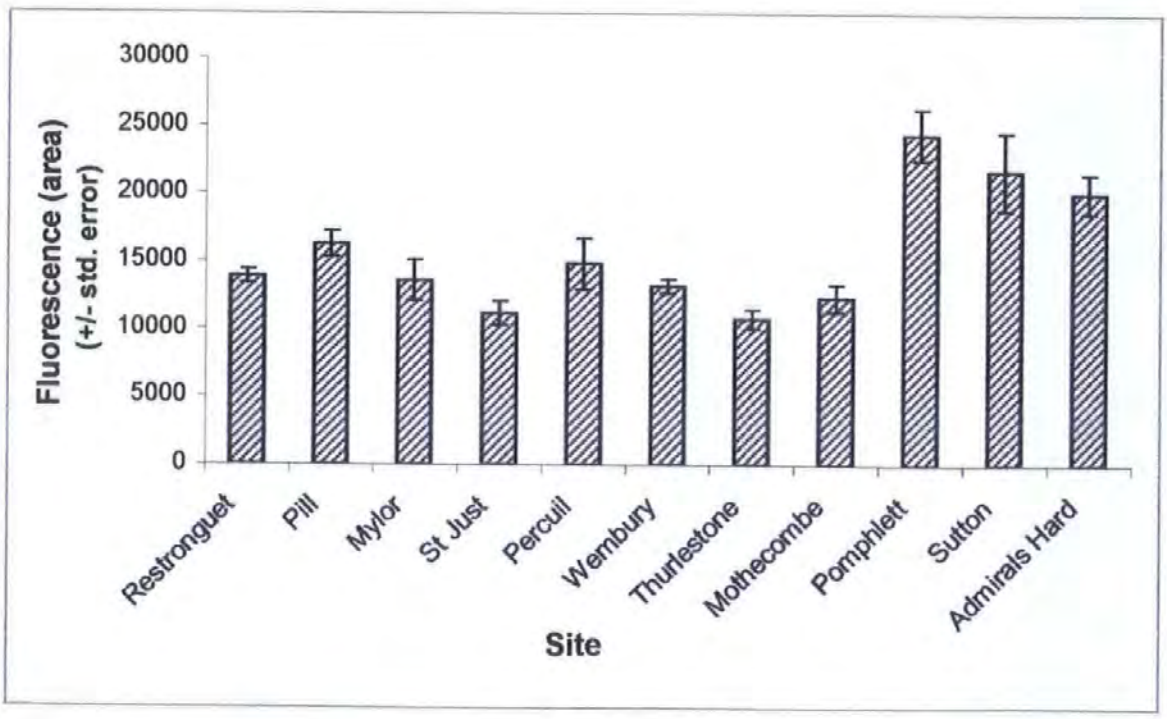

Figure 118: Mean fluorescence (area) of *E.intestinalis* collected from different sites after 48 hours acclimation

The results of the area above the fluorescence curve between F_0 and F_m (figure118) indicate that the 3 sites that are significantly different are Pomphlett Creek, Sutton Harbour and Admirals Hard. The fluorescence areas of the samples taken from these 3 sites are higher than the other sites. This was unexpected because the area above the fluorescence curve between F_0 and F_m is proportional to the pool size of the electron acceptors Q_A on the reducing side of Photosystem II and it would normally be decreased with the addition of a triazine herbicide. The shape of the fluorescence curve must, therefore, be different to that of the other sites in order for the area to be increased but this would have to be investigated further by the use of recording whole fluorescence traces in addition to recording individual parameters.

Copper and zinc content of the algae

	Metal content (μg/g dry weight)	
Site	**Copper**	**Zinc**
Wembury	5	25
Thurlestone	2	26
Restronguet	57	124
Pill	15	47
St. Just	14	89
Pomphlett	26	152
Admirals Hard	60	96
Sutton	67	143

Table 8: Copper and zinc content of *E.intestinalis* collected from different sites.

Results of the metal analysis of the *Enteromorpha intestinalis* collected from each site must be treated with caution as only one sample from each site was analysed. However,

the levels found in the Restronguet, Pill and St. Just samples are in the same order as published results of sediment analysis (Bryan and Gibbs, 1983, Williams *et al,* 1998) with Restonguet being the highest of the three sites. The levels of copper and zinc were lowest in the *E.intestinalis* collected from Wembury Bay and Thurlestone as expected. However, higher levels than expected both for copper and zinc were found in the algae collected from Sutton Harbour (levels of copper and zinc higher than that of Restronguet), Admirals Hard and Pomphlett Creek. In particular, the zinc levels at Pomphlett Creek are extremely high being comparable to Restronguet. This was unexpected but could possibly be due to zinc being used in some boat paints.

In vivo spectral properties of the algae

In vivo transmittance factor

Figure 119 illustrates that there is some variation between the mean *in vivo* transmittance factor of the *E.intestinalis* collected from the different sites. In order to examine this variation more closely, the 'clean'sites, 'metal polluted' sites and 'potentially organic' polluted sites were analysed separately (figures 120 – 124).

It can be seen from figure 120 that the *in vivo* transmittance factor of the *E.intestinalis* from Mothecombe is extremely similar to the Wembury (control) data whereas the Thurlestone data is slightly different. This is highlighted in figure 121 that illustrates how the *in vivo* transmittance factor is different to the Wembury (control) data. When comparing the sensitivity spectra, figures 121, 123 and 125 it is evident that there are clear differences in the *in vivo* transmittance factor of the different samples. Figure 125 shows that the in vivo transmittance factor of the algae collected from Sutton, Admiral's Hard and Pomphlett Creek, all 3 typically thought of as the organically polluted sites, exhibit the same sensitivity signature as each other but are very different to the data

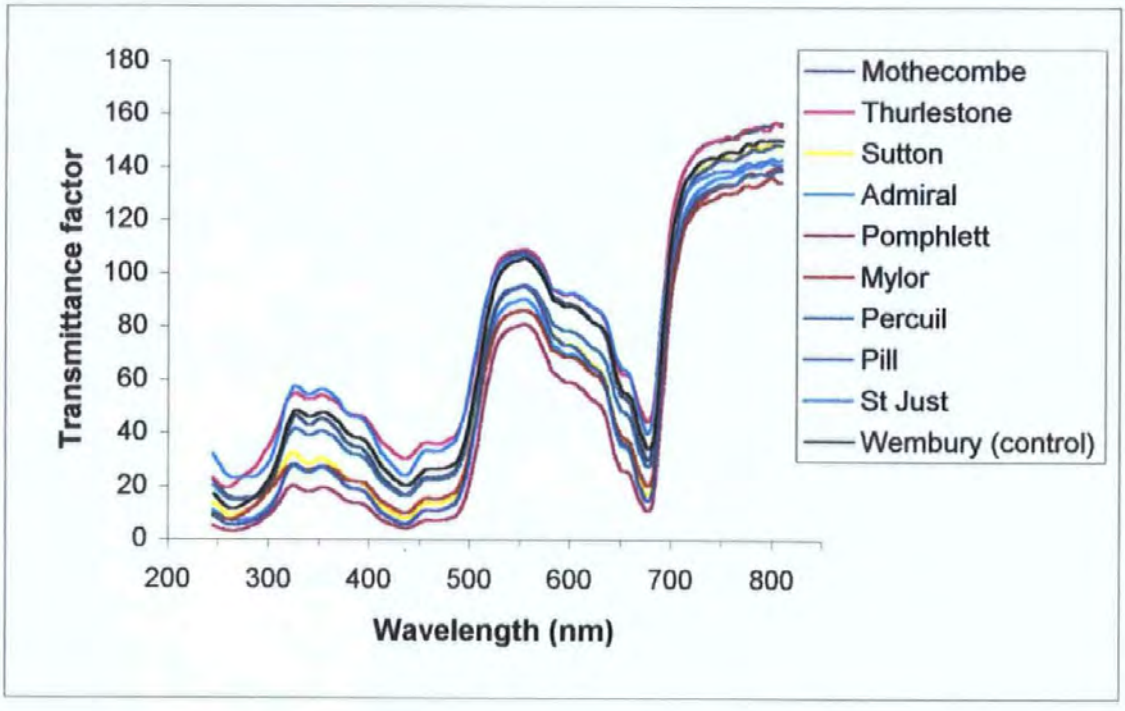

Figure 119: ***In vivo*** **transmittance factor of** ***E.intestinalis*** **collected from different sites**

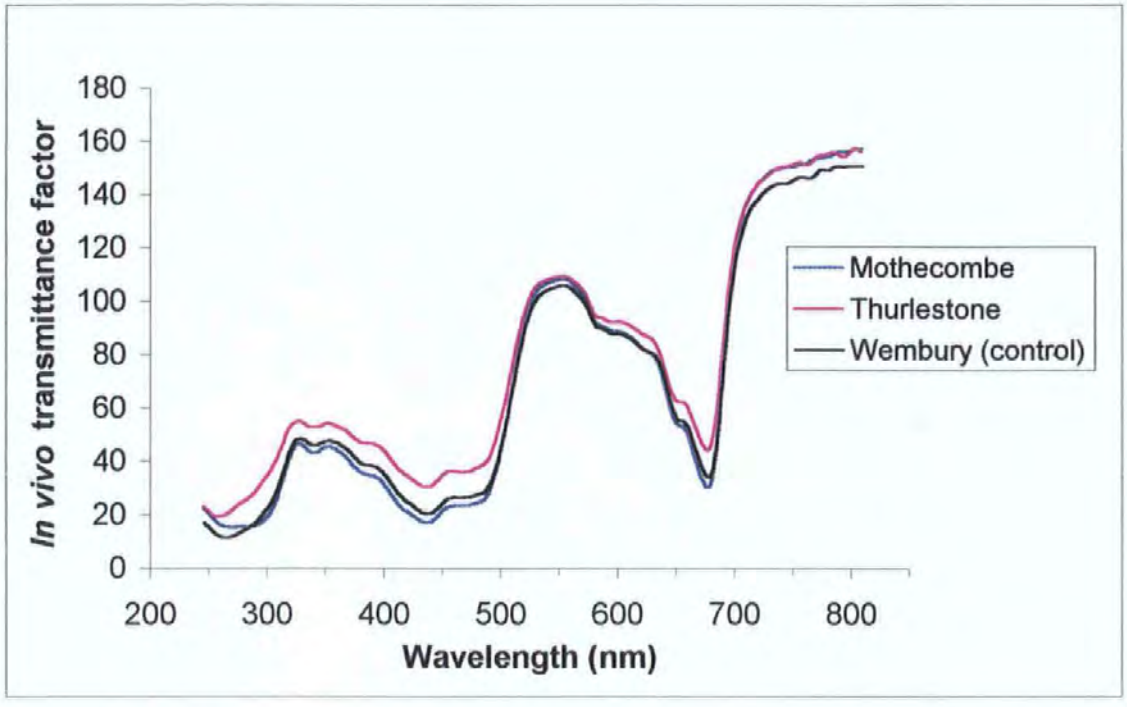

Figure 120: ***In vivo*** **transmittance factor of E.intestinalis collected from 'clean' sites**

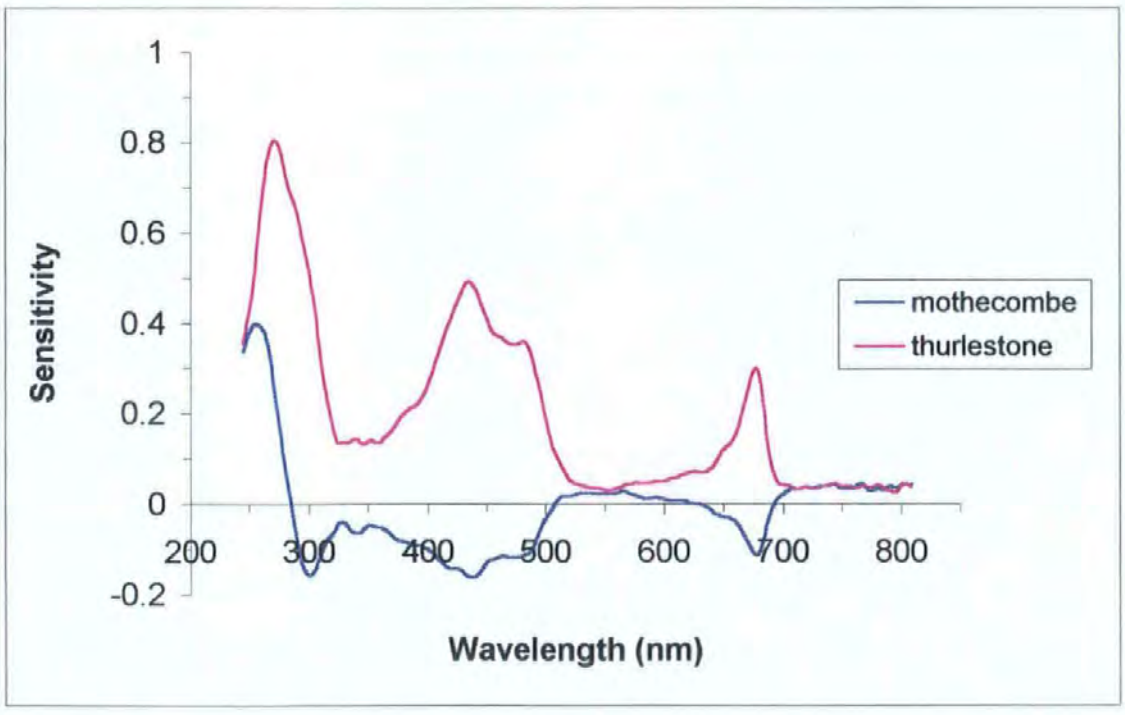

Figure 121: Sensitivity of *in vivo* transmittance factor of *E.intestinalis* collected from 'clean' sites

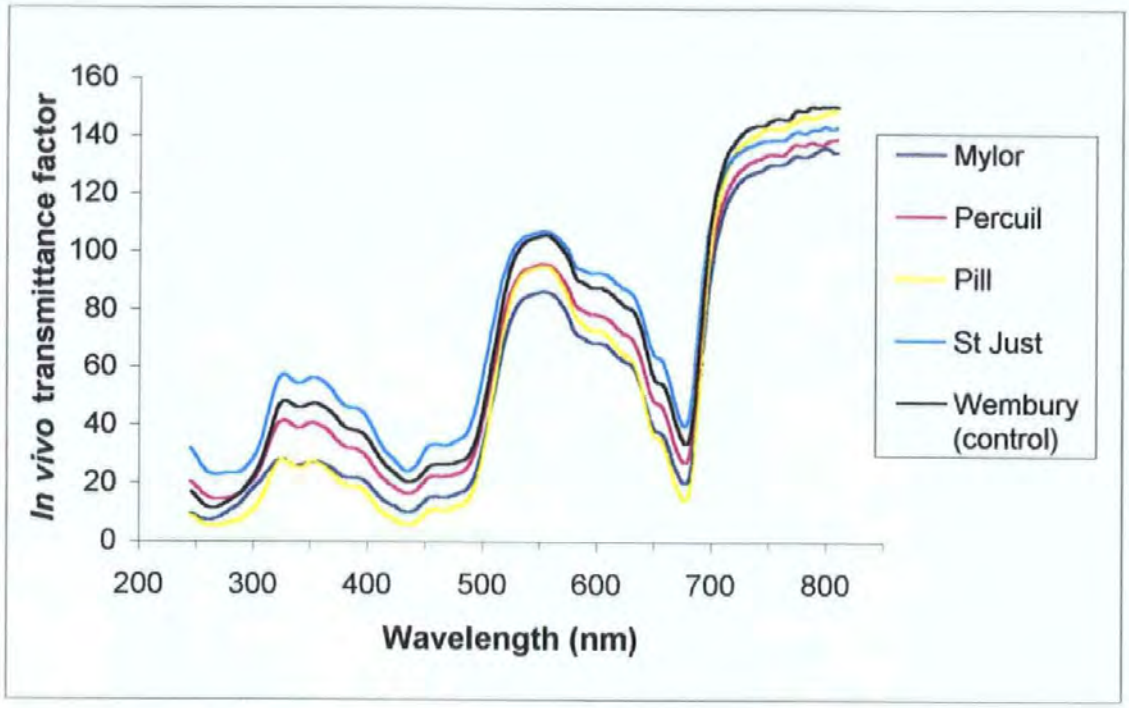

Figure 122: ***In vivo*** **transmittance factor of** ***E.intestinalis*** **collected from 'metal polluted' sites**

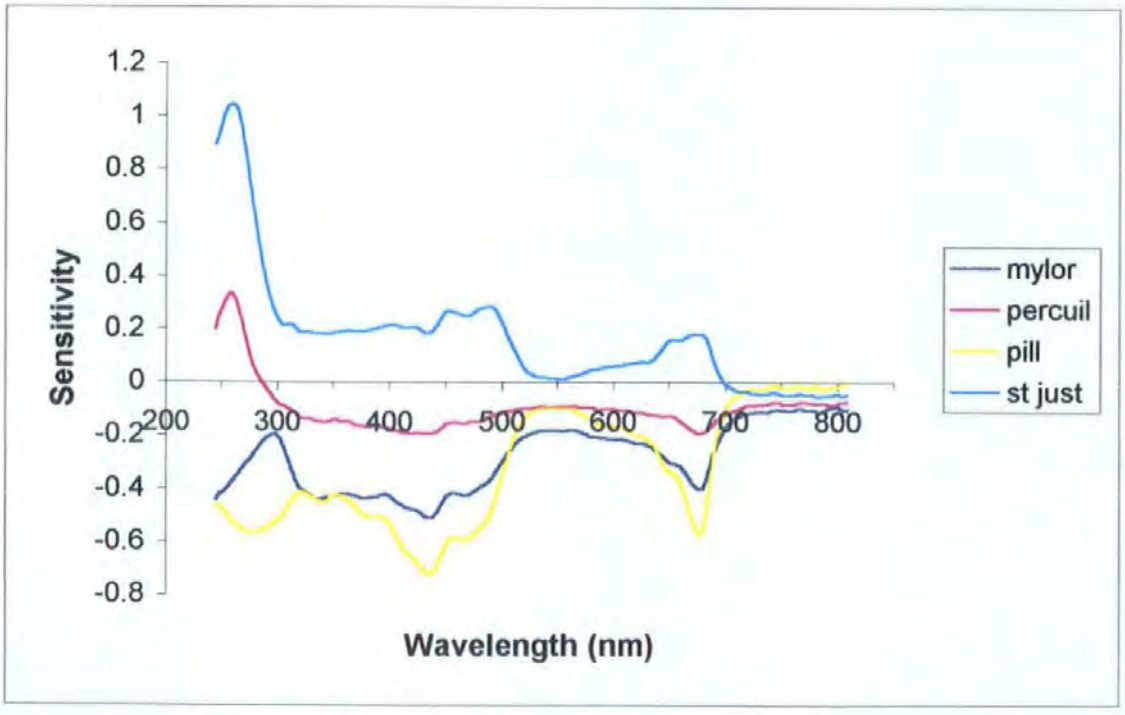

Figure 123: **Sensitivity of** ***in vivo*** **transmittance factor of** ***E.intestinalis*** **collected from 'metal polluted' sites**

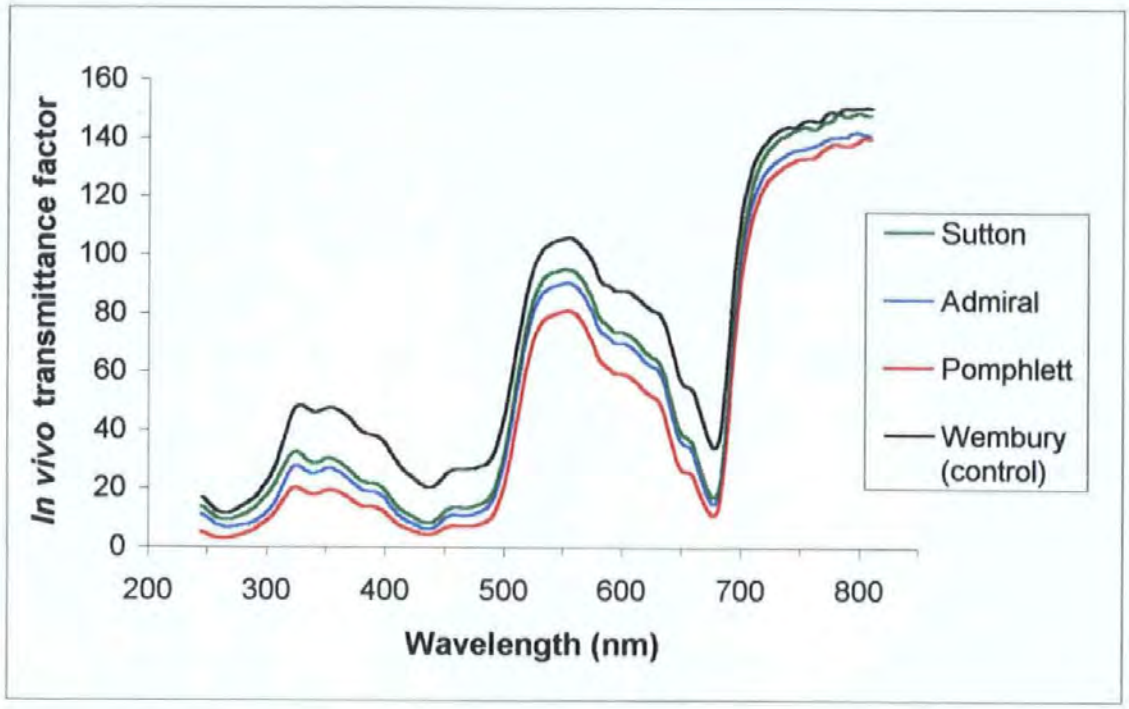

Figure 124: ***In vivo*** **transmittance factor of** ***E.intestinalis*** **collected from different potential 'organic' polluted sites**

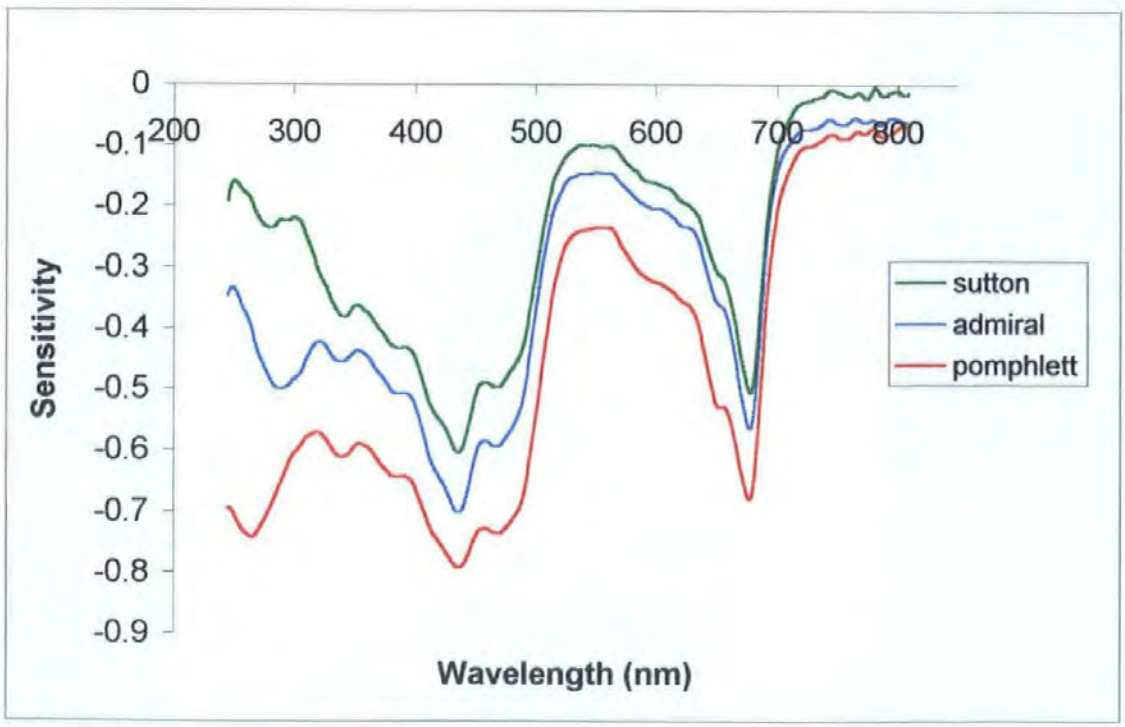

Figure 125: **Sensitivity of** ***in vivo*** **transmittance factor of** ***E.intestinalis*** **collected from different potential 'organic' polluted sites**

obtained from the other sites. Although, at this stage, it may not be possible to identify the particular pollutant types they have been exposed to, it is likely that the pollutants at these 3 sites are different to the other sites. This indicates that, at this stage, it is possible to discriminate between particular pollutants in the field using *in vivo* spectral properties.

Figure 121 appears to be showing a low copper signature when compared with Fig 20, Chapter 4, with increased *in vivo* transmittance at 440 and 680 nm in comparison to the control. This indicates that the samples obtained from this site have less chlorophyll than the control sections, which could be due to exposure to low levels of copper. Thurlestone is generally thought of as a clean site and the differences could be due to an alternative effect such as higher levels of nitrogen in the control site. Figure 125 illustrates that the *in vivo* transmittance factor is lowered at these 3 sites in relation to the control, especially at 440 and 680 nm. This indicates that, as less light is being transmitted at these wavelengths, more light must be reflected or absorbed. Examination of Figures 131 and 132, the *in vivo* reflectance graphs for these sites, does not show significant evidence for increased reflection at these wavelengths. Therefore, more light must be being absorbed, indicating that there is an increase in the chlorophyll content in relation to the control site, Wembury. This could possibly be due to high nutrient levels in the water or it may be linked with a shading effect caused by the murky water. Water samples would have to be taken to clarify this, but the technique has shown potential in discriminating between sites and, therefore presumably, pollutant exposure.

In vivo reflectance

Figures 126 - 132 relate to the *in vivo* reflectance of the samples from each site. The traces of *in vivo* reflectance for each site are relatively noisy, even with medium smoothing, but they do highlight some interesting data. As with the *in vivo* transmittance, the Thurlestone site (Figure 128) appears to be exhibiting a copper signature with the *in vivo* reflectance. The other main observation is that algae collected from Sutton, Admiral's Hard and Pomphlett Creek fall into a distinct group again, as with the *in vivo* transmittance, that is different from the results obtained for the other algae. Although it is not possible to identify the pollutants at these sites using the information already obtained, it is evident that the use of *in vivo* reflectance provides a distinction between the sites.

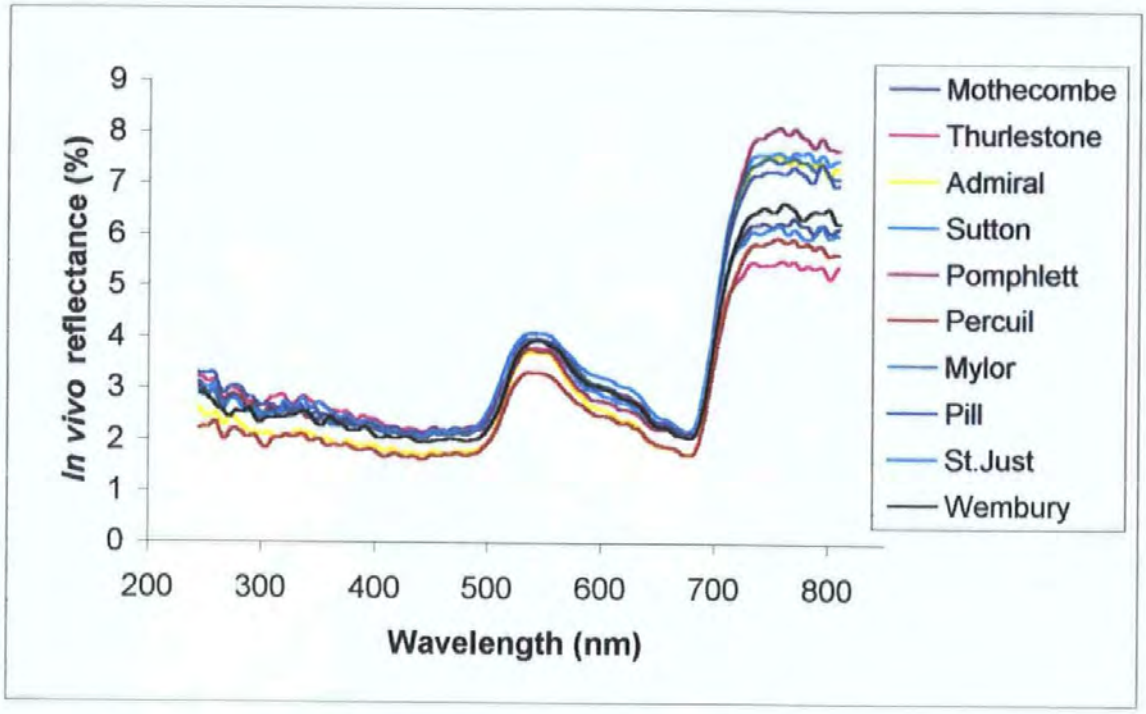

Figure 126: ***In vivo*** **reflectance of** ***E.intestinalis*** **collected from different sites**

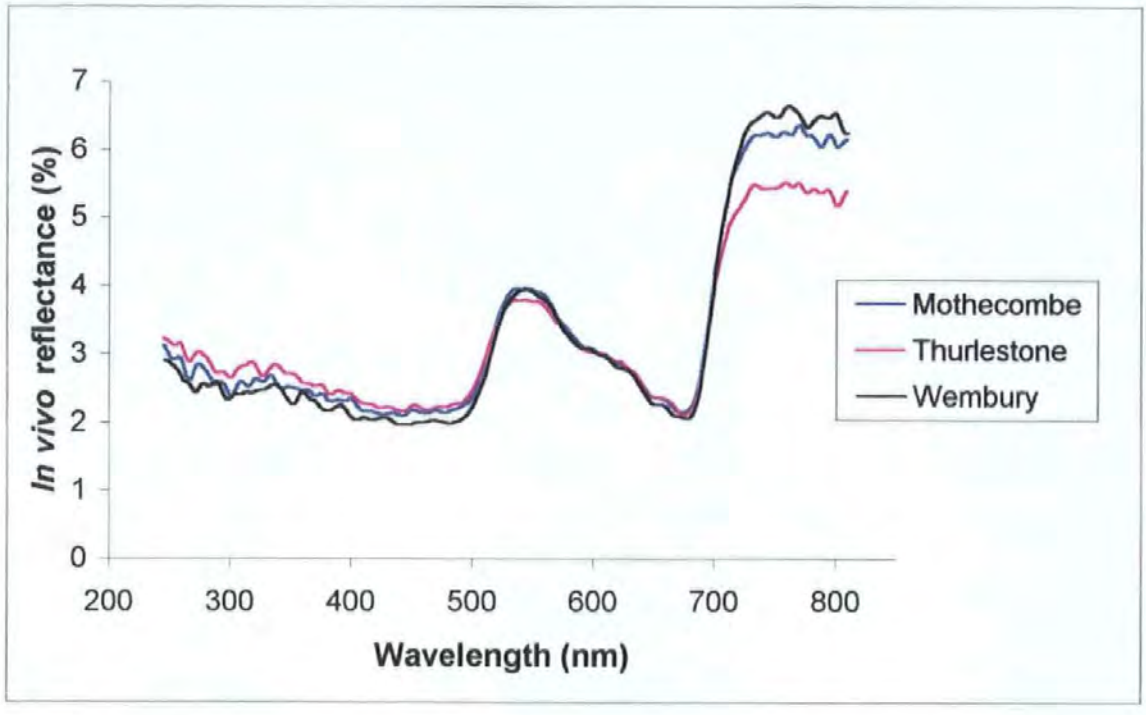

Figure 127: ***In vivo*** **reflectance of** ***E.intestinalis*** **collected from 'clean' sites**

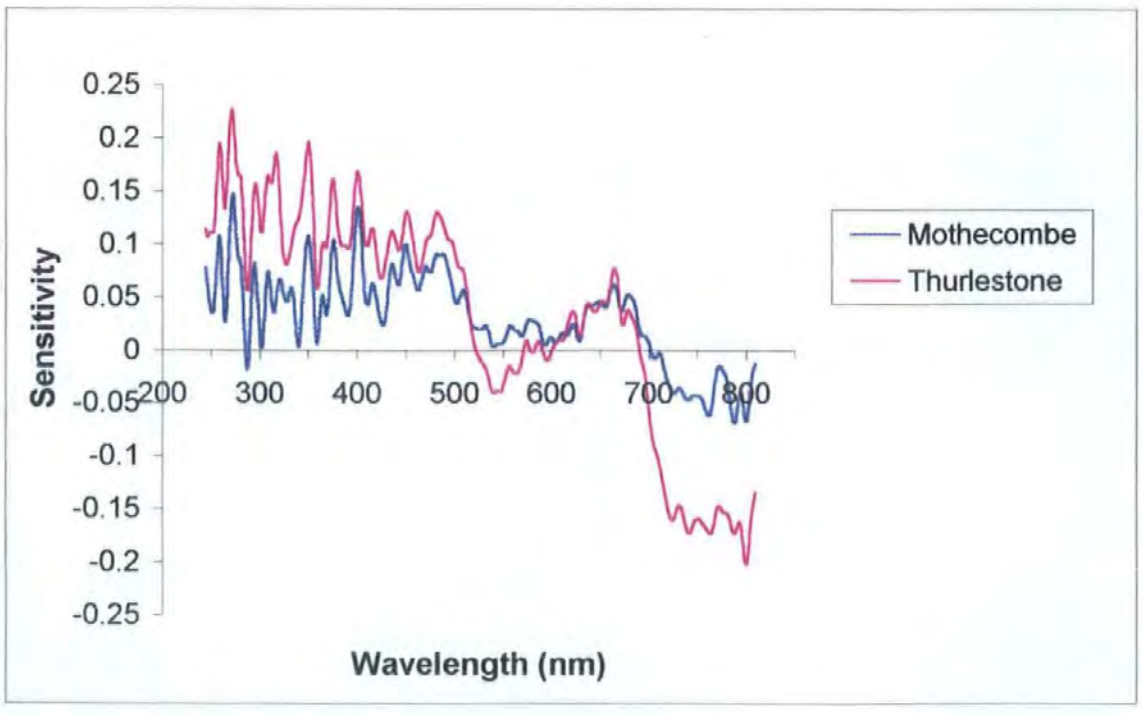

Figure 128: **Sensitivity of** ***in vivo*** **reflectance of** ***E.intestinalis*** **collected from 'clean' sites**

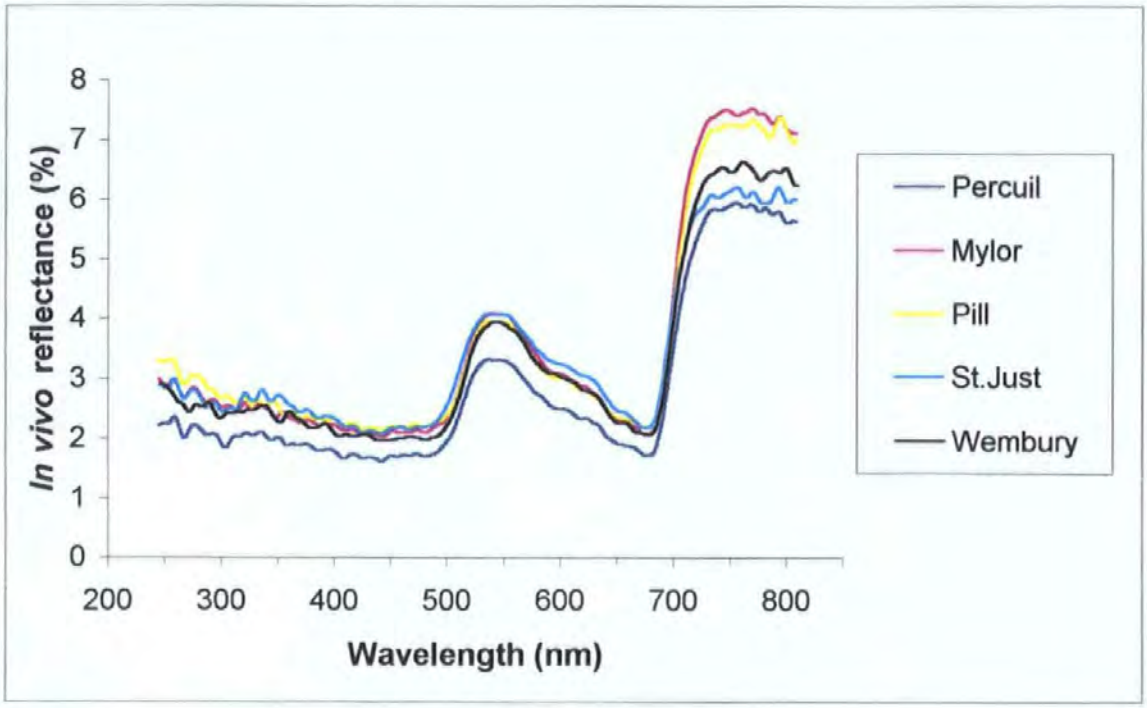

Figure 129: ***In vivo*** **reflectance of** ***E.intestinalis*** **collected from 'metal polluted' sites**

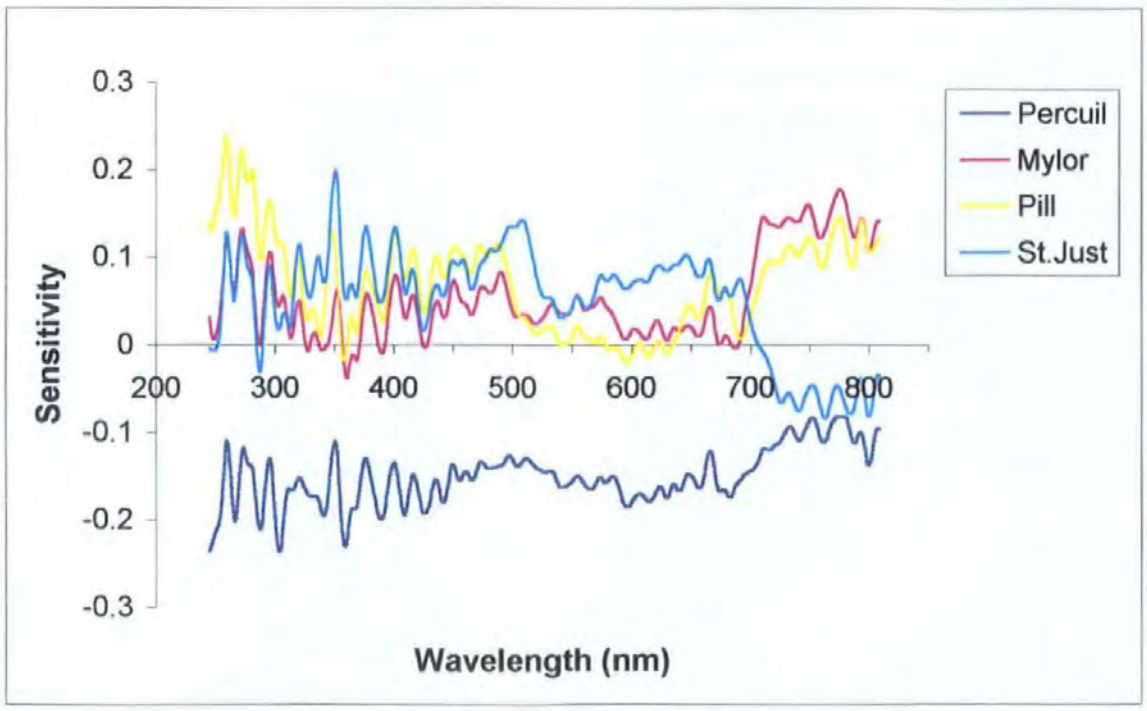

Figure 130: Sensitivity of ***in vivo*** **reflectance of** ***E.intestinalis*** **collected from 'metal polluted' sites**

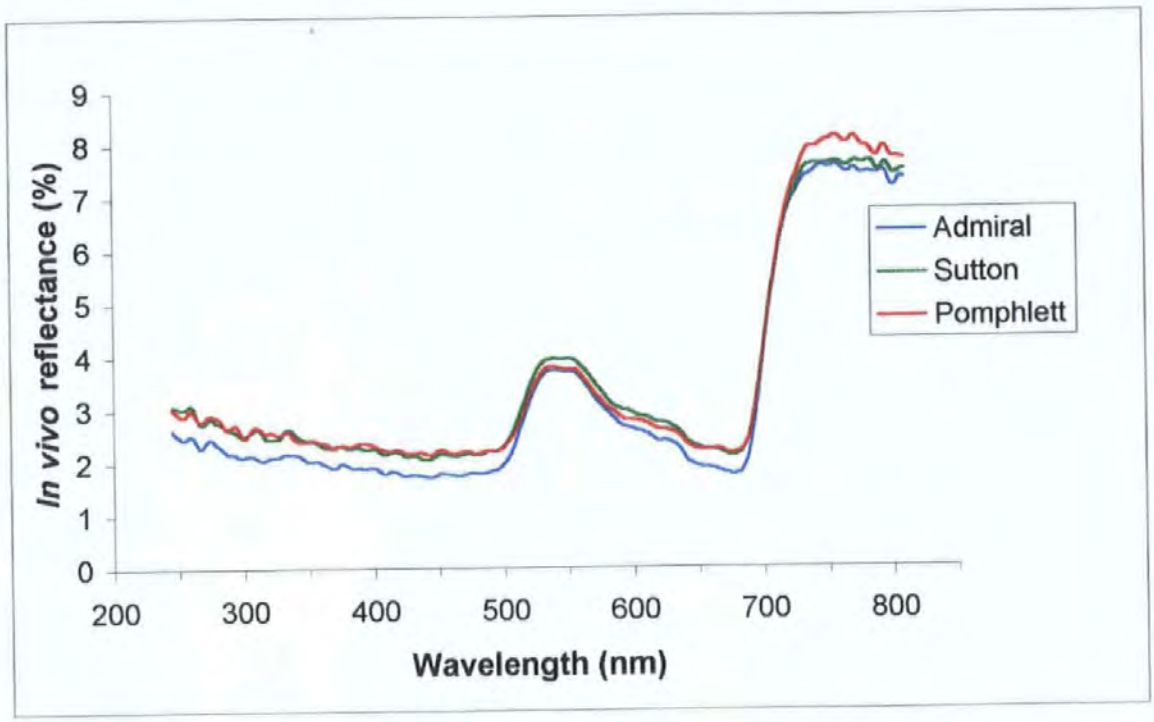

Figure 131: ***In vivo*** **reflectance of** ***E.intestinalis*** **collected from potential 'organic polluted' sites**

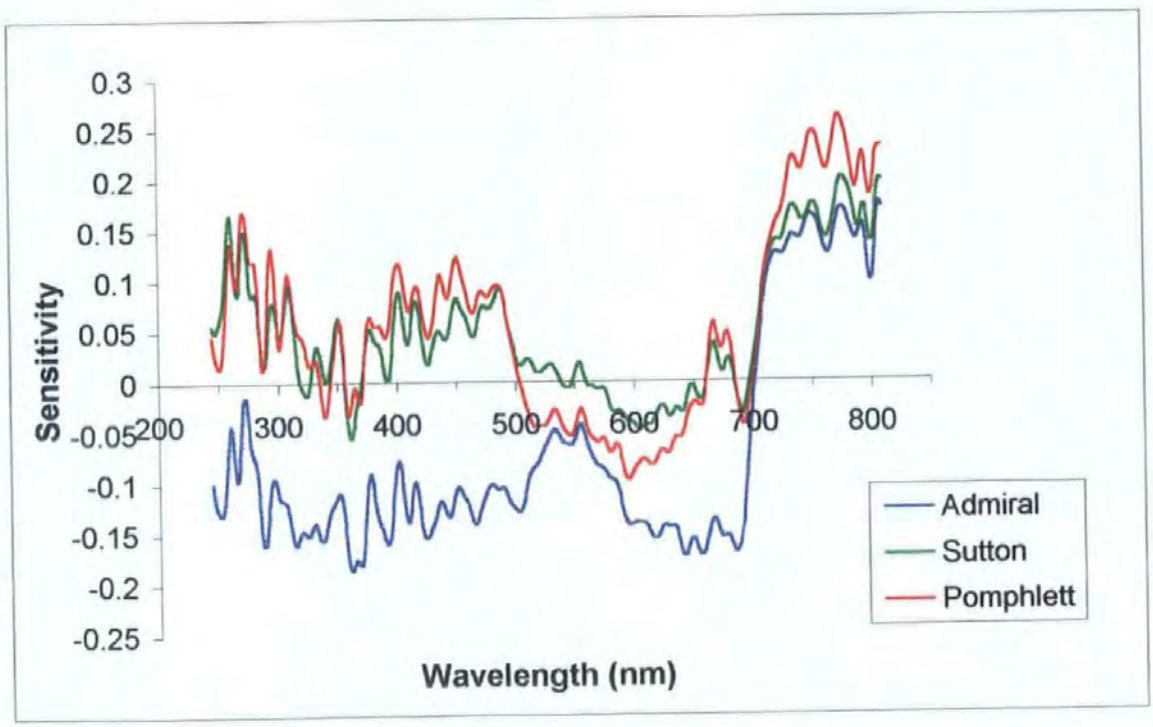

Figure 132: **Sensitivity of** ***in vivo*** **reflectance of** ***E.intestinalis*** **collected from potential 'organic polluted' sites**

Construction of 'web' diagrams and other manipulations of the data

The in vivo spectral results demonstrate that results obtained are certainly different for certain sites, with responses appearing to fall into distinct groups depending on the type of location and pollutant exposure. This indicates that they can be added to a growing list of biomarkers to study pollutant effect. Some of the main in vivo spectral parameters were chosen and plotted together as a web diagram with the fluorescence data obtained for each site (Figures 133 – 135). This revealed some interesting results with certain sites, in particular the 3 'organic' polluted (Figure 133) having a very distinctive shape. Once the appropriate parameters have been selected, it is important that the order of the axes remains the same each time they are plotted or this would change the shape of the web obtained. However, the use of the web diagrams are an extremely useful tool in observing change due to pollutant exposure as they provide an instant visual representation of the main parameters affected. The use of more than one parameter also provides a clearer insight into the mode of action of a pollutant.

The web diagrams for the sites sampled were plotted using a variety of in vivo spectral property responses together with fluorescence responses relevant to the control, Wembury (control = 1). It is evident that the web shapes obtained are different for some of the sites and, as mentioned above, this is extremely clear when comparing the responses of samples collected from Sutton, Admirals Hard and Pomphlett, all 3 being thought to exhibit possible organic pollution. The web shape of samples from these 3 sites is very distinctive with percentage *in vivo* transmittance at 440 nm and 680 nm being significantly less than the control and percentage *in vivo* transmittance ratio of 550:680 nm and area above the fluorescence curve being significantly increased. This shape of the web is also present, but to a lesser extent in samples collected from Mylor and Percuil. This could indicate that there are similar pollutants found at these sites in addition to the documented metal pollution.

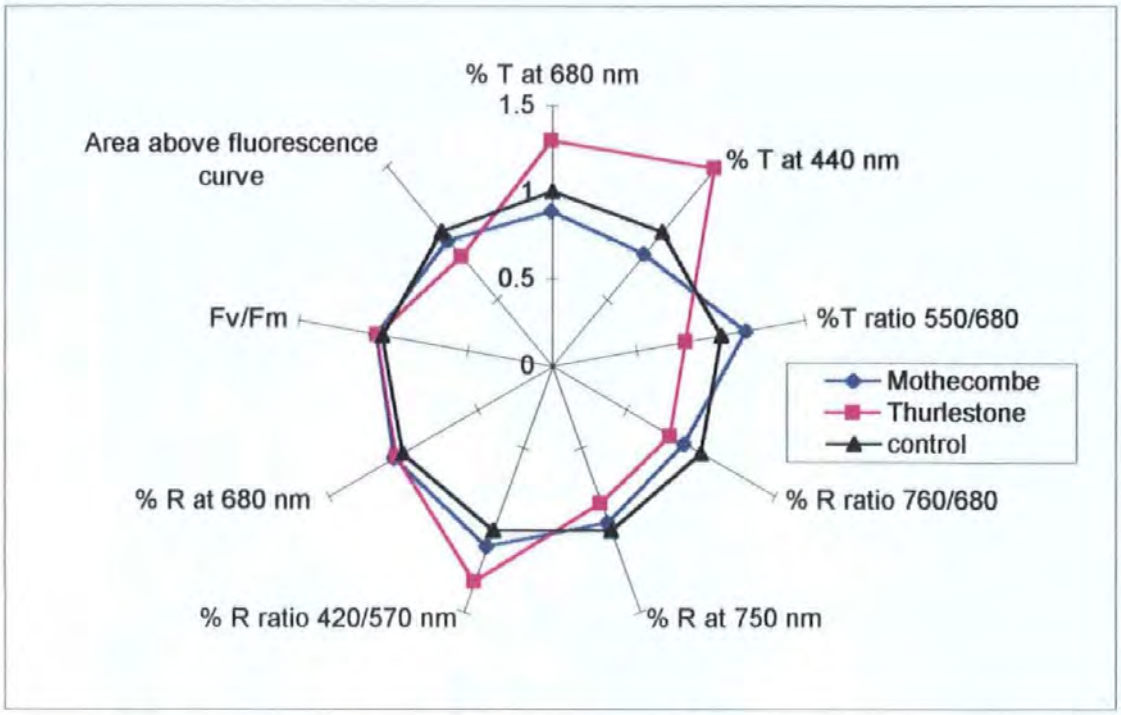

Figure 133: Comparison of selected parameters of *E.intestinalis* collected from 'clean' sites

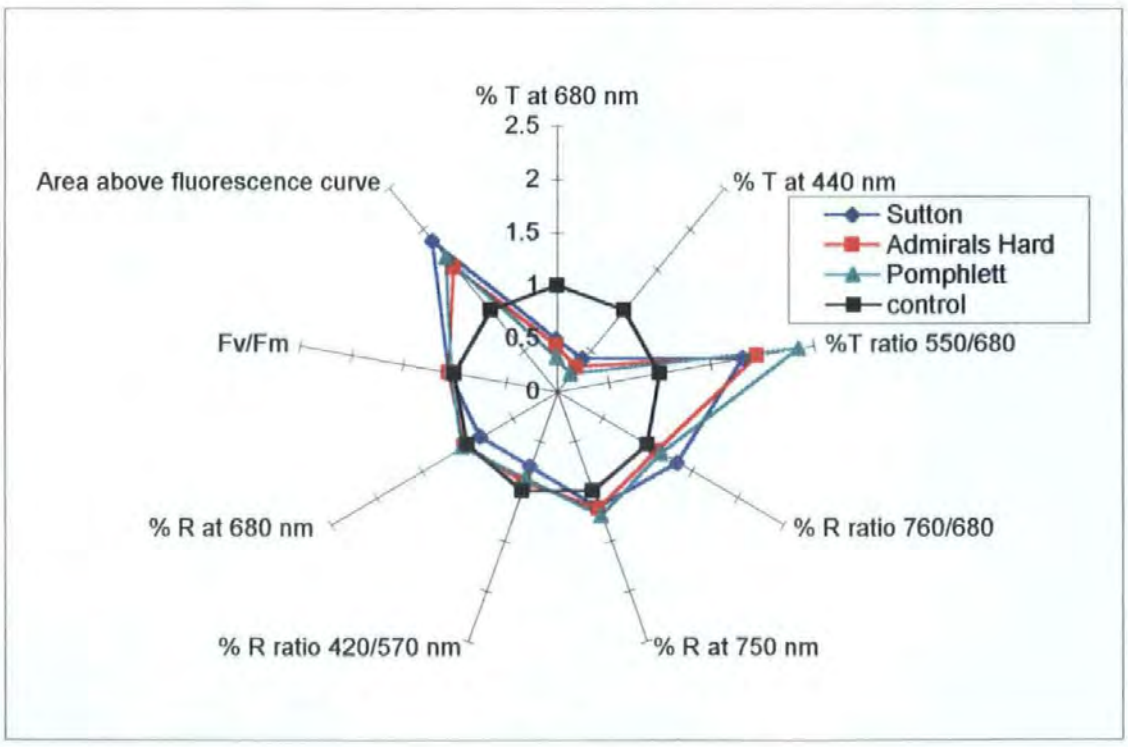

Figure 134: Comparison of selected parameters of *E.intestinalis* collected from potential 'organic' polluted sites

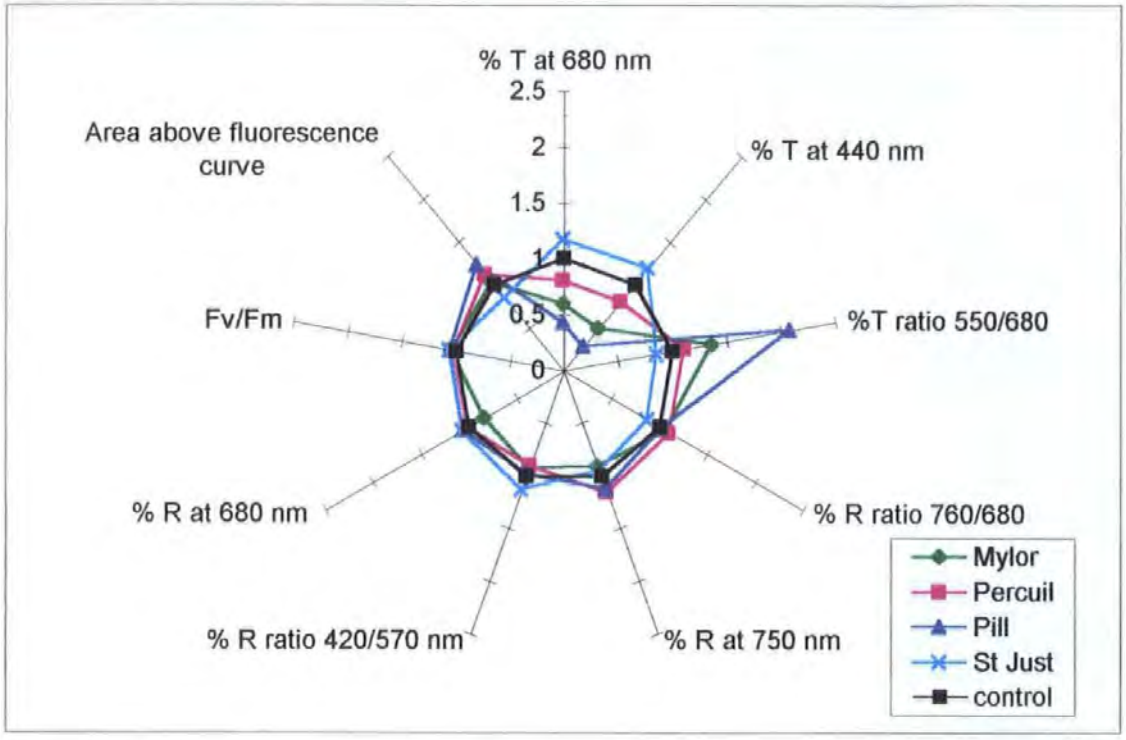

Figure 135: Comparison of selected parameters of *E.intestinalis* collected from 'metal polluted' sites

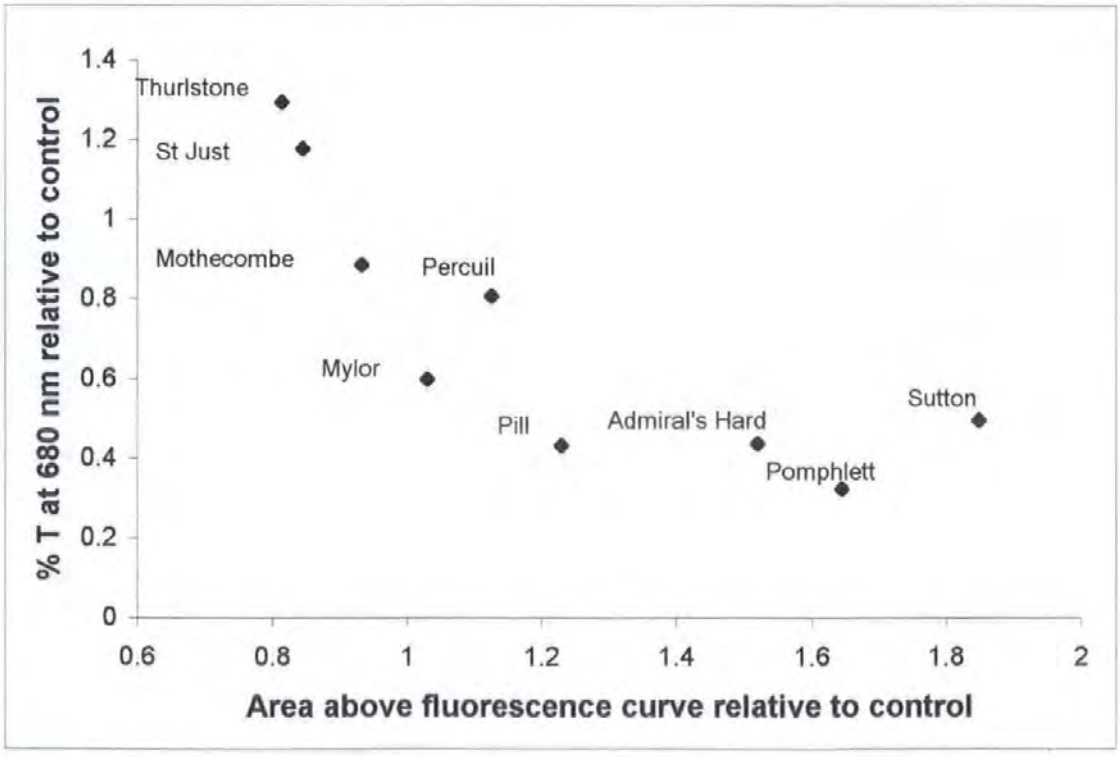

Figure 136: Separation of responses, using percentage *in vivo* transmittance factor at 680 nm and area above fluorescence curve, of *E.intestinalis* collected from different sites

The use of web diagrams can also be used to identify parameters that are most affected by pollutant exposure and therefore selected to produce plots of separation of responses. Examples of separation of response plots are shown in figures 136 - 138 with parameters selected from the in vivo spectral responses of the algae. These indicate that those sites thought of as either ' clean' or 'organically polluted' appear to fall into distinct groups in the plots. The other sites, 'metal polluted', fall between the two groups with samples collected from St. Just appearing to have a similar response to those collected from 'clean' sites. This was not unexpected as St. Just is one of the cleaner sites selected from the metal polluted areas according to the record of the gradient of metal pollution.

These results highlight the potential use of *in vivo* spectral properties of samples collected from the field. It is clearly evident that the samples of *E.intestinalis* have different *in vivo* spectral properties and that these can be used to distinguish between different sites and, possibly, provide an indication of the type of pollutant exposure that has occurred. It may be possible to select appropriate parameters from laboratory studies to provide 'standard webs' for different types of pollution and to use these to investigate pollutant effects at different sites where pollutant records may not already be known.

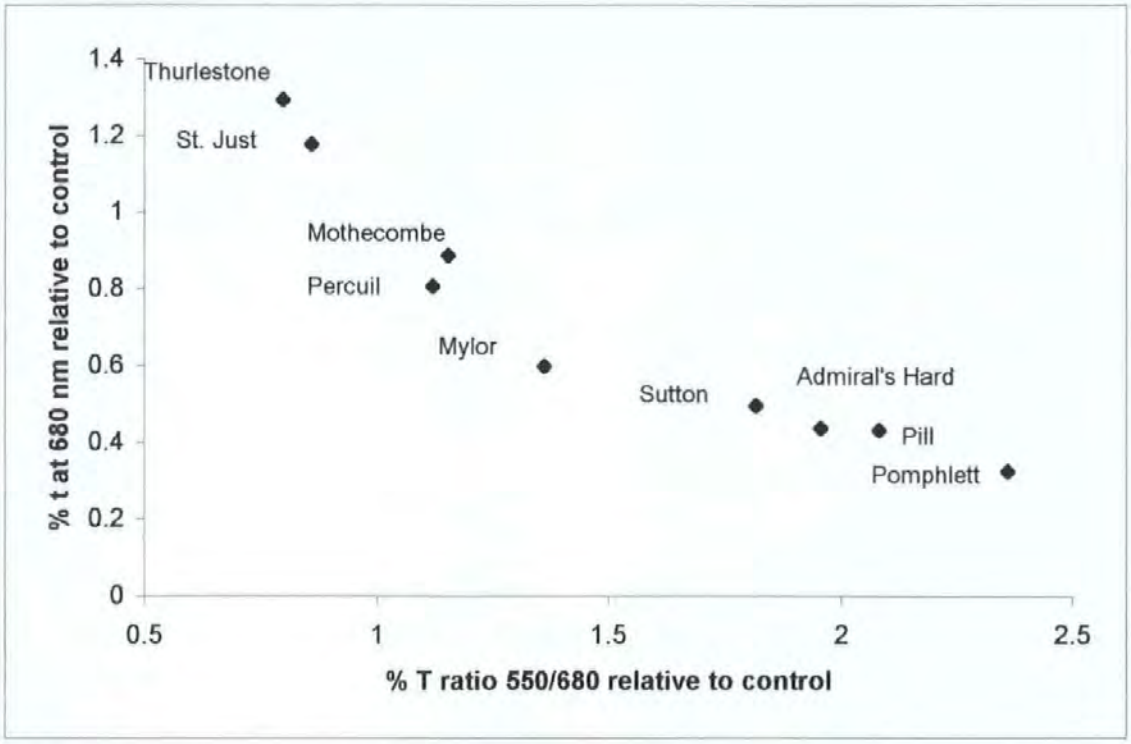

Figure 137: Separation of responses, using percentage *in vivo* transmittance factor at 680 nm and percentage *in vivo* transmittance factor ratio 550/680 nm, of *E.intestinalis* collected from different sites

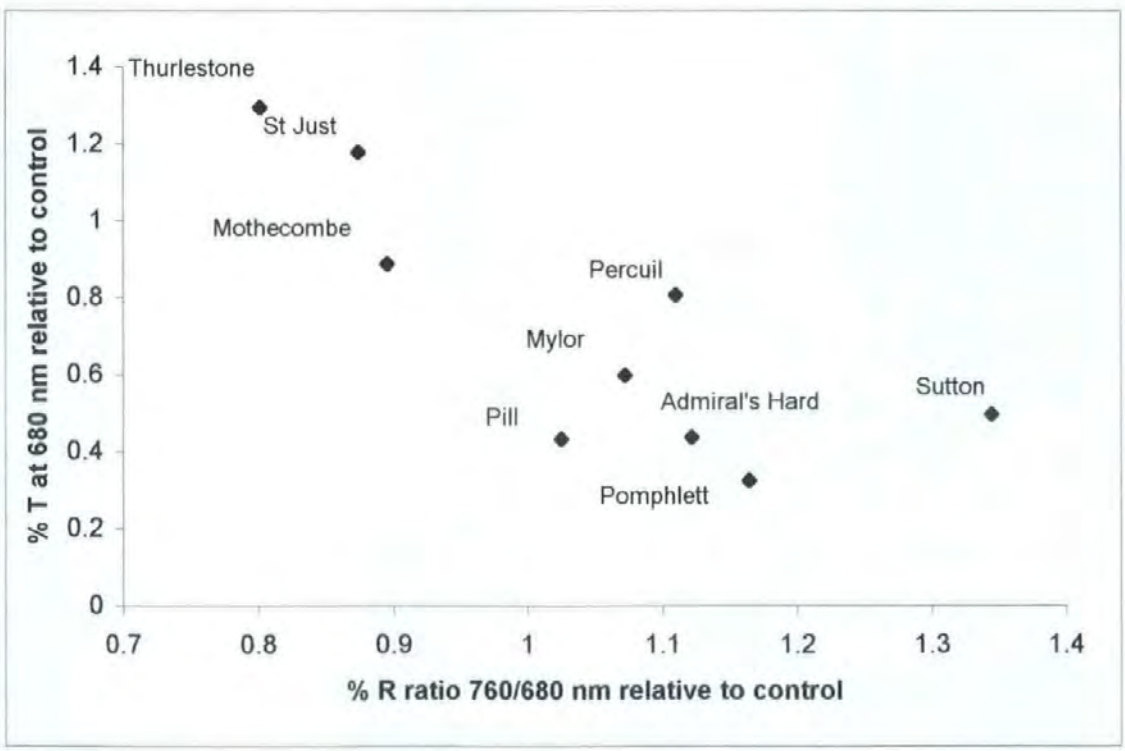

Figure 138: Separation of responses, using percentage *in vivo* transmittance factor at 680 nm and percentage *in vivo* reflectance ratio 760/680 nm, of *E.intestinalis* collected from different sites

8. REMOTE SENSING

8.1 INTRODUCTION

Algae are of major ecological importance in coastal regions and therefore they can be exploited as static environmental monitors. Traditional methods of mapping the distribution of intertidal macroalgae include aerial photos and fieldwork that can be both time consuming and costly if large areas are to be mapped. An alternative method could be the use of remote sensing methods involving reflectance measurements, as this could be used to monitor large areas of coastline over a relatively short period of time. This would enable areas of the intertidal region, which are only exposed for short periods of time depending on the tide, to be mapped effectively. In addition to mapping the location of algae, it may also be possible to monitor whether stress, due to pollutants, has occurred.

Remote sensing encompasses a variety of techniques but can be broadly defined as,

'the use of sensors to record images of the environment which can be interpreted to yield useful information'

(Curran, 1987)

Several major steps are involved including ground truthing, data acquisition and data processing to make maximum use of the information obtained. Many remote sensing techniques involve the use of the spectral response *ie.* the way in which an object reflects energy in different parts of the spectrum. This spectral response can then be used to identify the region of the spectrum that the objects can be distinguished in. An example of this is the differentiation of different types of vegetation by recording the reflectance in the red and infra-red regions of the spectra.

As mentioned previously (Chapter 1), the pigments, structure and water content affects the spectral response of vegetation with pigments affecting the 400 – 700 nm region, internal structure affecting the 700 – 1300 nm region and water content affecting the 1300 – 2500 nm region (Guyot and Guyon, 1989). Knowledge of a typical spectral response of vegetation can therefore be used extensively in remote sensing. Examples of this include vegetation mapping and change, chlorophyll estimation, and stress monitoring. Higher plants and algae have great potential for use with remote sensing because they have a rich variety of pigment components (Guillaumont *et al.* 1997). Multispectral remote sensing has been used to monitor crop types due to the individual spectral responses of different plant types with some of the spectral signatures being obtained from laboratory data (Barrett and Curtis, 1992). It has also been used to map *Enteromopha sps.* in estuaries in Oregon using the LANDSAT digital mapper (Murray, 1981). In addition to the mapping of crop species, remote sensing has also been used to monitor the effect of various crop diseases including corn leaf blight that is caused by a fungus, *Helminthosporium maydis.* The result of the study was that analysis of the remotely sensed data did provide enough information to detect moderate to severe outbreaks of infection (Barrett and Curtis, 1992). It is thought that spectral signatures of vegetation, which can be obtained in the laboratory using an integrating sphere or in the field using a handheld spectroradiometer, are both repeatable and predictable (Guillaumont *et al*, 1997).

As mentioned above, there are several applications that involve remote sensing in vegetation studies. These include vegetation mapping (Penuelas *et al* 1993, Singh, 1987) chlorophyll estimation (Chapelle *et al* 1992), the normalised vegetation index (Guillaumont *et al*, 1997) and the red edge effect (Munden et al, 1994, Filella and Penuelas, 1994, Vogelmann, 1993) and stress (Jackson, 1986). Some of these techniques rely on the use of band ratios. An example of this is the Normalised Difference Vegetation Index (NDVI).

$$NDVI = \frac{R_{NIR} - R_{RED}}{R_{NIR} + R_{RED}}$$

where R_{NIR} = Reflectance value at infrared wavelengths

and R_{RED} = Reflectance value at red wavelength

The NDVI is frequently used as it provides an indication of the vegetation density and has been used in mapping macroalgae (Bajjouk *et al* 1996).

The aim of this study was primarily to assess whether algal types could be identified using remotely sensed images and whether data obtained in the laboratory could be linked with the data obtained remotely. Comparisons of data obtained using the Compact Airborne Spectrographic Imager (CASI) and the Airborne Thematic Mapper (ATM) were also made to assess whether it was more beneficial to select specific wavebands to expand the possibility of being able to use the data to monitor the algal dynamics using remote sensing methods.

8.2 METHODS

8.2.1 FIELD SITE

The region that was investigated during this study was the intertidal area of the Tweed estuary and the surrounding coastal environment. This area was chosen as it coincided with the Land-Ocean Interaction Study (LOIS), therefore flights were already being performed, and preliminary observations revealed that the area had a diverse variety and large stands of seaweed that were suitable for remote sensing activities. The LOIS, 1994, study was concerned with

' innovative studies on the dynamic properties of the land and ocean boundaries to the North Sea to determine how materials are transformed and transported within river

catchments and at the land-atmosphere-ocean interfaces, and how these interfacial interfaces processes affect estuarine and shelf sea ecology and water quality as well as coastal dynamics.'

(LOIS newsletter 1994)

A map of the region chosen is shown in figure 139

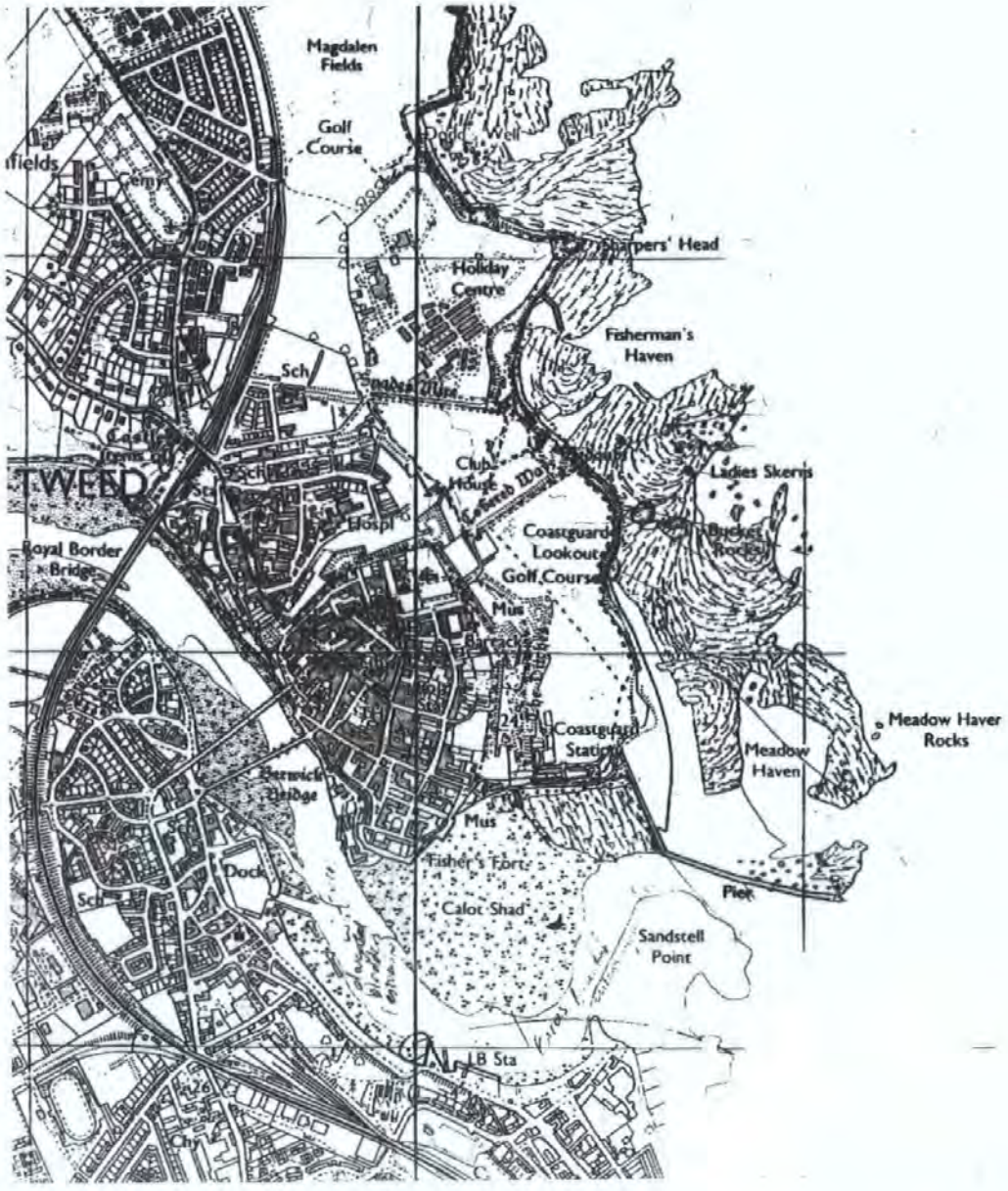

Figure 139: Region of Berwick upon Tweed (adapted from Ordnance Survey Map)

8.2.2 GROUND-TRUTHING

Before remote sensing can be performed, it is essential that groundtruthing of the area is carried out. Ground truthing of the area, usually done manually and in great detail to identify the major species and their respective positions, has several important functions. It is important that certain parts of a remotely sensed image can be linked to an area to enable the image to be interpreted correctly. Secondly, the data collected by remote sensing is collected from 'pixels' and these pixels may have more than one vegetation type in them. The smaller the pixel sizes, the better, which is why aircraft sensors are now frequently used for many studies. The problem of mixed pixels can be overcome by various data processing techniques including principal component analysis and mixture modelling. However, for these techniques to be used, pixels containing 100 % of a certain vegetation or substrate are required. These pixels are called 'endmembers' and their position is linked to the final images obtained by a combination of extensive groundtruthing and the use of a Global Positioning System (GPS).

Pixels containing endmembers were identified for green and brown macroalgae with a region of 100 % coverage with *E.intestinalis* being chosen from the coastal area and an area of mixed fucoids and *Ascophyllum* chosen from the estuarine area. Photos of these endmembers, to clarify both composition and area were taken in addition to the position being recorded with the GPS. Quadrats were taken for each endmember region and these were photographed individually to assess total coverage. Endmembers of sand, water and rock were also identified, photographed and recorded using the GPS to enable them to be identified in the images obtained.

Plate 9: Endmember of *E.intestinalis*

Plate 10: Endmember of *Ascophyllum nodosum*

In addition to endmember selection, the region was also examined generally to determine additional species present. This is important as the presence or absence of particular species could be linked to certain kinds of exposure. Photos of species identified were taken, together with panoramic views of the entire region, to ensure the remotely sensed images could be interpreted accurately. In addition, any distinctive features of the region, including large rockpools and unusual rock formations were photographed and their position recorded with the GPS. All features identified were also identified on a map of the region and transects were used to clarify positions with respect to the map.

8.2.3 SENSORS USED

There are a variety of sensors that can be used for collecting data, each type having its advantages and disadvantages. These can include the cost, amount of data processing required, spectral and spatial characteristics. Remote sensing of the chosen area was carried out using a Compact Airborne Spectrographic Imager (CASI) and the Airborne Thematic Mapper (ATM). The altitude of the flight for both the ATM and CASI was 1353m and a low tide of 0.7 m at Berwick upon Tweed was at 9.37 GMT. The CASI data was obtained at 11.35 GMT and the ATM data was acquired at 10.10 GMT. The flight path incorporated a large coastal area and the mouth of the estuary. Further flights were performed covering further up the estuary and coastline but these are not analysed here. In addition to the CASI and ATM images, aerial photographs of the same regions were also taken.

The ATM is a scanner with sensors that acquire data from 11 spectral bands.

	Wavelength (nm)
Band 1	420 – 450
Band 2	450 – 520
Band 3	520 – 600
Band 4	605 – 625
Band 5	630 – 690
Band 6	695 – 750
Band 7	760 – 900
Band 8	910 – 1050
Band 9	1550 – 1750
Band 10	2080 – 2350
Band 11	8500 – 13000

The most relevant bands for use in vegetation studies are bands 1 – 7.

The CASI is a multispectral imager, developed in 1989, light enough for use on a small aircraft where the bandwidths can be chosen to suit an individual study. It can be used in spatial (imaging) or spectral (multispectrometer) mode. Spectral mode has the advantage that the full spectral information from 430 nm – 870 nm is collected but the number of pixels along the line of scene is then reduced from 578 to just 39. An alternative is to use the CASI in spatial mode. This restricts the spectral information obtained to a limited number of bands, usually 8 or 12, but the pixel size is much smaller, resulting in information being collected from up to 578 points (LOIS, 1994).

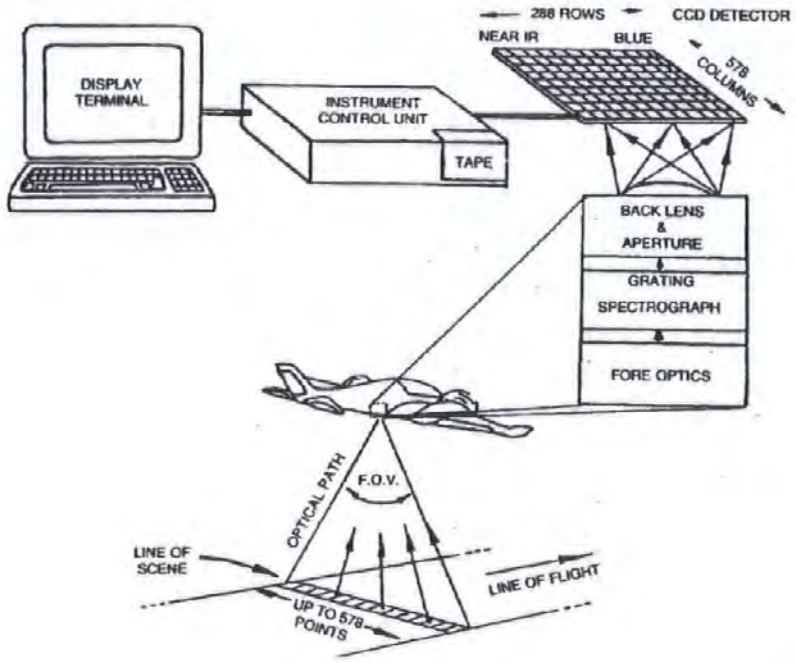

Figure 140: CASI data acquisition (from LOIS, 1994)

The CASI used was set to obtain a high spectral resolution and the spectral bands chosen were based on those used in a study, by Zacharias *et al* (1992), to determine a miltispectral bandset for the remote sensing of intertidal seaweeds.

	Wavelength (nm)
Band 1	436.2 – 450.3
Band 2	547.7 – 562.0
Band 3	662.0 – 674.5
Band 4	678.1 – 688.3
Band 5	748.3 – 753.7
Band 6	847.8 – 882.3

The data was obtained and preprocessed, including being radiometrically corrected, by the Natural Environment Research Council (NERC). Several flights were obtained using both

the CASI and ATM and two of the images were chosen for comparison. The area of the estuary was chosen for comparison because the amounts of seaweed were large and, therefore, mixed pixels would not be as much of a problem as for the data recorded on the rocky coastline. True and false colour composites were produced to ascertain whether it was possible to identify the different seaweed types.

8.3 RESULTS AND DISCUSSION

8.3.1 GROUND TRUTHING

Berwick upon Tweed is varied with an outer coastline and an estuary area. The dominant species in the estuary area were brown and green algae consisting primarily of *Fucus sps.*, *Ascophylum nodosum* and *Enteromorpha sps.* There were, in addition to these types, some red algae on the intertidal area of the exposed coastline but these were not investigated using the remote sensing, as there were not sufficient quantities present. The outer coastline had an unusual rock formation which appeared to affect the distribution of the seaweed (Plate 11)

The outer coastline was more exposed than the estuary and there was a diversity of algal species with large amounts of *Fucus sps.*, *Ascophylum nodosum* and *Enteromorpha sps.* The endmember for Enteromorpha (Plate 9) was selected from this area.

Plate 11: Rock formation at Berwick upon Tweed

Plate 12: Outer coastline of Berwick upon Tweed

The exposed areas of the estuary, as mentioned above, had very large areas of *Fucus* and *Ascophyllum* species. It also had an unusual rock formation, with the rocks arranged in a striplike fashion. The *Fucus* and *Ascophyllum* were primarily attached to the long strips of rock and there were large quantities of *Enteromorpha sps.* in between. Endmembers for *Fucus* and *Ascophyllum* were selected from this area. It was also necessary to take endmembers for other substrates *ie.* sand and rock. At low tide, it was apparent that the mudflats of the estuary were occupied primarily by *Enteromorpha sps.* and *Cladophora sps.*

The aerial photographs of the region clearly distinguished between certain areas and types of vegetation. Altough they may not offer the same potential of monitoring pollutant stress as the CASI and ATM images, they still have many advantageous features including being more accessible and cheaper to obtain than a CASI image. They also offer potential for monitoring change in species coverage over time or spatially as they can be placed on an image analyser and the percentage coverage of different algal types can be mapped. Plate 15 is an example of one of the aerial photos obtained.

Plate 13: Calot Shad – the mouth of the estuary of Berwick upon Tweed

Plate 14: Seaweed near the mouth of the estuary

Plate 15: Aerial photo of Berwick upon Tweed estuary and outer coastline

8.3.2 COMPARISON OF CASI AND ATM IMAGES

The ATM and CASI images obtained of the mouth of the Tweed estuary were obtained as follows:

Figure 141 – Near true colour composite CASI image with band 4 (678.1 – 688.3 nm), band 2 (547.7 – 562.0 nm) and band 1 (436.2 – 450.3 nm) loaded into red, green and blue respectively.

Figure 142 – False colour composite CASI image with band 5 (748.3 – 753.7 nm), band 4 (678.1 – 688.3 nm) and band 2 (547.7 – 562.0 nm) loaded into red, green and blue respectively.

Figure 143 – Near true colour composite ATM image with band 5 (639 – 690 nm), band 3 (520 – 600 nm) and band 2 (450 – 520 nm) loaded into red, green and blue respeectively.

Figure 144 – False colour composite ATM image with band 7 (760 – 900 nm), band 4 (605 – 625 nm) and band 2 (450 – 520 nm) loaded into red, green and blue respectively

It can be seen that in both the ATM and the CASI true colour images the vegetation is visible but it is difficult to distinguish it from other components in the image. However, in the false colour composites the bands were selected so that the vegetation would appear as different shades of red and the different areas of algae can be clearly seen. Ground truthing revealed that the bright red area in the mouth of the estuary is composed of phaeophytes of which *Fucus* species and *Ascophyllum nodosum* were dominant. The paler red region in the estuary mouth was composed of the chlorophyte, *Enteromorpha sps.*

It appears that the CASI images are not as sharp as the ATM images because a slight aircraft roll occurred whilst collecting the data. However, there is more contrast between the vegetation types in the CASI images due to being able to select the bands required for the CASI data collection based on previous knowledge of vegetation. Another consideration when comparing the ATM and CASI images presented is that the flight path

Figure 141: Near true colour composite CASI image of Berwick upon Tweed

Figure 142: False colour composite CASI image of Berwick upon Tweed

Figure 143: Near true colour composite ATM image of Berwick upon Tweed

Figure 144: False colour composite ATM image of Berwick upon Tweed

continued further upstream and the tide is lower in the ATM image so more algae is exposed. This highlights the importance of timing of the flights over a coastal region where certain areas are only exposed for short periods of time.

The images produced could be processed further to provide more information but they do indicate that it is possible to distinguish between the main algal types. Figures 145 and 146 show the use of applying principal component analysis to a similar image. This CASI image was obtained in a different study, part of the Humber estuary, at 3 km altitude. The first image is a near-true colour composite obtained by loading band 7 (662-676 nm), band 5 (547-562 nm) and band 3 (482-498 nm) into red, green and blue respectively. The area in the rectangle is a glacial moraine, which is submerged at high tide and is covered by *Fucus vesiculosis* and *Enteromorpha intestinalis*. The second image is the application of a principal components analysis to produce a colour composite consisting of the first three principal components, derived from the data, loaded into the red, green and blue respectively. In this image the red areas are *Fucus vesiculosis* and the yellow areas are *Enteromorpha intestinalis* (images and processing courtesy of R.Murphy).

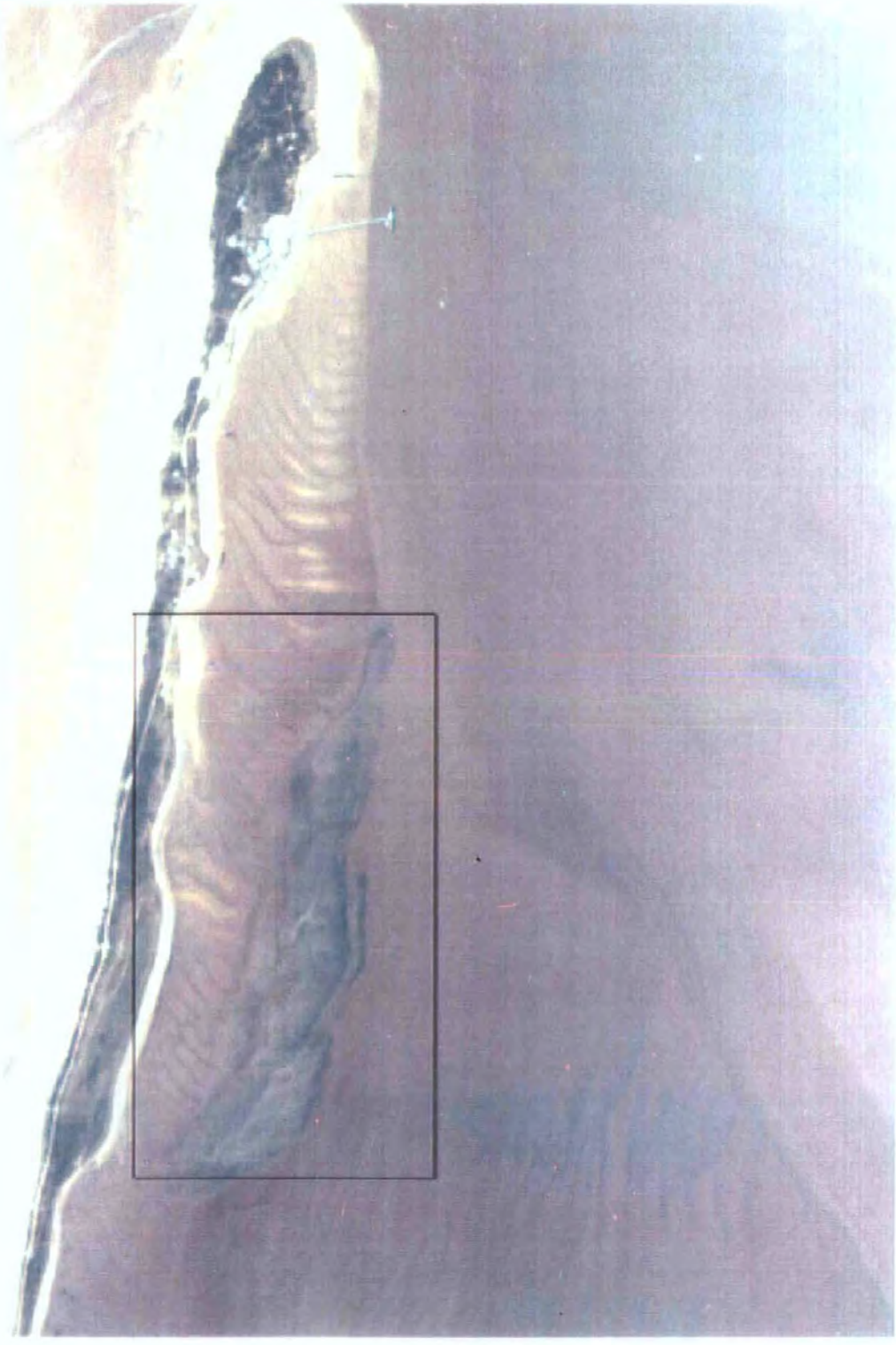

Figure 145: Near true colour composite CASI image of Spurn Point

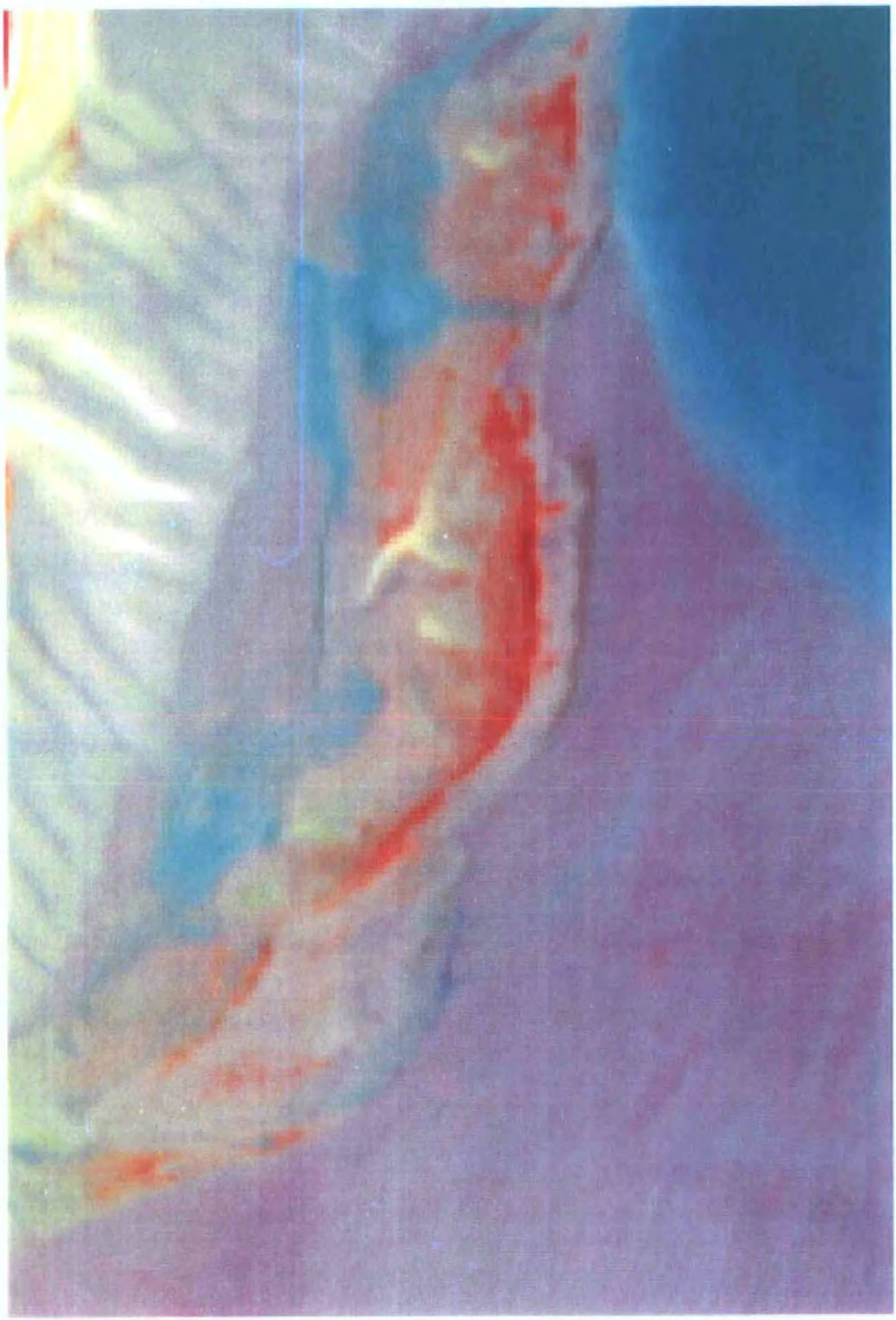

Figure 146: Application of a principal components analysis to the CASI Image of Spurn Point, Humber, Estuary

Bajjouk *et al*, 1996, successfully applied remote sensing techniques to mapping and classifying intertidal seaweed. Remote sensing, therefore, can be used to monitor algal type and distribution as the major species could be identified in the images produced but further work is needed to classify the images. However, as there is a high spatial variability of the algae in the intertidal region the problem of spectral mixing of image components could occur. This could result in mixed pixels as the remotely sensed signals from several spectrally distinct components are integrated over an area of ground represented by each image pixel. The classification system used would therefore have to take this into account. This problem was encountered by Bajjouk *et al* (1996) but it was found that, in addition to identifying the green, red and brown algae, it was also possible to distinguish between 2 groups of brown algae consisting of one group containing *Fucus* and *Ascophyllum* species and a second group containing *Himanthalia* and *Laminaria* species. The use of remote sensing to monitor algal populations could be extremely useful as large areas could be mapped repeatedly leading to a better understanding of changes in intertidal populations over time and, possibly, due to exposure to stresses such as pollution incidents.

The images of the estuary could be used in several ways. These include mapping the seaweed along the length of the estuary and mapping the change in the seaweed composition over longer time periods.

8.4 COMPARISON OF LABORATORY AND CASI DATA

In order to assess the possibility of linking the data obtained in the laboratory studies with that from remote sensing, the effects of copper and Irgarol 1051 on the in vivo reflectance of *Enteromorpha intestinalis* at the midpoint wavelengths of the CASI bands were plotted

(figure 147). As discussed in the respective chapters, there is a difference in the *in vivo* reflectance signatures of *Enteromorpha intestinalis* exposed to copper and Irgarol 1051 and this is highlighted when the midpoint bands of the CASI are plotted. By choosing certain ratios it may be possible to detect changes caused by each pollutant. A suitable ratio in this case appears to be the 420/750 nm ratio as it is significantly different for the pollutants and the control. The 420/750 nm *in vivo* reflectance ratio is 0.19 for the control, 0.33 for copper exposed samples and 0.40 for the *Enteromorpha intestinalis* exposed to Irgarol 1051. This results in a 73 % increase in the ratio for copper and 110 % for Irgarol 1051 indicating that a rise in this *in vivo* reflectance ratio could be used to diagnose that the algae has been exposed to a pollutant. In order to then discriminate between exposure to Irgarol 1051 and copper the actual value of *in vivo* reflectance change at 750 nm could possibly be used as it is apparent from figure 147 that Irgarol 1051 causes a considerable increase at this wavelength in contrast to copper which does not.

The use of remote sensing to monitor changes due to pollutant effects obviously requires more extensive investigation but it certainly offers potential. The ultimate aim would be to use the knowledge of spectral changes, exhibited by macro-algae in response to pollutant effects under controlled laboratory conditions, to assess the use of detecting such changes in CASI data. This could therefore lead to the construction of validated thematic maps of pollutant stressing of macro-algal communities over large intertidal regions.

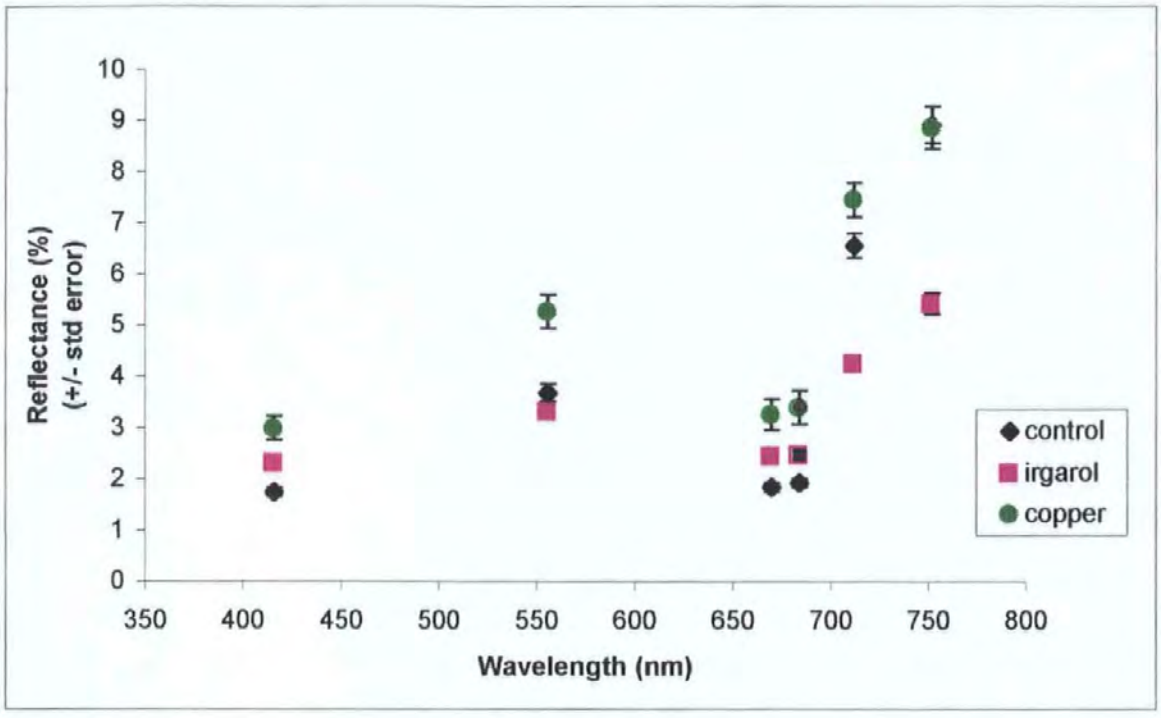

Figure 147: **Effect of Irgarol 1051 (250 ug/l) and copper (500 ug/l) on reflectance of *Enteromorpha intestinalis* at the midpoint wavelengths of the CASI bands selected**

9 DISCUSSION

- **Do spectral changes offer appropriate quantitative parameters for monitoring pollutant stress?**

The results obtained during these studies indicate that spectral changes certainly have the potential to offer quantitative parameters for monitoring pollutant stress, clearly indicating that the *in vivo* spectral properties of *E.intestinalis* change when exposure to a pollutant has occurred. In addition to the *in vivo* spectral properties changing with exposure to a pollutant, it is also evident that these methods can be used to detect different types of pollutant damage. The *in vivo* optical properties of *E.intestinalis* treated with copper (Chapter 4) and Irgarol 1051 (Chapter 5) are very different from each other with copper appearing to affect the pigments, especially chlorophyll, and Irgarol 1051 affecting the structure of the algae. The results also indicate that the method of using *in vivo* spectral properties offers potential with the use of ratios and other mathematical derivations of the data obtained.

- **Do certain wavelengths, ratios and other derivations offer the possibility of resolving damage to particular stresses?**

The results obtained using *in vivo* spectral properties can, as illustrated in the result section of each chapter, be converted into different ratios and derivations. These manipulations of the data extend the use of the technique, as they highlight certain aspects that may not be immediately obvious. Certain changes, especially pigment changes, can be determined relatively easily by examination of the optical properties themselves and confirmed by the use of the sensitivity spectra. The sensitivity spectra, as explained previously, highlight the wavelengths that are most affected by exposure to the pollutant and are a manipulation frequently used in higher plant studies (Carter 1994). Other manipulations, including spectra derivatives and ratios, are also extremely useful.

Ratios are used routinely during remote sensing studies on higher plants to detect vegetation change or exposure to stress. These include universal ratios, for example the Normal Vegetation Index utilising the reflectance values obtained in the red and infrared regions and the red edge effect used to detect higher plant stress. Ratios offer certain advantages including providing a consistent method for measuring change, even when using different equipment (Chapter 2) and offering the possibility of quantifying an effect of pollutant exposure.

- **What mathematical interpretations of the data obtained appear to be most useful in detecting stress by particular pollutants?**

The manipulation of the *in vivo* spectral data obtained is a useful tool that offers potential for identifying the effect of pollutants on the algae. One of the most useful derivations is the production of sensitivity spectra, devised by Carter (1994), as these indicate the wavelengths that are most sensitive to exposure of the pollutant. However, care must be taken with their interpretation. Figures 148 and 149 show the sensitivity spectra produced by exposure to copper, *in vivo* transmittance factor peaks, and Irgarol 1051, *in vivo* reflectance troughs, respectively. It should be noted that the important factor is the position of the peaks as opposed to whether they are positive or negative. A negative peak, or trough, indicates that a wavelength is affected but is reduced by exposure to a pollutant whereas a positive peak indicates an increase at that wavelength due to a particular pollutant.

These sensitivity spectra offer the potential of identifying wavelengths that are most affected, therefore indicating how the pollutant is exerting an effect. They also allow the use of ratios, which are instrument independent, and may be linked to remote methods of detection. The selection of suitable wavelengths and ratios offers the potential to produce dose response curves, therefore enabling EC_{50} values to be calculated for specific

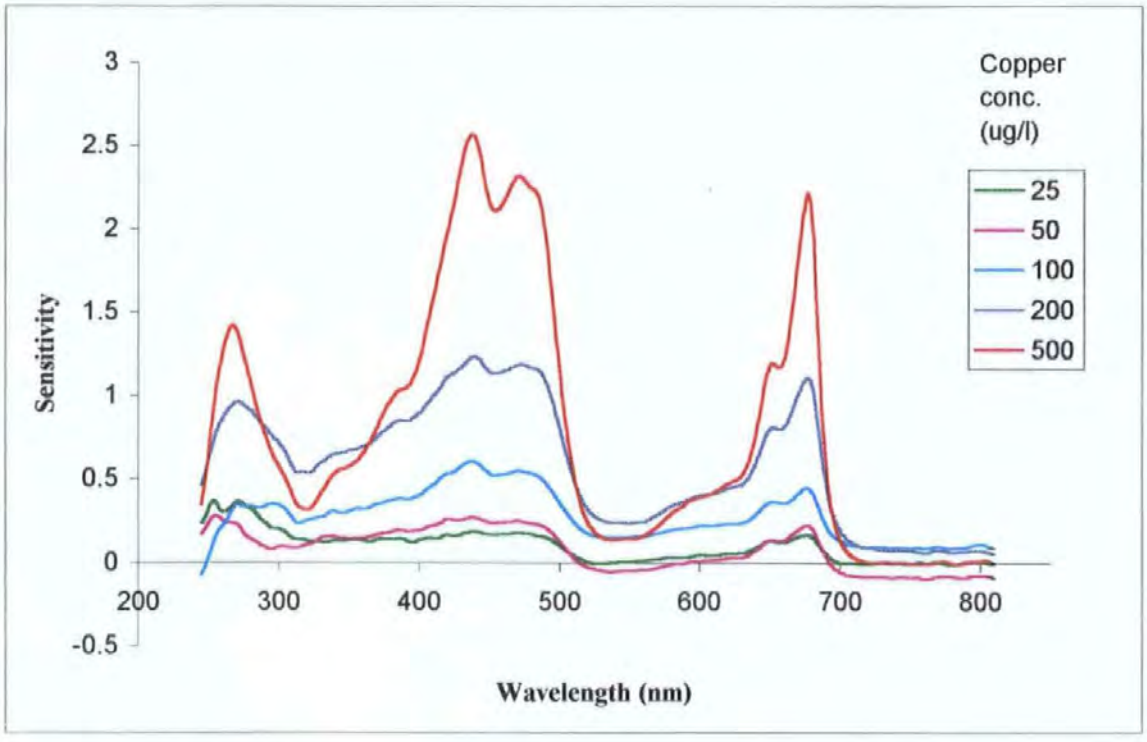

Figure 148: **Sensitivity to copper, with increasing concentration, of *in vivo* transmittance factor of *E.intestinalis* exposed for 7 days**

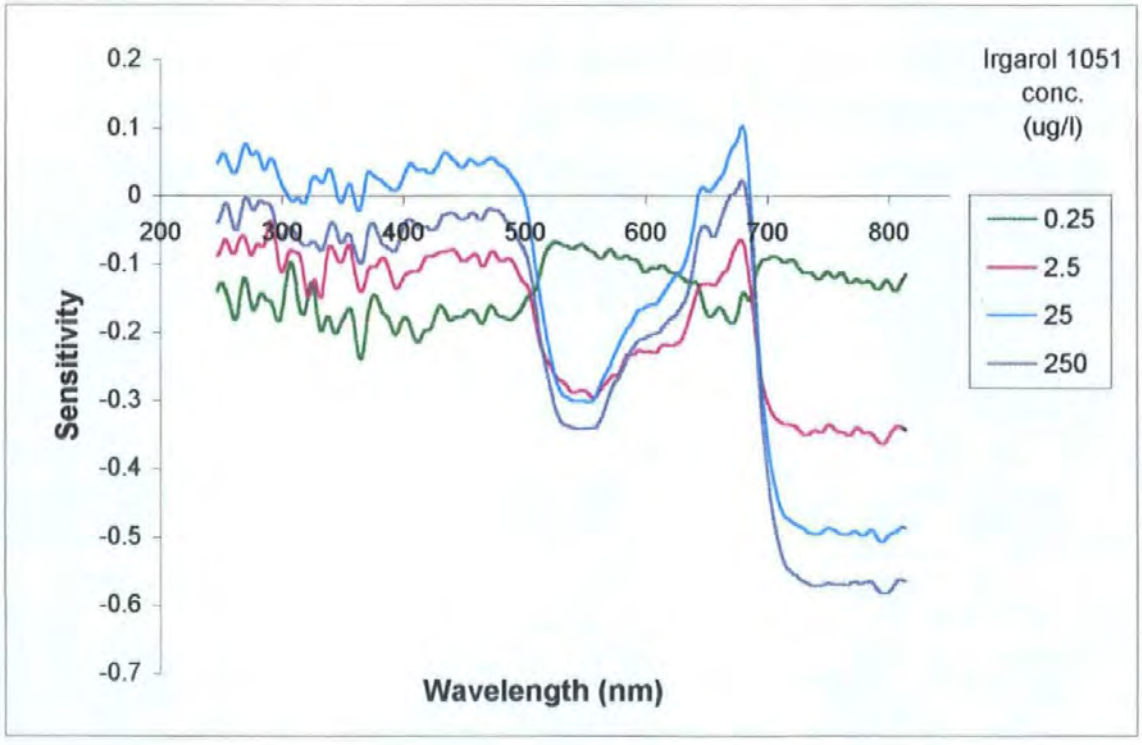

Figure 149: **Sensitivity to Irgarol 1051, with increasing concentration, of *in vivo* reflectance of *E.intestinalis* after 7 days exposure**

pollutants. These values can then be compared with different, more established, methods and may prove that use of this method may be more sensitive than other methods. This is illustrated in the copper exposure results where it was found that the use of the *in vivo* spectral properties was more sensitive as an indicator of copper exposure than F_v/F_m.

- **Can the use of *in vivo* spectral properties allow the detection, quantification and discrimination between pollutants in a mixture?**

The results obtained when *E.intestinalis* was exposed to a mixture of copper and Irgarol 1051 show that it is possible to use the method of *in vivo* spectral properties to discriminate between exposure to different pollutants in a mixture. The spectra obtained during the Copper and Irgarol 1051 mixture studies show a combination of the individual signatures that were produced for each pollutant. This is illustrated in figure 80, which clearly shows that the shape of the *in vivo* reflectance is similar to that obtained by Irgarol 1051 with specific wavelengths being affected. However, there is also a general raising of the entire sensitivity spectra, which corresponds with copper exposure. Contaminants in marine systems are likely to occur as complex mixtures, rather than as individual pollutants, resulting in a combination of effects on marine organisms. Although some pollutants have an additive effect, in particular non-specific narcotics, some pollutants will have more specific action, for example copper. The method of using *in vivo* spectral properties is, therefore, extremely promising, as not only can it detect toxic effects, it can also provide information about the system of the algae that has been affected. This, as seen in the copper and Irgarol 1051 mixture, allows the discrimination between different pollutants in a mixture.

Selection of certain parameters offers the potential to extend the method to use in complex mixtures. Figures 150 – 153 show the web diagrams produced when the certain parameters were chosen and plotted for copper, Irgarol 1051 and alcohol exposure. These

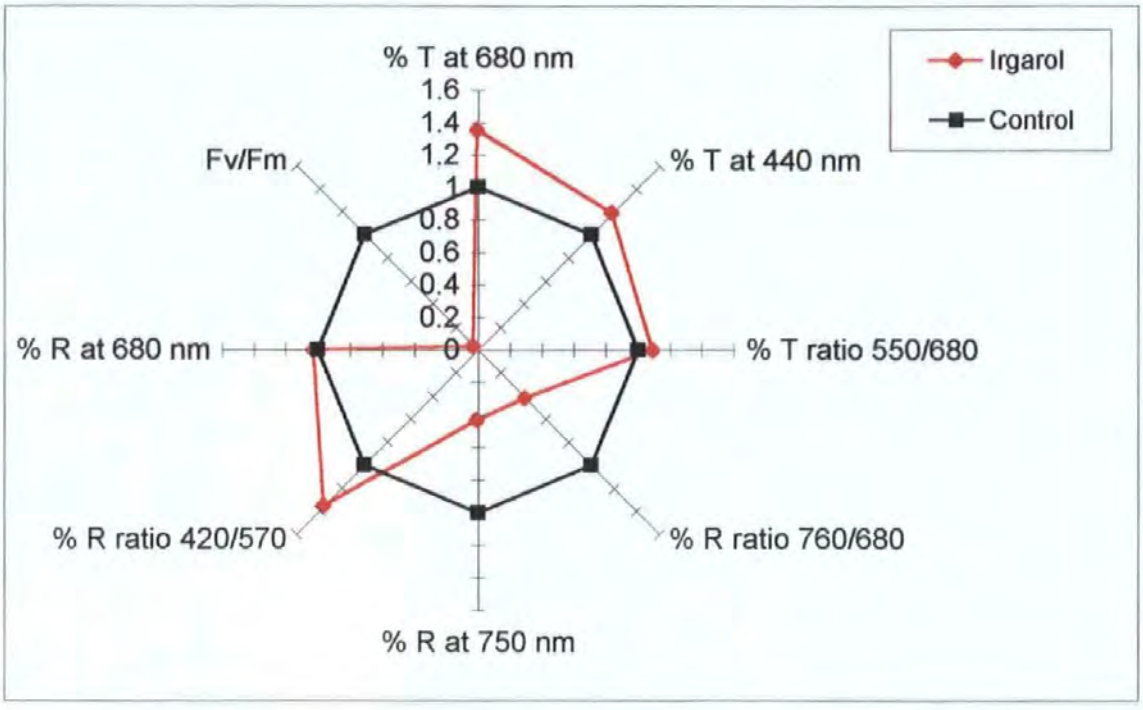

Figure 150: Comparison of selected parameters of *E.intestinalis* exposed to 2500 ug Irgarol after 7 days

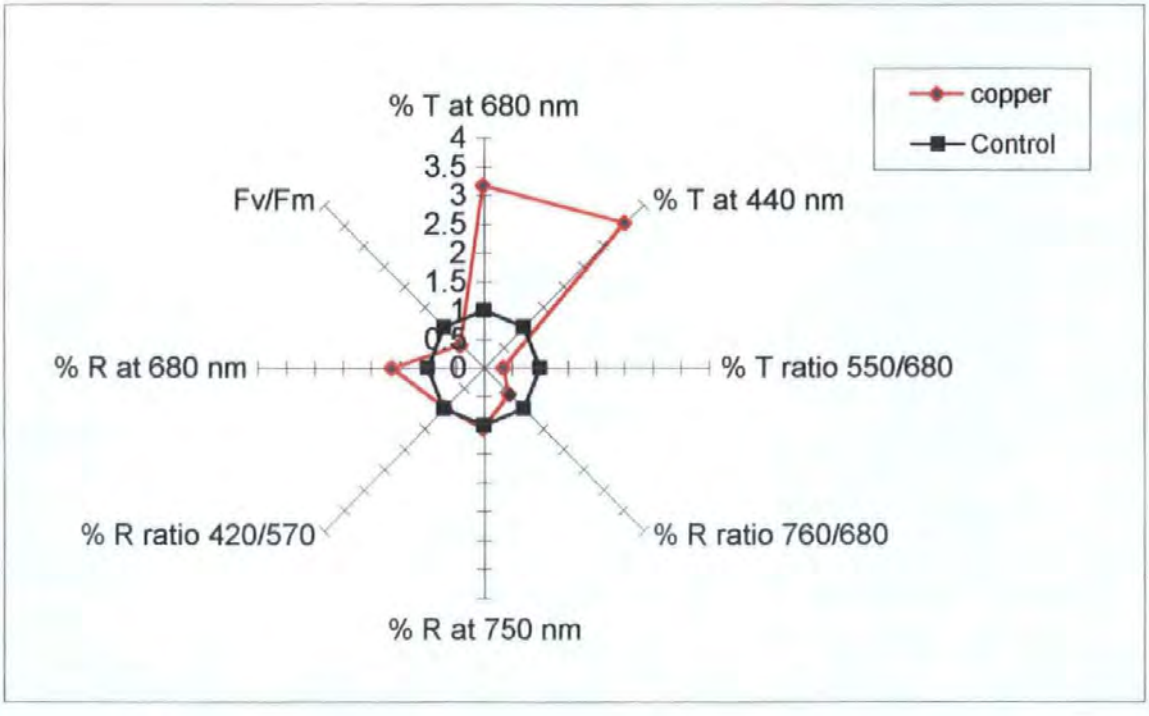

Figure 151: Comparison of selected parameters of *E.intestinalis* exposed to 500 ug/l Copper for 7 days

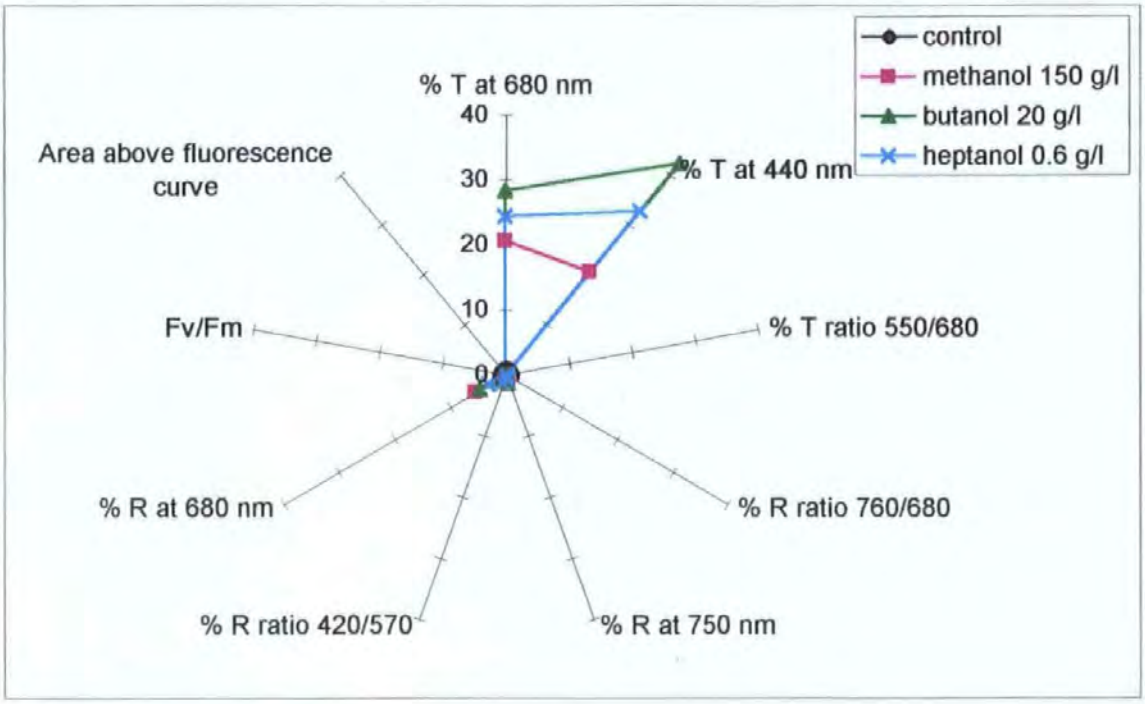

Figure 152: Comparison of selected parameters of *E.intestinalis* exposed to methanol, butanol and heptanol

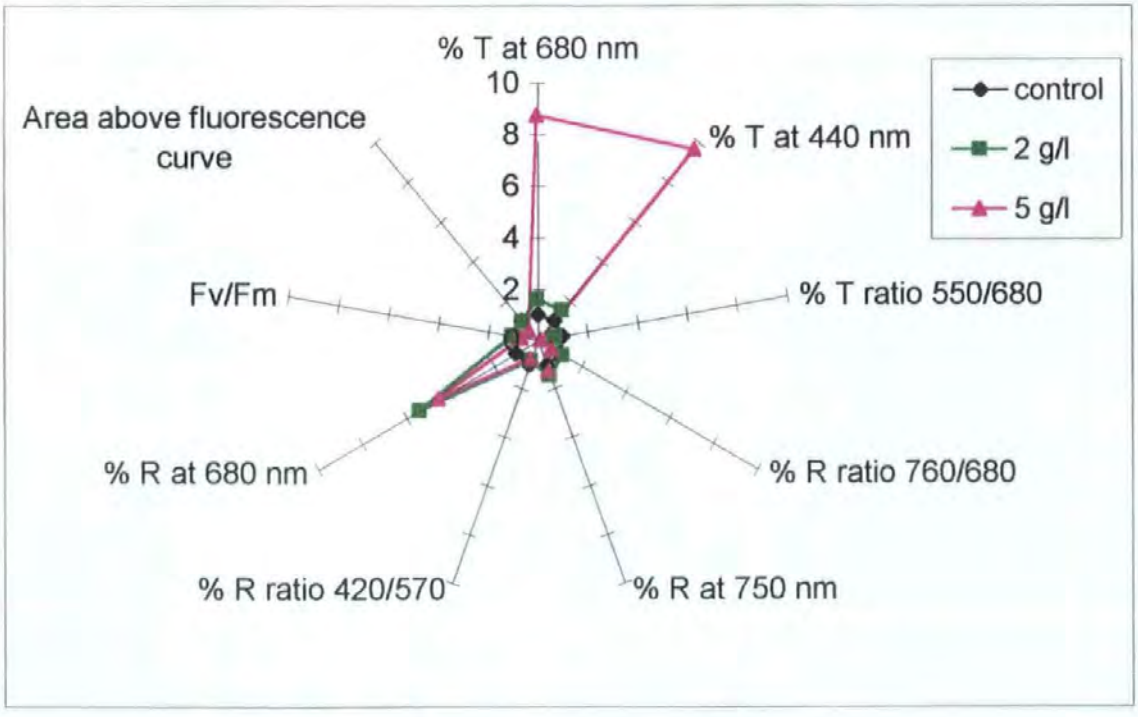

Figure 153: Comparison of selected parameters of *E.intestinalis* exposed to butanol for 96 hours

are examples of parameters that could be chosen and it is clear that the pattern obtained is different for each pollutant. When using these visual descriptors of the effects of the pollutant, it is vital that the parameters are chosen carefully and the axes are displayed in the same order each time. The pattern obtained therefore not only depends on the pollutant used, but also on the order the parameters are plotted, as this would significantly affect the final pattern.

Figure 150, the web diagram illustrating the effect of Irgarol 1051 on various parameters of *E.intestinalis*, produces a distinctive pattern identifying the dramatic reduction in Fv/Fm and percentage *in vivo* reflectance at 750 and 760 nm. From the use of this diagram it is immediately apparent that Irgarol 1051 has affected Photosystem II of the algae and lowered *in vivo* reflectance in the region that indicates structural changes have occurred. Very little change is apparent in the *in vivo* parameters that would indicate pigments are the primary systems being affected *ie.* change at 680 and 440 nm. This therefore illustrates the potential of this type of method to display the data, in addition to appropriate parameters being chosen, in particular including *in vivo* spectral properties. The effect of copper, figure 151, results in a completely different pattern than that produced for Irgarol 1051. It is apparent from this web diagram, that the primary system the copper is affecting involves pigments, as the main differences are seen in the *in vivo* spectral properties at 680 and 440 nm. The web illustrates a large increase for both *in vivo* reflectance and transmittance at 680 nm and also transmittance at 440 nm, indicating that there is less chlorophyll present which could be due to changes in pigment turnover or actual pigment breakdown. Figure 152 shows that exposure to an alcohol produces the same pattern regardless of which alcohol (figure 152) or which concentration (figure 153) is used.

It is possible that, in addition to certain ratios being able to discriminate between different pollutants in a mixture, selection of appropriate parameters plotted as a web diagram may

produce a certain shape, indicating certain pollutants in the mixture, resulting in the production of 'web' figures that emphasise the potential of using *in vivo* spectral properties to monitor pollution.

- **Does the method exhibit a high degree of repeatability?**

Many of the studies presented were performed several times to ensure repeatability. Although the pigment constitution of *E.intestinalis* is unlikely to differ significantly during a season, it is possible that different amounts of chlorophyll may be present due to light or nutrient variations in the field. The use of ratios and sensitivity spectra eliminate this problem, as they identify the wavelengths affected or reflect any changes relative to other parts of the spectra, therefore increasing the reliability of the method.

- **Is there a concentration effect on the sensitivity signatures?**

It was apparent that the concentration produces a marked effect on the shape of the sensitivity signature if excessively high concentrations of pollutants are used, resulting in complete narcosis of the algae. The concentrations used throughout these studies resulted in a sensitivity signature that increased in magnitude with increasing concentration and that the maximum concentrations used were higher than would usually be found in the environment. Figs 148 and 149 illustrate the effect of concentration on *in vivo* transmittance factor due to copper exposure and *in vivo* reflectance due to Irgarol 1051 exposure. It is evident that the signature remains the same regardless of the concentration and that the higher concentrations exhibit more noticeable peaks or troughs, but that these are at the same wavelengths. This demonstrates that the use of *in vivo* spectral properties would be useful as a biomonitoring tool as it is not dependent on concentration.

- **Can the data obtained under laboratory conditions be extended to situations in the field?**

The laboratory data obtained, as mentioned above, demonstrates the potential of the use of *in vivo* spectral properties in identifying damage by specific pollutants. Certain pollutants, for example copper, Irgarol 1051 and the series of alcohols, exhibit very different signatures. The Irgarol 1051 and copper mixture experiment also resulted in the detection of the individual pollutants. This indicates that these results could be very useful if they are extrapolated into situations in the field. The samples collected from chosen sites in the field had different *in vivo* spectral properties and this was highlighted by the use of the 'web' diagrams in Chapter 7. Even though, at this stage, it was not possible to determine the actual pollutants at the sites, it was certainly possible to group the algae according to their response and therefore, to the group of pollutants to which they had been exposed. Pollutants in the marine environment are likely to be complex mixtures. Clearly, to determine specific pollutants it would be necessary to test a wider range of pollutants and pollutant mixtures, however, results obtained do indicate with further research, this method would be suitable.

- **Can changes observed in the spectra form the basis of remote detection?**

Remote detection often uses the reflectance of vegetation to determine species present and any vegetation changes. The results obtained indicate that certain pollutants result in a particular *in vivo* reflectance signature. This could certainly be used in conjunction with more traditional remote sensing studies to determine stress in marine macroalgae. Remote sensing studies have resulted in the technique advancing and smaller pixel sizes can now be used, which is a beneficial development for studies involving marine macroalgae. This, together with methods to solve the problem of mixed pixels, should result in the mapping of marine macroalgae. This, in conjunction with studies involving *in vivo* spectral properties could result in the mapping of stress incurred by the algae. In addition, there is

the potential that laboratory data could be used to select the CASI wavebands, resulting in suitable bands being chosen to map pollutant stressing of the algae remotely.

- **Is the use of *in vivo* spectral changes an appropriate biomonitor?**

The results obtained during these studies, indicate that the use of *in vivo* spectral properties is certainly an appropriate biomonitor to add to the expanding range of biomonitors already used. It is a relatively quick, inexpensive method and is non-invasive. The technique has also demonstrated it can determine certain pollutants and, in addition to identifying whether stressing has occurred, it can also be used to determine the systems in the algae that have been affected. Not only can it be used to determine a specific pollutant, it can also determine whether the algae have been exposed to a mixture of pollutants as exhibited in the copper-Irgarol 1051 study.

The results from using *in vivo* spectrophotometry can, as discussed earlier, be mathematically manipulated in a variety of ways resulting in ratios, derivatives and sensitivity graphs, which are instrument independent, and thus are not dependent on absolute values. In addition, the selection of appropriate parameters can be used carefully to produce web diagrams that give an immediate representation of the effect of the pollutant on the algae. The results obtained during the alcohol studies, resulted in a QSAR that was extremely similar in slope and R^2 value to QSARs already published for *E.intestinalis* using ion leakage and neutral red retention., but the results also provide more information than these two methods. This, together with the technique being non-invasive, allows the same algae to be studied over a time period. The selection of appropriate parameters can provide an indication of the actual effect the pollutant has exerted.

A useful biomonitor should be able to detect changes at the molecular, biochemical or physiological level of the organism and the use of *in vivo* spectral properties can evidently

detect physiological changes. Other advantages of this technique include being instrument non-specific, relatively quick and is able to be repeated on the same pieces of seaweed without affecting the results, enabling time-courses to be plotted. It also has the potential to be extrapolated to the field, particularly in remote sensing studies which would enable thematic maps of pollutant stressed algae to be produced. The combination of these factors indicates that the use of *in vivo* spectral properties should be added to the growing list of biomonitors.

Opportunities for further work:

- **Extend studies to incorporate prolonged exposure to pollutants at lower concentrations.**

These studies involved relatively acute concentrations of pollutants for short periods of time. Even though the time was long enough to determine EC_{50}'s for the pollutants and various parameters, the concentrations were higher than would be found typically in marine systems. There would obviously be situations where acute levels of pollutants would be found, for example after a major pollution incident or near sites of certain activities such as mining, but situations involving longer, chronic exposure should also be considered.

- **What is the basis for any changes in the spectra?**

As mentioned above, the results enable the systems that are affected by the pollutant to be determined, with the main basis for change being either due to pigments or structure of the algae. Although it is difficult to tell, using the results, exactly how Irgarol 1051 is affecting the structure of *E.intestinalis*, from the increased transmission and reduced reflection in certain regions of the spectrum, it is evident that pigments are not the primary site affected. Other studies in higher plants, have shown that thylakoid movement has an effect on reflectance but this is unlikely to be the cause of any differences encountered

with *E.intestinalis* because of it's structure. The thallus of *E.intestinalis* is only one cell thick which would largely eliminate this problem and electron microscopy examining the effect of Irgarol 1051 on *E.intestinalis* indicated that the thylakoids were not affected. The fact that *E.intestinalis* is cylindrical, however, may offer some explanation as air bubbles which may be invisible to the naked eye inside the 'tube', which could increase reflectance, may be destroyed due to the thallus of *E.intestinalis* 'collapsing' inwards, possibly due to membrane damage.

Knowledge of the *in vivo* absorptance of the different algal pigments enables the method to be used to identify whether the pigments are being affected. The results obtained in the copper studies (Chapter 4) clearly indicate that chlorophyll is being affected, as the sensitivity spectrum is extremely similar to the absorption spectra of chlorophyll. These results are in agreement with a study by Lewis *et al* 1997, who found, by measuring the amount of chlorophyll, that copper exposure did reduce chlorophyll levels in *E.intestinalis*. It would be useful to perform pigment analyses in conjunction with *in vivo* spectral properties to gain a greater understanding of the systems affected by pollutant exposure.

- **Use of laboratory work and hand held spectroradiometer to ascertain particular wavelengths affected by pollutants to enable the CASI to be programmed to detect particular pollutant damage.**

The results obtained during this research indicate that the laboratory data can be extrapolated to be useful for remote sensing. This could be extended to include the use of a hand-held spectroradiometer, which could measure the *in vivo* spectral properties of large areas of algae as opposed to single fronds. By comparing this data with the laboratory data useful information would be gained to identify the particular wavelengths, or wavebands, for programming the CASI in order to detect particular pollutant damage. This, in turn, could result in large coastal regions being sensed remotely, after accurate ground truthing,

to ascertain levels of pollutant stress and, potentially, identification of pollutants encountered.

- **Use of a field spectroradiometer in addition to CASI to look for spectral shifts identified in laboratory work to relate the results to a wider analysis of ecotoxicological damage**

The laboratory studies provide information about specific pollutants, however this could be extended to use in the field with a hand-held spectroradiometer. The field spectroradiometer could be used to relate the laboratory work with spectral shifts resulting from pollutant stress in the field and this, in turn, could be extrapolated to use with the CASI for remote sensing studies.

- **SEM, TEM and light microscope investigation into the basis of the structural changes observed**

The results indicate that some of the effects of the pollutants observed are due to pigment changes (copper) and/or structural changes (Irgarol 1051). The changes due to the pigment effects are relatively easy to identify by using a combination of the *in vivo* spectral properties and manipulations of the data. However, changes due to structure are not so easy to identify specifically. To determine what actual structural changes were occurring due to pollutant damage, it would be useful to use a combination of SEM, TEM and light microscopy to identify the basis of the change. This could determine whether the changes observed are due to changes in the air spaces, damage to membranes, chloroplast structure or general lysis of the cell.

- **Development of image analysis protocols for quantifying coloured lesions with the aim of identifying spectral and surface alterations specific to particular pollutants**

It is possible that some pollutants would affect the surface of the algae and produce coloured lesions or spectral alterations. Image analysis could be used to quantify these and to investigate whether they are specific to particular pollutants.

- **Effects on the algae due to factors other than pollutants**

The results of any information obtained using biomonitoring techniques have to be interpreted with caution, as there would usually be a variety of factors that would cause differences encountered in the algae. This is one reason why it is useful to employ a range of techniques to maximise information obtained concerning the pollutant's mode of action and effects of stress on the algae. The results of this study proved to be repeatable, regardless of season and precautions were taken to ensure the algae was always acclimated and not in a reproductive state. However, other factors should be tested to ascertain whether they affect the results. This is particularly applicable for remote sensing methods, where the same control over the algae cannot be guaranteed. For instance, there could be some algae that were in a reproductive state and this would have to be taken into account when viewing the results. One of these factors would be the thickness of the algae as these laboratory studies only involved using one algal frond, whereas beds of algae, several fronds deep, could be encountered in the field. This should be tested, however it is unlikely to be as much of a problem as encountered in higher plant studies, where for example the problem of leaf angle and height of crop would have to be considered. Another factor which may need to be taken into account, is desiccation of algae. A series of experiments that involve measuring the *in vivo* spectral properties of algal fronds undergoing a process of desiccation, should be performed to determine if this has any effect on the data obtained. This is unlikely to be a problem with the laboratory studies, as the algae is held under controlled conditions and 'Intelliscan' was used to minimise the

time taken for spectral measurements. However, it would be more pertinent to remote sensing, especially on studies that involved longer flight paths undertaken during monitoring surveys. This would apply in particular to higher shore species, as they would be uncovered by water for long periods of time if the flight were performed at low tide, to incorporate as much of the intertidal region as possible.

- **Other species**

The use of *E.intestinalis* in this research indicates that the technique involving *in vivo* spectral properties offers excellent potential as a monitoring system. The *in vivo* signatures obtained spectrally are different for the pollutants to which the algae were exposed. However, as mentioned previously, *E.intestinalis* has a relatively simple tubular structure just one cell thick. Many studies have been carried out on higher plants and it would be extremely interesting to investigate the effect of pollutants on the *in vivo* optical properties of other species of algae. The fucoids, in particular, would potentially show differences in their *in vivo* optical signatures because they have a much more complex structure incorporating more layers and air spaces than *E.intestinalis*. Red algae, which have a complex pigment composition in comparison with *E.intestinalis* could also provide useful information for use in monitoring. A further reason for using additional species is that *E.intestinalis* is fairly resilient to pollution, which partly explains its cosmopolitan distribution. Other species, could therefore, be more sensitive to certain pollutants and may exhibit changes at more subtle levels of pollution.

Overall, although further research is needed, this study certainly demonstrates that the use if *in vivo* spectral properties has the potential to be a useful tool in monitoring stress in marine algae. It is sensitive, repeatable, gives an indication of the algal system affected and has the possibility of extension to remote methods. All these factors indicate that it is a suitable biomonitor to increase understanding of pollutants and their biological effects.

APPENDIX 1

	Element		Instant Ocean mg/l	Natural Seawater Mg/L
Major Elements	Cl	Chlorine	19600	19000
	Ca	Calcium	420	400
	K	Potassium	360	380
	Mg	Magnesium	1400	1350
	Na	Sodium	10400	10500
Minor and Trace Elements	B	Boron	5.5	4.5
	Ba	Barium	<0.05	0.03
	Cd	Cadmium	<0.01	0.0001
	Co	Cobalt	<0.03	0.00008
	Li	Lithium	0.18	0.18
	Mo	Molybdenum	2.2	0.1
	Ni	Nickel	<0.4	0.007
	P	Phosphorus	0.19	0.07
	Pb	Lead	<0.08	0.00003
	Ru	Ruthenium	<0.1	0.12
	Sb	Antimony	<0.2	0.0003
	Si	Silicon	0.34	2.0
	Sn	Tin	0.15	0.0008
	Sr	Strontium	7.1	8.5
	V	Vanadium	<0.04	0.002
	Al	Aluminium	<0.04	0.01
	Cu	**Copper**	**0.011**	**0.003**
	Fe	Iron	0.012	0.003
	Mn	Manganese	0.004	0.002
	Zn	Zinc	0.018	0.01

Table 9: Composition of Instant Ocean compared with natural seawater (taken from Seascope, Volume 7, 1990

REFERENCES

Armstrong R.A. 1993. Remote sensing of submerged vegetation canopies for biomass estimation. *International Journal of Remote Sensing.* **14** (3): 621-627

Ashton, F.M. and Cratfs, A.S. 1981. *Mode of action of herbicides.* John Wiley and sons, New York

Bajjouk, T., Guillaumont, B. and Populus, J. 1996. Application of airborne imaging spectrometry data to intertidal seaweed classification and mapping. *Hydrobiologia* **326/327**:463-471

Barbier, M., Joly, D., Saliot, A. and Tourres, D. 1973. Hydrocarbons from sea water. *Deep Sea Research* **20**:305-314

Barreiro, R., Real, C., Carballeira, A. 1993. Heavy metal accumulation in *Fucusceranoides* in a small estuary in North-West Spain. *Marine environmental Research.* **36** (1):39-61

Baret, F. and Guyot, G., 1991. Potentials and limits of vegetation indices for LAI and APAR assessment. *Remote Sensing of Environment* **35**:161-173

Barrett, E.C. and Curtis, L.F. 1992. *Introduction to Environmental Remote Sensing.* 3rd ed., Chapman and Hall, London.

Barrett, E.C. and Curtis, L.F. 1995. *Introduction to Environmental Remote Sensing,* Chapman and Hall, London p329

Bartley, C.E., 1957. Simazine and related triazines as herbicides. *Ag. Chem.* **12**:34-36,113-115

Bester, K., Huehnerfuss, H., Brockman, U. and Rick, H.J. 1995. Biological effects of triazine herbicide contamination on marine phytoplankton. *Arch. Environ. Contam. Toxicol.* **29**(3): 277-283

Bjorkman, O. and Demmig, B. 1987. Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77-K among vascular plants of diverse origins. *Planta* **170** (4) : 489-504

Bolhar-Nordenkampf,H.R. and Őquist,G. 1993 Chlorophyll fluorescence as a tool in photosynthesis research. In: Hall,D.O., Scurlock,J.M.O., Bolhar-Nordenkampf,H.R., Leegood,E.C. and Long,S.P. (Eds) *Photosynthesis and production in a changing environment: a field and laboratory manual.* Chapman and Hall, London.

Britton,G. 1983. *The biochemistry of natural pigments,* Cambridge University Press, London. pp. 14

Bryan, G.W. and P.E. Gibbs. 1983. *Heavy metals in the Fal Estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms.* Occasional Publication Number 2, Marine Biological Association of the United Kingdom

Bryan, G.W. and Langston, W.J.. 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution* **76**:89-131

Burrows, E.M. 1971. Assessment of pollution effects by the use of algae. *Proceedings of the Royal Society of London B* **177**:295-306

Burrows, E.M. 1991. *Seaweeds of the British Isles: a collaborative project of the BPS and the British Natural History Museum. Volume 2. The Chlorophyta.* NHM Publications

Carter, G.A., Mitchell, R.J., Chappelka, A.H. and Brewer, C.H. 1992. Response of leaf spectral reflectance in Loblolly Pine to increased atmospheric ozone and precipitation acidity. *Journal of Experimental Botany.* **43** (249): 577-584

Carter, G.A. 1993. Responses of leaf spectral reflectance to plant stress. *American Journal of Botany* **80**: 239-243

Carter, G.A. 1994. Ratios of leaf reflectances in narrow wavebands as indicators of plant stress. *International Journal of Remote Sensing* **15** (3): 697-703

Chapman, D and Kimstach, V. 1996. Selection of water quality variables. In: Chapman, D. (Ed.) *Water Quality Assessments: A Guide To The Use Of Biota, Sediments And Water In Environmental Monitoring*, pp.59 -126. University Press, Cambridge.

Chappelle, E.W., Kim, M.S. and McMurtrey, J.E. 1992. Ratio analysis of reflectance spectra (RARS): An algorithm for the remote estimation of the concentrations of chlorophyll a, chlorophyll b, and carotenoids in soybean leaves. *Remote Sensing of the Environment* **39**: 239-247

Chester, R. 1990. *Marine Geochemistry*. Chapman and Hall, London. pp. 347.

Ciba Geigy. 1995. *IRGAROl 1051 in antifouling paints, Technical Information Bulletin.* Ciba Geigy, Basel, Switzerland

Correa, J.A., Gonzalez, P., Sanchez, P., Munoz, J. and Orellana, M.C.. 1996. Copper-algae interactions: inheritance or adaptation? *Environmental Monitoring and Assessment* **40**:41-54

Curran, P.J. 1987. *Principles of Remote Sensing,* Longman, London.

Dearden, J.C. 1985. Partitioning and lipophilicity in quantitative structure-activity relationships. *Environmental Health Perspect.* **61**: 203-228

Deysher,L.E. 1993. Evaluation of remote sensing techniques for monitoring giant kelp populations. *Hydrobiologia.* **260/261**:307-312

Dennis, T.D. and Turpin, D.H. (Eds) 1990. *Plant Physiology, Biochemistry and Molecular Biology.* Longman Group, London.

Depledge, M.H., 1993. Ecotoxicology:A Science or a Management Tool? *Ambio* **22** (1): 51-52

Depledge, M.H., Bjerregaard, P. and Weeks, J.M. 1994. Heavy metals In *Handbook of Ecotoxicology Volume 2*. P. Calow (ed). Blackwell Scientific Publications, Oxford

Deysher, L.E. 1993. Evaluation of remote sensing techniques for monitoring giant kelp populations. *Hydrobiologia*. **260/261**: 307-312

Dierickx, P.J. and Van De Vyver, I.E. 1991. Correlation of the neutral red uptake inhibition assay of cultured fathead minnow fish cells with fish lethality tests. *Bulletin of Environmental Contamination and Toxicology*. **46**: 649-653

Dodge, A. 1989. *Herbicides and plant metabolism*. Cambridge University Press

Donkin, P., Widdows. J., Evans, S.V., and Brimsley, M.D. 1991. QSARs for the sublethal responses of marine mussels (Mytilus edulis). *The Science of the Total Environment*. **109/110** : 461-476

Donkin, P. 1994. Quantitative structure activity relationships. In: Calow, P. (Ed) *Handbook of ecotoxicology*. Blackwell Scientific Publications, Oxford, pp 321 – 347

Fedtke, C. 1982. *Biochemistry and Physiology of Herbicide Action*. Springer-Verlag, Berlin.

Filella, I. and Peñuelas, J. 1994. The red edge position and shape as indicators of plant chlorophyll content, biomass and hydric status. *International Journal of Remote Sensing*. **15**(7): 1459-1470

Forbes, V.E. and Forbes, T.L. 1994. *Ecotoxicology in theory and practice*. Chapman and Hall, London

Gausman, H.W, Allen, W.A., Escobar, D.E., Rodriguez, R.R. and Cardenas, R. 1971. Age effects of cotton leaves on light reflectance, transmittance, and absorptance and on water content and thickness. *Agronomy Journal*. **63** : 465-469

Geider,R.J. and Osborne,B.A. 1992. *Algal photosynthesis: the measurement of algal gas exchange, current phycology 2*. Chapman and Hall, London

Geyer, H.J. and Muir, D.C.G., 1993. New results and considerations on the bioconcentration of the superlipophilic persistent chemicals octachlorodibenzo-p-dioxin (OCDD) and mirex in aquatic organisms, In : Mansour, M. (Ed) *Fate and prediction of environmental chemicals in soils, plants and aquatic systems*, pp. 185 – 197, Lewis Publishers, USA

Gibbs, P.E. and Bryan, G.W. 1994. Biomonitoring of Tributylt in (TBT) pollution using the imposex response of neogastropod molluscs. In : *Biomonitoring of coastal waters and estuaries*. K.J.M. Kramer (ed). CRC Press

Gledhill, M., Nimmon, M., Hill, S.J. and Brown, M.T. 1997. The toxicity of copper (II) species to marine algae, with particular reference to macroalgae. *J. Phycol*. **33**:2-11

Goodman,C., Newall,M. and Russell,G. 1976. Rapid screening for copper tolerance in ship-fouling algae. *International Biodeterioration Bulletin*. **12**:81-83

Gough, M.A., Fothergill, J. and Hendrie, J.D. 1994. A survey of Southern England coastal waters for the s-triazine antifouling compound Irgarol 1051. *Marine Pollution Bulletin* **28**(10):613-620

Guillaumont, B., Callens, L. and Dion, P. 1993. Spatioal distribution and quantification of *Fucus* species and *Ascophyllum nodosum* beds in intertidal zones using spot imagery. *Hydobiologia.* **260/261**: 297-305

Guillaumont, B., Bajjouk, T. and Talec, P. 1997. Seaweed and remote sensing: A critical review of sensors and data processing. In: **Round, F.E. amd Chapman, D.J.** (Eds) *Progress in Phycological Research, Volume 12.* Biopress Ltd. Bristol

Guyot, G. and Guyon, D. 1989. Factors affecting the spectral response of forest canopies: a review. *Geocarto International.* **3**: 3-18

Hanelt, D., Wiencke, C. and Karsten, U. 1997. Photoinhibition and recovery after high light stress in different developmental and life-history stages of *Laminaria saccharina* (Phaeophyta) *Journal of Phycology* **33**: 387-395

Hansatech, 1995. *Operating Instructions for Plant Efficiency Analyser.* Hansatech Instruments, Norfolk, England

Hansch, C., Kim, D., Leo, A.J., Novellino, E., Silipo, C. and Vittoria, A. 1989. Toward a quantitative comparative toxicology of organic compounds. *CRC Critical Reviews in Toxicology.* **19** (3): 185-226

Hardy, J.T., Crecelius, E.A., Antrim, L.D., Broadhurst, V.L., Apts, C.W., Gurtisen, J.M. and Fortman, T.L. 1987. The sea-surface microlayer of Puget Sound: Part II. Concentrations of contaminants and relation to toxicity. *Marine Environmental Research.* **23**: 251-271

Hersh, C.M. and Crumpton, W.G. 1987. Determination of growth rate depression of some green algae by atrazine. *Bull. Environ. Contam. Toxicol.* **39**(6): 1041-1048

HMSO 1993. *Pesticides 1993- pesticides approved under the Control of Pesticides regulations, 1986.* Reference Book 500, HMSO Publications Centre, London

Horler, D.N.H., Barber, J. and Barringer, A.R. 1980. Effects of heavy metals on the absorbance and reflectance spectra of plants. *International Journal of Remote Sensing* **1**: 121-136

Ikemori, M. and Arasaki, S. 1977. Photosynthetic pigments in marine algae. I. Two-dimensional paper chromatographic separation of chlorophylls and carotenoids from green algae and sea grasses. *Bulletin Japanese Society Phycology* **25** (2):54-65

Jackson, P.J., Unkefer, P.J., Delhaize, E. and Robinson, N.J. 1990. Mechanisms of trace metal tolerance in plants. In: Katterman, F. (Ed). *Environmental injury to plants.* Academic Press

Jackson, R.D. 1986. Remote sensing of biotic and abiotic plant stress. *Annual Review of Phytopathology* **24**: 265-287

Khorram, S., Cheshire, H., Geraci, A.L. and La Rosa, G. 1991. Water quality mapping of Augusta Bay, Italy from Landsat-TM data. *International Journal of Remote Sensing.* **12**:803-808

Klumpp, D.W. and Peterson, P.J. 1979. Arsenic and other trace elements in the waters and organisms of an estuary in SW England. *Environmental Pollution* **19**:11-20

Labsphere, 1995, RSA-UC-40 Reflectance Spectroscopy Accessory Manual, Labsphere, North Sutton, USA

Landis, W.G. and Yu, M. 1995. *Introduction to environmental toxicology: impacts of chemicals upon ecological systems.* CRC Press, Florida

Law, R.J. 1981. Hydrocarbon concentrations in water and sediments from UK marine waters, determined by fluorescence spectroscopy. *Marine Pollution Bulletin* **12**:153-157

Law, R.J., Waldock, M.J., Allchin, C.R., Laslett, R.E. and K.J. Bailey. 1994. Contaminants in seawater around England and Wales: results from monitoring surveys, 1900-1992. *Marine Pollution Bulletin* **28**(11):668-675

Leal, M.C.F., Vasconelos, M.T., Sousa-Pinto, I. and Cabral, J.P.S. 1997. Biomonitoring with benthic macroalgae and direct assay of heavy metals in seawater of the Oporto coast (northwest Portugal). *Marine Pollution Bulletin.* **34** (12): 1006-1015)

Lewis, M.M. 1994. Species composition related to spectral classification in an Australian spinifex hummock grassland. *International Journal of Remote Sensing.* **15** (16) 3223-3239

Lewis, S.A., May, S.J., Donkin, M.E. and Depledge, M.H. 1998. The influence of copper and heatshock on the physiology and cellular stress response of *Enteromorpha intestinalis*. ***Marine Environmental Research.*** **46**(1-5): 421-424

Liu, D., Pacepavicius, G.J., Maguire, R.J., Lau, Y.L., Okamura, H. and Aoyama, I. 1999. Mercuric chloride-catalysed hydrolysis of the new antifouling compound Irgarol 1051. *Water Research.* **33**(1): 155-163(1): 155-163

Lillesand, T.M. and Kiefer, R.W. 1994. *Remote sensing and image interpretation.* John Wiley and Sons, New York

Lipnick, R.L. 1991. Outliers:their origin and use in the classification of molecular mechanisms of toxicity. *Science of the Total Environment.* **109/110**: 131-153

Lipnick, R.L. 1995. Structure-Activity Relationships. In: Rand, G.M. (Ed) *Fundamentals of Aquatic Toxicology: Effects, Environmental fate and risk assessment.* 2nd Edition. Taylor and Francis Publishers, Washington

Livingstone, D.R. and Goldfarb, P.S. 1998. Biomonitoring in the aquatic environment: use of cytochrome P4501A and other molecular biomarkers in fish and mussels. In: Lynch, J.M. and Wiseman, A. (Eds) *Environmental Biomonitoring – The Biotechnology Ecotoxicology Interface.* pp101-129.Cambridge University Press, UK.

Lobban, C.S. and Harrison, P.J. 1994. *Seaweed ecology and physiology.* Cambridge University Press, New York

LOIS 1994. *Land-Ocean Interaction Study.* Issue no.1. West Hoe, Plymouth

Lorenzoni, G. 1962. The stimulating effect of simazine at high dilutions. *Maydica* 7: 115-124

Magalhães, M.J., Sequeira, E.M. and Lucas, M.D. 1985. Copper and zinc in vineyards of Central Portugal. *Water, Air, Soil Pollution.* **26**:1-27

Matheson, W. and Ringrose, S. 1994. The development of image processing techniques to assess changes in green vegetation cover along a climatic gradient through Norhern Territory, Australia. *International Journal of Remote Sensing.* **15** (1): 17-47

May S.J., Price, D.N., Donkin, M.E. and Lane, S.D. 1998. Modelling the effects of non-specific narcotic hydrocarbon pollutants on green macroalgae. *Journal of Experimental Botany; S.E.B. Conference Abstracts.*

Miller,W.E., Peterson,S.A., Greene,J.C. and Callahan, C.A. 1985. Comparative toxicology of laboratory organisms for assessing hazardous waste sites. *Journal of Environmental Qual.* **14**:569-574

Moriarty, F. 1983. *Ecotoxicology: The Study of Pollutants in Ecosystems.* Academic Press, London

Munden, R., Curran, P.J. amd Catt, J.A. 1994. The relationship between red edge and chlorophyll concentration in the Broadbalk winter wheat experiment at Rothamsted. *International Journal of Remote Sensing.* **15**(3): 705-709

Murray, R. 1981. Eelgrass mapping from LANDSAT digital data. *Estuaries.* **4** (3) 268

Newman, M.C. 1998. *Fundamentals of ecotoxicology.* Sleeping bear/Ann Arbour Press, Chelsea (USA)

Nieboer, E. and Richardson, D.H.S. 1980. The replacement of the nondescript term 'heavy metal' by a biologically and chemically significant classification of metal ions. *Environmental Pollution Bulletin.* **1**: 3 1980

NRA (National Rivers Authority) 1994. *Contaminants entering the sea - a report on contaminant levels entering the seas of England and Wales 1990-1993.* Water Quality Series N° 24

Nybakken, J.W. 1997. *Marine Biology, An Ecological Approach.* Addison-Wesley Educational Publishers Inc., USA

Peñuelas, J., Gamon, J.A., Griffin, K.L. and Field, C.B. 1993. Assessing community type, plant biomass, pigment composition, and photosynthetic efficiency of aquatic vegetation from spectral reflectance. *Remote Sensing of the Environment.* **46**: 110-118

Phillips, D.J.H. 1980. *Quantitative Aquatic Biological Indicators.* Applied Science, London

Phillips, D.J.H. 1977. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments – a review. *Environmental Pollution.* **13**: 281-317

Phillips, D.J.H. 1994. Macrophytes as biomonitors of trace metals. In *Biomonitoring of coastal waters and estuaries.* K.J.M. Kramer (ed). CRC Press

Poole, L.J and Raven, J.A. 1997. *The Biology of Enteromorpha.* In: **Round, F.E. amd Chapman, D.J.** (Eds) *Progress in Phycological Research, Volume 12.* Biopress Ltd. Bristol

Premila, V.E. and Rao, M.U. 1997. Effect of crude oil on the growth and reproduction of some benthic marine algae of Viasakhapatnam coastline. *Indian Journal Marine Science* **26** (2): 195-200

Rai, L.C., Gaur, J.P. and Kumar, H.D. 1981. Phycology and heavy-metal pollution. *Biol. Rev.* **56**:99-151

Rana, B.C. and Srinivas, S.S. 1992. Effect of atrazine on chlorophyll and biochemical contents of water hyacinth. *Indian Journal of Ecology.* **19**(1): 115-117

Readman, J.W., Kwong, L.I.W., Grondin, D., Bartocci, J., Villeneuve, J.P. and Mee, L.D. 1993. Coastal water contamination from a triazine herbicide used in antifouling paints. *Environ. Sci. Technol.* **27**:1940-1942

Riedel-de Haën. 1994. Atrazine (Pestenal) Data sheet

Reynolds, C.F. 1984. *The ecology of freshwater plankton.* Cambridge University Press, Cambridge, MA, USA

Salisbury, F.B. and Ross, C.W. 1985. *Plant Physiology.* Wadsworth Publishing Company, America.

Say, P.J., I.G. Burrows and Whitton, B.A. 1986. *Use of estuarine and marine algae to monitor heavy metals. Final report from Northern Environmental Consultants Ltd. to the Department of the Environment, UK.* Northern Environmental Consultants Ltd., Consett, Co. Durham

Say , P.J., Burrows, I.G. & Whitton, B.A. 1990. *Enteromorpha* as a monitor of heavy metals in estuaries. *Hydrobiologia* **195**:119-126

Scanlan, C.M. and Wilkinson, M., 1987. The use of seaweeds in biocide toxicity testing. Part 1. The sensitivity of different stages in the life history of *Fucus* and of other algae to certain biocides. *Marine Environmental Research,* **21**:11-29

Scarlett, A., Donkin, M.E., Fileman, T.W. and Donkin, P. 1997. Occurrence of the marine antifouling agent Irgarol 1051 within the Plymouth Sound locality: implications for the green macroalga *Enteromorpha intestinalis. Marine Pollution Bulletin* **34**(8):645-651

Scheuert, I. 1993. Transport and transformation of pesticides in soil. In: Mansour, M. (Ed) *Fate and prediction of environmental chemicals in soils, plants and aquatic systems,* pp. 1-22.Lewis Publishers, USA

Schild, R., Donkin, P., Donkin, M.E. and D.N. Price. 1995. A QSAR for measuring sublethal responses in the marine macroalga *Enteromorpha intestinalis. SAR and QSAR in Environmental Research* **4**:147-154

Schild, R. 1996. *Evaluation of techniques for the biomonitoring of pollutants in members of the Ulvaceae.* Ph.D. Thesis. University of Plymouth

Schultz, T.W., Arnold, L.M., Wilke, T.S. and Moulton, M.P. 1990. Relationships of quantitative structure-activity for normal aliphaitic alcohols. *Ecotoxicology and Environmental Safety.* **19**: 243-253

Singh, A. 1987. Spectral separability of tropical forest cover classes. *International Journal of Remote Sensing.* **8**(7): 971-979

Skipnes, O., Roald, T. and Haug, A. 1975. Uptake of zinc and strontium by brown algae. *Physiol. Plant.* **34**:314-320

Somerville, C.R. 1990. The biochemical basis for plant improvement. In: Dennis , D.T. and Turpin, D.H. (Eds.) *Plant physiology, biochemistry and molecular biology,* pp. 490 – 501. Longman Singapore Publishers (Pte) Ltd., Singapore

Somerfield, P.J., Gee, J.M. and R.M. Warwick. 1994. Soft sediment meiofaunal community structure in relation to a long-term heavy metal gradient in the Fal estuary system. *Mar. Ecol. Prog. Ser.* **105**:79-88

Tait, R.V. and Dipper, F.A., 1998. *Elements of marine ecology.* Reed Elsevier plc., UK.

Taiz,L and Zeiger,E. 1991. *Plant Physiology.* The Benjamin/Cummings Publishing Company, California

Terry, L.A. and Edyvean, R.G.J. 1983. Methods of assessing the influence of fouling algae on the corrosion of offshore steel structures. *British Phycology Journal.* **18** (2):211

Tewari, A., Thampan, S. and Joshi, H.V. 1990. Effect of chlor-alkali industry effluent on the growth and biochemical composition of two marine macroalgae. *Marine Pollution Bulletin* **21**(1):33-38

Thomas,J.M., Skalski,J.R., Cline,J.F., McShane,M.C., Simpson,J.C., Miller,W.E., Peterson,S.A., Callahan,C.A., and Greene,J.C. 1986. Characterisation of chemical waste site contamination and determination of its extent using bioassays. *Environmental toxicological Chemistry.* **5**:487-501

Toler,R.W., Smith, B.D. and Harlan,J.C. 1981. Use of aerial color infrared to evaluate crop disease. *Plant Disease.* **65**:24-31

Tolosa, I., Readman, J.W., Blaevoet, A., Ghilini, S., Bartocci, J. and Horvat, M. 1996. Contamination of Mediterranean (Côte d'Azur) coastal waters by organotins and Irgarol 1051 used in antifouling paints. *Marine Pollution Bulletin* **32**(4):335-341

Townshend, J.R.G. 1981. *Terrain Analysis and Remote Sensing.* Allen and Unwin, London

Valentini, R., Cecchi, G., Mazzinghi, P., Scarascia Mugnozza, G., Agati, G., Bazzani, M., De Angelis, P., Fusi, F., Matteucci, G. and Raimondi, V. 1994. Remote sening of chlorophyll *a* fluorescence of vegetation canopies: 2. Physiological significance of fluorescence signal in response to environmental stress. *Remote Sensing of Environment.* **47**: 29-35

Vogelmann, J.E., Rock, B.N. and Moss, D.M. 1993. Red edge spectral measurements from sugar maple leaves. *International Journal of Remote Sensing* **14** (8): 1563-1575

Vymazel, J. 1995. *Algal and element cycling in wetlands.* Lewis publishers.

Wang,W and Freemark,K. 1995. The use of plants for environmental monitoring and assessment. *Ecotoxicology and environmental safety.* **30**:289-301

Whitton, B.A. 1984. Algae as monitors of heavy metals in freshwaters. In: Schubert. *Algae as ecological indicators.* Academic press Inc., London

Widdows, J. and Donkin, P. 1991. Role of physiological energetics in ecotoxicology. *Comp. Biochem. Physiol.* **100C**: 69-75

Wilkinson, M. 1992. Marine toxicology -use of algae. *A paper presented at international Environment 92*

Williams, P.R., Attrill, M.J. and Nimmo, M. 1998. Heavy metal concentrations and bioaccumulation within the Fal estuary, UK: A Reappraisal. *Marine Pollution Bulletin.* **36** (8): 643-645

Yilmaz, K., Yilmaz, A., Yemenicioglu, S., Sur, M., Salihoglu, I., Karabulut, Z., Telli Karakoc, F., Hatipoglu, E., Gaines, A.F., Phillips, D. and Hewer, A. 1998. Polynuclear Aromatic Hydrocarbons (PAHs) in the Eastern Mediterranean Sea. *Marine Pollution Bulletin* **36** (11): 922-925

Zacharias, M., Niemann, O. and Borstad, G. 1992. An assessment and classification of a multispectral bandset for the remote sensing of intertidal seaweeds. *Canadian Journal of Remote Sensing.* **18**(4): 263-274

Zaroogian, G., Heltshe, J.F. and Johnson, M. 1985. Estimation of toxicity to marine species with structure-activity models developed to estimate toxicity to freshwater fish. *Aquatic Toxicology.* **6**: 251-270