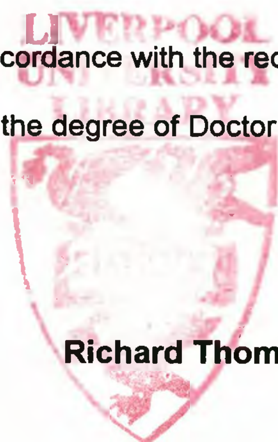
The ecology of epilithic microalgae on Manx shores

Thesis submitted in accordance with the requirements of Liverpool University

for the degree of Doctor of Philosophy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This Thesis is dedicated to those who helped me to change my career,

and become first a student and then a marine biologist.

Thank you, I may need your help again.

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ABSTRACT: The Ecology of Epilithic Microalgae on Manx Shores

Richard Thompson

Microalgae such as diatoms and cyanobacteria colonise surfaces which are immersed in shallow waters, forming a surface layer or 'biofilm'. These microalgae are important for nutrient cycling and primary production. However, partly because of difficulties in examining microbial films, little is known of the ecology of these algae.

Epilithic microalgae have been examined directly by microscopy. Alternatively, extracted chlorophyll has been used to provide an index of biomass. This study reviews methods of chlorophyll extraction and proposes a standard method using cold methanol. Seasonal variations in microalgal abundance have been recorded, with reduced biomass during the summer. The causes of this seasonality are not known, but emersion stresses, and removal of algae by grazing have been suggested. Limpets are the major grazing molluscs on many shores. However, little is known of seasonal variations in their activity. A simple technique for quantifying limpet grazing activity was developed using discs of wax recessed into the shore to record marks made by limpet radulae during feeding.

A combination of direct counts and chlorophyll determinations were used to record microalgal abundance on both sheltered and wave exposed shores. During the winter microalgae were less abundant on sheltered shores than on exposed shores. However, general trends in abundance were not apparent between shores of differing exposure, or between tidal levels. A more detailed study at Port St Mary monitored monthly variations in microalgal abundance. Here microalgal standing stock was negatively correlated with variations in limpet density between shore levels. The algae being least abundant on the mid shore where grazer density was greatest. There were clear seasonal variations on all shores, standing stock increased during the early spring and declined dramatically during the summer. These variations were negatively correlated with insolation and temperature, but not with seasonal changes in grazing intensity. Seasonal variations in limpet grazing activity were positively correlated with sea temperature. A factorial experiment at Port St Mary indicated that insolation stress and grazing intensity reduced microalgal biomass in equal proportions during the summer. Areas of shore where grazer density and insolation were reduced had increased microalgal abundance and enhanced growth of *Fucus* germlings.

Microbial films influence settlement and survival of other shore dwellers, facilitating settlement of some organisms whilst inhibiting that of others. Previous work has mostly examined settlement on artificial substrates or considered single species interactions. The influence of epilithic microalgal films was examined using settlement of *Semibalanus balanoides* cyprids. In choice chambers, cyprids settled preferentially on filmed rock from the mid shore. At a scale of a few centimetres settlement was enhanced by the presence of a microalgal film but not by presence of adult barnacles. On the shore, at a slightly greater scale, cyprids selected areas which were nearest to conspecifics but still preferred filmed to unfilmed surfaces.

Some problems associated with assessing productivity, from measurements of standing stock are discussed, together with difficulties in experimental design for manipulative ecology. Interactions between 'bottom-up' and 'top-down' regulation of communities are considered and several models are presented which describe variations in microalgal abundance in terms of both grazing activity and emersion stresses.

SECTION I

General Introduction and Study Sites

CHAPTER ONE

General Introduction

1.1 INTRODUCTION

Biofilms form on surfaces immersed in water and are composed of cyanobacteria, fungi, diatoms, Protozoans, larvae, and the early stages of macroalgae; some of these organisms secrete mucilage or extracellular polymers which helps to bind the cells together forming a film (Costerton *et al.*, 1978; Wahl, 1989; Christensen and Characklis, 1990; Wigglesworth-Cooksey and Cooksey, 1992). This film acts as an interface between the underlying substratum and the surrounding water (Meadows, 1963) and can be important to habitat selection by settling larvae (Wilson, 1955; Meadows, 1963; Crisp, 1974; Mihm *et al.*, 1981; Maki *et al.*, 1988; Maki *et al.*, 1992; Wieczorek *et al.*, 1995) and survival of macroalgal propagules (Huang and Boney, 1983; Huang and Boney, 1984; Huang and Boney, 1985a; Amsler and Neushul, 1989; Amsler and Neushul, 1990). Biofilms may also influence species succession by facilitation, inhibition or tolerance (Connell and Slatyer, 1977).

Microalgae are a major component of biofilms in shallow waters such as streams (Cox, 1990; Rout and Gaur, 1994), rivers (Lock, 1981; Hamilton and Duthie, 1984; Lock, 1993; Shamsudin and Sleigh, 1994), lakes (Burkholder and Wetzel, 1989; Hawes and Smith, 1994), shallow seas (Cahoon and Cooke, 1992) and on rocky (Aleem, 1950; Castenholz, 1963; Hill and Hawkins, 1991) or sedimentary shores (Colijn and de Jonge, 1984; Zhu *et al.*, 1994). They are important to primary production (Fielding *et al.*, 1988; Gilbert, 1991; Kristensen, 1993; Pinckney and Zingmark, 1993; Yallop *et al.*, 1994), nutrient recycling (Paul and Duthie, 1989; Lock, 1993), and provide food for grazers such as molluscs, urchins and crustaceans (Pace *et al.*, 1979; Eichenberger *et al.*, 1983; Underwood, 1984a; Hawkins *et al.*, 1989; Becker, 1994; Newell *et al.*, 1995). Microalgal production is also passed on to higher trophic levels by grazers such as molluscs and crustaceans. Juvenile fishes, for example, eat epifaunal crustaceans (Edgar and Shaw, 1995), whilst oyster catchers feed on limpets (Hockey and Branch, 1984).

This thesis investigates the role of microalgal biofilms in the ecology of rocky shores.

1.2 BIOFILM FORMATION

The principal stages of film formation are common to both natural and artificial substrata (Wahl, 1989). Initially this process is governed by physical factors but, as the film develops, biological factors become increasingly important and subsequent communities of fouling organisms may vary considerably from one location to another. Film formation has been reviewed by Wahl (1989) and can generally be considered as having four overlapping phases: biochemical conditioning, colonisation by bacteria, colonisation by unicellular eukaryotes and finally colonisation by multicellular organisms (Figure 1).

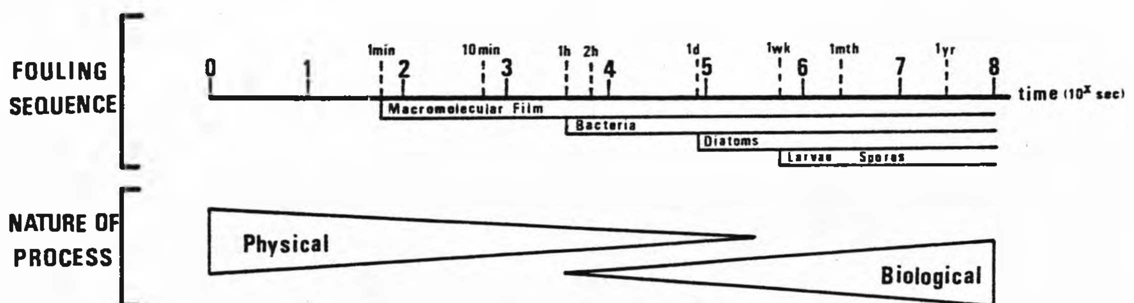


Figure 1) Highly schematised colonisation sequence leading to establishment of a fouling community (from Wahl, 1989).

1.2.1 Biochemical Conditioning

When a substratum is submerged in water biochemical conditioning begins immediately; glycoproteins, proteo-glucans, polysaccharides and other organic compounds present in the water adsorb on to the surface. The process is driven by a reduction in free energy at the surface/liquid interface, and so the comparatively high energy solid/liquid interface becomes replaced by an organic macromolecular layer of lower energy. This causes the physical and chemical surface properties of various substrata to converge. In other words, the surface energy of low-energy hydrophobic surfaces increases and that of higher energy hydrophilic surfaces decreases (Becker and Wahl, 1991). This process of biochemical conditioning is almost identical in most environments the common feature in all cases being the medium which is a dilute aqueous solution of proteins, sugars and salts (Baier, 1984).

1.2.2 Colonisation by Bacteria

Colonisation begins within an hour of immersion (Little, 1984). Generally rod-shaped bacteria arrive first, followed by coccoid, then stalked and finally filamentous forms. Colonisation consists of a reversible adsorption phase followed by irreversible adhesion. Adsorption is governed by physical forces: Brownian motion, electrostatic interactions, gravity and van-der-Vaal forces (Dexter, 1978; Fletcher and Loeb, 1979; Walt *et al.*, 1985). Adhesion occurs when covalent bonds form between the bacteria and the organic film (Wahl, 1989). Attachment is similar for yeasts (Douglas, 1985), unicellular algae (Tosteson *et al.*, 1983), and algal spores (Fletcher and Baier, 1984). The developing bacterial layer of living and dead cells, is described as a primary film (Wahl, 1989).

1.2.3 Colonisation by Unicellular Eukaryotes

Colonisation by diatoms, Protozoa and yeasts usually occurs several days after a surface has become immersed (Wahl, 1989). Motile benthic diatoms normally arrive first. These attach by mucus secretions and may densely

cover large areas of substratum (Cooksey *et al.*, 1984; Ferreira and Seeliger, 1985). The Protozoa which colonise are either sessile filter feeding forms, or motile predators feeding on other micro-organisms within the film. Colonising yeasts rely on the film for their nutrition and are important for recycling nutrients from dead and dying cells (Cuomo *et al.*, 1985). Subsequent development of the film may proceed by increases in the abundance of photosynthetic overstorey species (Paul and Duthie, 1989).

1.2.4 Colonisation by Multicellular Organisms

Colonisation by multicellular organisms has been described by numerous authors (Meadows, 1963; Crisp, 1974; Sutherland, 1974; Chracklis, 1981; Fletcher and Barier, 1984). The process may be considerably affected by the surface energy and topography of a substratum and there are often distinct differences between surfaces (Edyvean *et al.*, 1985; Roberts *et al.*, 1991; but see also Henschel *et al.*, 1990; Becker and Wahl, 1991 for examples of little overall differences in fouling assemblages between surfaces). For example, Mihm *et al.* (1981) found initial rates of settlement of bryozoan larvae were similar on plastic, which has a low surface energy, and glass, which has a higher surface energy. However, once these surfaces had become colonised by bacteria, glass became attractive and plastic unattractive to further settlement.

The effect of surface topography has been examined in marine and freshwater habitats, and in both, biofilm formation was enhanced on rugose surfaces. Colonisation frequently commenced in small pits and then in some instances extended outward over smoother areas (Hamilton and Duthie, 1984; Petraitis, 1990; Baker, 1992).

On a broader scale, colonisation may vary seasonally as a result of changes in weather conditions and variability in the supply of propagules and larvae caused by reproductive cycles (e.g. Gaines and Bertness, 1992; Turner and Todd, 1993). Local variations may also be caused by differences in the

supply of organisms from the water column. Bacteria, for example, segregate into distinct populations in response to physical, chemical and biological factors. Different suites of marine bacteria are associated with macroalgae (Rieper Kirchner, 1989), sewage, hydrocarbons (Bruns *et al.*, 1991; Floodgate, 1991; Venkateswaran and Harayama, 1995) and with inshore and offshore waters (Davis *et al.*, 1983). Where a variety of different habitats occur in close proximity within a freshwater lake, for example, microbial diversity can be higher than in more homogenous environments (Earle *et al.*, 1988).

1.3 FOULING AND APPLIED ASPECTS OF BIOFILMS

Biofilms also form on man-made structures such as cooling systems, oil platforms, piers and boats (Rao, 1989; Rao *et al.*, 1993). Here they are detrimental since they represent the first stage in macrofouling (Wahl, 1989). Fouling on ships hulls increases drag, for a large ship this may cost an additional $400 per hour in fuel usage (Bohandler, 1991; Cooksey and Wigglesworth Cooksey, 1995). Oil platforms and similar structures must be engineered to withstand the additional drag and weight of fouling organisms. Some aquatic bacteria and microalgae may also increase corrosion of metal and concrete structures (Terry and Edyvean, 1981; Edyvean and Terry, 1983; Patil *et al.*, 1988). Hence, fouling creates a continual requirement for surfaces to be cleaned or protected by antifouling chemicals that will inhibit colonisation.

Benthic microalgae have also been harnessed by man for the purification of water (Takasaki *et al.*, 1988), mine waste detoxification, metal recovery (Bender and Ibeanusi, 1987; Whitlock, 1990; Kang, 1994), and potential use for oil-spill cleansing (Sorkhoh *et al.*, 1992). Some microalgae have been cultured and utilised as a food source for human consumption (e.g. the freshwater cyanobacteria *Spirulina*) and as a feed stock for aquaculture

(Venkataraman, 1989). Recently there has also been interest in microbiota as a source of antibiotics, cancer reducing agents and other pharmaceuticals (Metting and Pyne, 1986; Kellam and Walker, 1989; Miura and Matsunaga, 1989; Noue *et al.*, 1989; Gerwick *et al.*, 1993).

1.4 BENTHIC MICROALGAE

Benthic microalgae such as diatoms and cyanobacteria are ubiquitous to biofilms in shallow aquatic habitats and are found in freshwater streams (Hamilton and Duthie, 1984; Marker *et al.*, 1986; Lock, 1993) and lakes (Burkholder and Wetzel, 1985; Hawes and Smith, 1994), and in marine habitats (Castenholz, 1963; Nicotri, 1977; MacLulich, 1987; Hill and Hawkins, 1991). They live on a variety of natural and artificial hard substrata (e.g. Korte and Blinn, 1983; Edyvean *et al.*, 1985) and also on and amongst sediment particles (e.g. Colijn, 1983; Yallop *et al.*, 1994). These algae are an important yet frequently overlooked source of primary production. In intertidal sediments and in the shallow subtidal they may contribute 20 to 30% of primary production (Moriarty *et al.*, 1985; Klumpp *et al.*, 1987; Fielding *et al.*, 1988; Polunin and Klumpp, 1992; Kristensen, 1993; Schreiber and Pennock, 1995). Recent work on rocky shores suggests intertidal epilithic microalgae may make an equally important contribution to inshore primary production (Dye and White, 1991; Fuji *et al.*, 1991; Bustamante *et al.*, 1995).

Microalgae have a considerable influence on the ecology of benthic communities. They are a major source of nutrient recycling in freshwater streams (Hamilton and Duthie, 1984; Paul and Duthie, 1989; Paul *et al.*, 1991) and in marine sediments (Rysgaard *et al.*, 1995). On rocky shores grazing of microalgae has a considerable impact on community structure since it removes macroalgal propagules and invertebrate larvae, thereby

influencing recruitment to populations (Southward, 1964; Hawkins, 1981; Hawkins, 1983; Hartnoll and Hawkins, 1985; Jernakoff, 1985).

Removal of limpets, for instance, from a two metre wide strip of rocky shore on the Isle of Man caused an initial increase in the abundance of microalgae such as diatoms. After a month this was followed by a dense growth of macroalgae which persisted for several years. Limpets gradually migrated into the cleared area and, after five years, macroalgal cover declined as a result of increased grazing (Jones, 1948; Lodge, 1948; Southward, 1956). Other workers have manipulated the abundance of grazers by removal or exclusion with cages or toxic paints (Castenholz, 1963; Hawkins, 1981; Dye and White, 1991). Without exception, reduction in grazing led to an increase in algal growth (Southward, 1964; Jones and Kain, 1967; Cowen *et al.*, 1982; Hatcher and Larkum, 1983; Farrell, 1988; Eekhout *et al.*, 1992; Dye, 1995; Littler *et al.*, 1995). However, the extent of algal proliferation varied depending on the relative importance of other regulating factors such as recruitment, nutrient availability, light and environmental stresses associated, for example, with low tide exposure (Hay, 1979; Underwood, 1980; Underwood, 1981a; Williams, 1994; Prince, 1995).

In freshwater streams, grazing of microalgae limits succession by *Cladophora* and other microalgae (Eichenberger *et al.*, 1983; Power *et al.*, 1988; Creed, 1994), whilst on coral reefs grazing limits succession by filamentous algae and may stimulate growth of calcareous reef building species (Scott and Russ, 1987; Littler *et al.*, 1995).

Microalgae also grow epiphytically on other aquatic organisms such as sea grasses (Klumpp *et al.*, 1992; Klumpp *et al.*, 1993), macroalgae (Huang and Boney, 1985b), molluscs and crustaceans (Round, 1971). The constituents of these epiphytic and epizooic communities may be quite different from those found on the surrounding substratum (Thompson *et al.*, 1996) and possibly provide a food resource for grazing species that would be unable to

survive elsewhere in the surrounding habitat. In this respect biofilms are an important consideration for assessment of biodiversity, yet surprisingly most studies of species diversity on hard substrata do not consider microbiota (but see Thompson *et al.* 1996).

1.5 THE ECOLOGY OF EPILITHIC MICROALGAE ON ROCKY SHORES

In the intertidal zone, conditions alternate between those experienced during high tide when the shore is immersed and those encountered whilst the tide is out. In this highly variable environment the distribution of organisms is regulated by both physical and biological factors. Physical extremes are largely caused by the duration of exposure at low tide. Conversely, biological interactions primarily act whilst the tide is in (Lewis, 1964; Underwood, 1980; Underwood, 1981a). Macroalgae can reduce emersion stresses experienced by the shore dwellers living beneath their canopy (Hawkins, 1983).

This thesis considers microalgae such as diatoms and cyanobacteria (Figures 2 and 3) in the rocky intertidal. With the exception of a few pioneering studies (Aleem, 1950; Castenholz, 1963) biofilms in this habitat have only recently been studied in detail (Underwood, 1984c; MacLulich, 1986; MacLulich, 1987; Hill and Hawkins, 1990; Dye and White, 1991; Hill and Hawkins, 1991; Bustamante *et al.*, 1995). In addition, the majority of these studies have been at moderately exposed locations and sheltered shores have received little attention.

Seaweeds and other macrobiota are physiologically and structurally adapted to withstand the extremes of temperature, insolation and desiccation experienced during low tide (Wolcott, 1973; Schonbeck and Norton, 1978; Schonbeck and Norton, 1980; Chapman, 1995). By comparison, microalgae appear relatively ill-equipped to cope with these stresses, and can become photoinhibited (Stahl *et al.*, 1985; Lamontagne *et al.*, 1989). On sheltered

shores conditions are mediated either by canopy-forming algae such as *Ascophyllum nodosum*, or by sediment particles which attenuate light and trap moisture (Blanchard and Gall, 1994; Kuhl and Jorgensen, 1994). The potential mediating effects of canopy-forming algae on the abundance of epilithic microalgae on sheltered shores has never been examined. By contrast, on moderately wave exposed shores the rock surface is predominantly exposed to the air and, consequently, microalgae living there may experience extreme diel changes in conditions.

Despite the harshness of the intertidal environment, epilithic algae persist throughout the year from polar regions, where they are covered by ice during the winter (Holt, 1980; Archibald *et al.*, 1983; Burkholder and Wetzel, 1985; Palmisano *et al.*, 1985; Kirst and Wiencke, 1995), to the sub-tropics where temperatures on the rock surface may exceed 50°C (Williams, 1994). Clearly, the severity of low tide stresses will vary spatially, both geographically and locally, and temporally between seasons.

Seasonal changes in environmental conditions may account for the variations in the abundance and species composition of microalgae that have been recorded by several researchers (Aleem, 1950; Castenholz, 1963; MacLulich, 1987; Hill and Hawkins, 1991). In the Antarctic, abundance was maximal in mid summer (Gilbert, 1991; Asmus and Bauerfeind, 1994), whilst in temperate and tropical locations abundance was maximal in the winter and early spring, then declined during the summer (Aleem, 1950; Castenholz, 1963; MacLulich, 1987; Hill and Hawkins, 1991; Hansen and Skilleter, 1994). There are no records of seasonal variations in the abundance of intertidal microalgae from tropical regions, but work in the shallow subtidal has showed that abundance was maximal during the winter. Collectively, these observations suggest optimal conditions for microalgal growth may vary with season and with latitude.

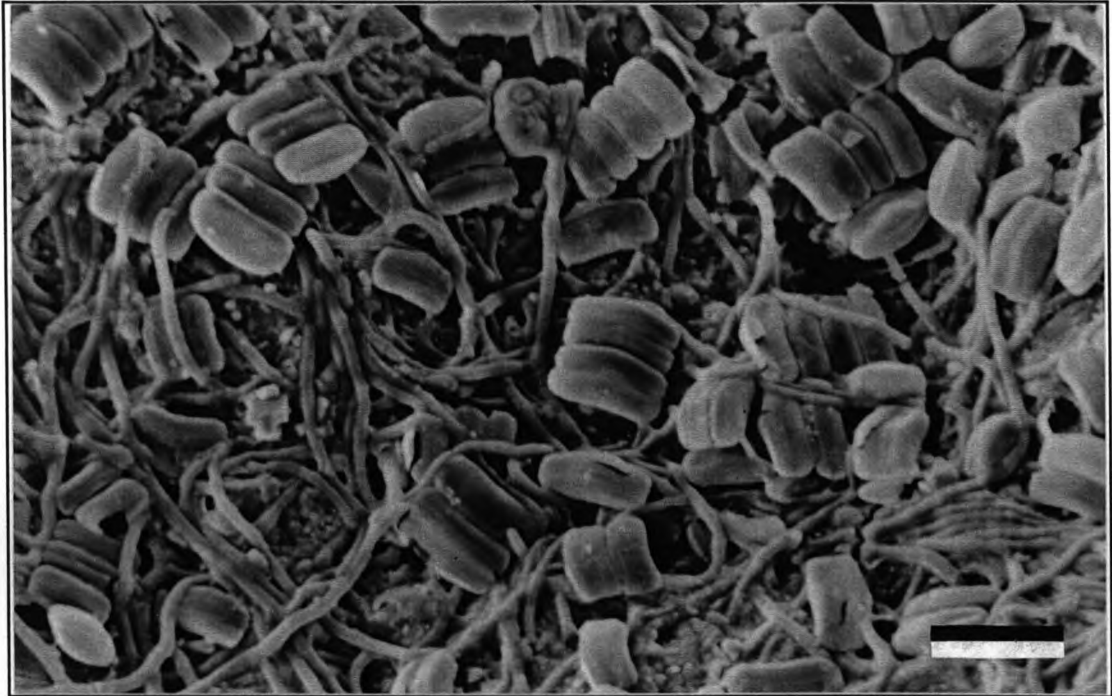


Figure 2) Scanning electron micrograph of epilithic microalgae showing an area densely colonised by diatoms and cyanobacterial filaments. Scale bar = 25 µm.

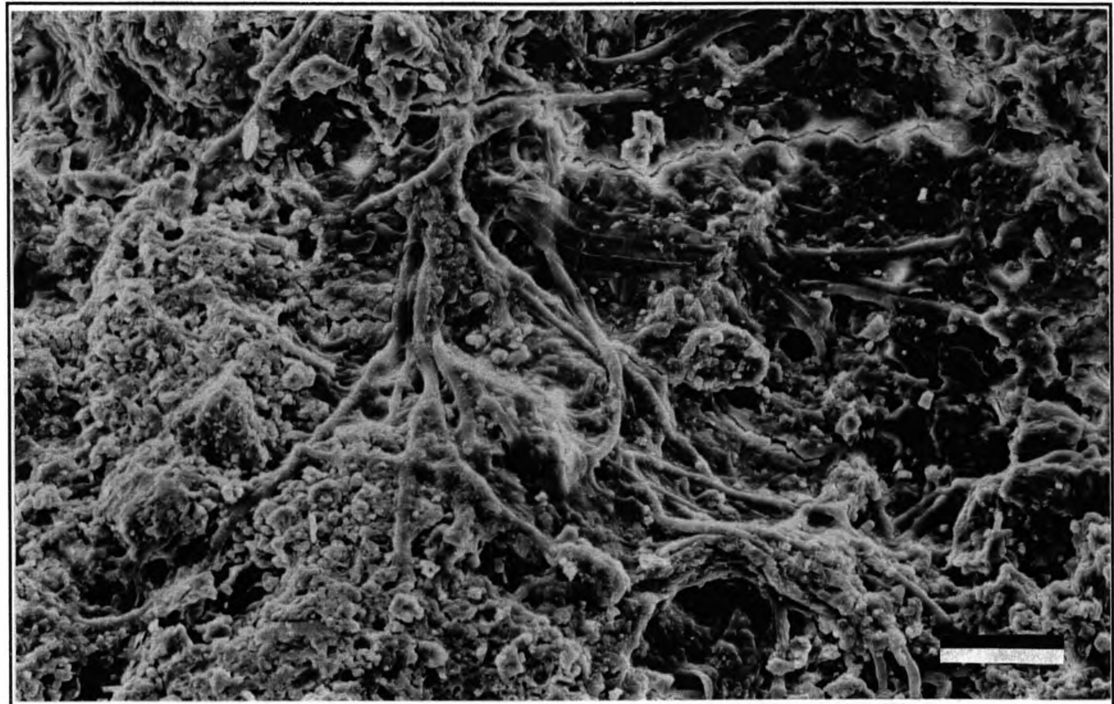


Figure 3) Scanning electron micrograph of epilithic microalgae showing a typical area of biofilm. Diatoms and cyanobacterial filaments are partially obscured by dehydrated mucilage. Scale bar = 25 µm.

Favourable conditions for microalgal growth are most probably caused by an interaction of physico-chemical and biological variables such as nutrient availability, insolation, desiccation, thermal stress and grazing activity (Underwood, 1984c). For algae on rocky shores and in soft sediments light and temperature have frequently been suggested as major regulating influences (Aleem, 1950; Castenholz, 1963; Thom and Albright, 1990; Kristensen, 1993). However, their importance has yet to be established conclusively by experimental work.

On rocky shores in the north eastern Atlantic limpets are the principal herbivorous molluscs. Limpets have a specialised feeding apparatus called a radula which rasps epilithic algae and some of the underlying rock surface from the shore (Hawkins *et al.*, 1989; Fretter and Graham, 1994). Their grazing increases variability in microalgal abundance at various spatial scales. On Manx shores limpets are most abundant at mid-tide level (Southward, 1951; Hawkins, 1979) and their grazing would be expected to reduce microalgal abundance considerably at that level. At a more localised scale, limpets intensively graze small patches of algae during foraging excursions. In addition, variability within grazed areas is considerable, with about 50% of the microalgae passing between the limpets teeth (R. C. T. unpublished data, see Figure 4).

Somewhat surprisingly, correlations between the grazing activity of molluscs and standing stock have seldom been examined. Two studies in which these factors were considered did not reveal any correlation between variations in the abundance of grazers and seasonal variations in standing stock (Underwood, 1984c; Dye and White, 1991). However, in these studies, grazing activity was estimated by recording changes in the abundance of molluscs. This approach did not take account of variations in feeding rate between seasons, and is therefore of limited value.

Figure 4) Rock surface on the upper shore at Port St Mary, showing areas of epilithic algae remaining after limpet grazing. Scale bar = 1cm.

Quantifying the feeding rate of molluscs is difficult in the field. A variety of techniques have been used to asses limpet foraging (e.g. direct observation: Hartnoll and Wright, 1977; Boyden and Zeldis, 1979; recording noises made during feeding: Kitting, 1979; Little *et al.*, 1990;and time lapse photography: Chelazzi *et al.*, 1994b) but these methods are both time consuming and costly. Seasonal patterns in foraging activity have not been examined.

Spatial variations in the abundance of microflora have also been reported with increasing abundance or productivity towards the lower shore (Aleem, 1950; Round, 1971; Underwood, 1984c; Dye and White, 1991). These variations are also frequently attributed to differences in the relative impact of low tide stresses such as insolation and temperature experienced between different shore heights (Castenholz, 1963; Underwood, 1984c). Underwood (1984c) considered microalgal abundance may also be regulated by differences in the abundance of grazers between tidal levels but did not find any correlation between these factors. Similarly, Dye and

White (1991), working in southern Africa, found little correlation between standing crop and grazer abundance at different tidal levels. The relative importance of each these possible regulating factors has not been tested in field conditions.

Biofilms are the first point of contact for larvae and propagules settling on the shore and may have a considerable influence on colonisation by macrobiota such as algae (Norton, 1983; Huang and Boney, 1985a; Dillon *et al.*, 1989) and invertebrates (e.g. Crisp and Meadows, 1963; Keough and Raimondi, 1995; Wieczorek *et al.*, 1995), thereby influencing species successions. However, the majority of research has either been laboratory based or has used artificial settlement surfaces. The overall significance of natural intertidal biofilms to settlement and habitat selection by macrobiota is not known.

1.6 ENUMERATING EPILITHIC MICROALGAE

Several techniques are available to study microalgal films. Abundance can be estimated directly by counting organisms on the surface of rock chippings chiselled from the shore (Hill and Hawkins, 1990) or by counting cells removed from the rock surface by brushing or scraping (MacLulich, 1986). Neither of these methods are ideal, brushing and scraping are difficult to apply on hard substrata, and result in loss of the orientation of cells within the film. Cells can be examined on the surface of rock chips using scanning electron microscopy (SEM), but this method only permits the surface of the film to be resolved and gives no indication of abundance cells in underlying layers (MacLulich, 1986; Hill and Hawkins, 1990).

Fluorescence microscopy provides an alternative to SEM and conventional light microscopy. Here, the autofluorescence of chlorophyll is utilised to resolve chloroplasts, whilst other components of the cell can be stained so

that they also fluoresce (Jones, 1974; Sherr and Sherr, 1983). However, once again this technique is restricted to the surface layer of the film. Biofilms growing on epoxy resin blocks in freshwater streams, have been section and examined using transmission electron microscopy (Lock *et al.*, 1984), but this technique could not be used for films growing directly on rock surfaces. Recently, progress has been made using a combination of fluorescence and confocal microscopy (Lawrence *et al.*, 1991; Verity *et al.*, 1996, Section IV). This method allows the film to be viewed as a series of thin horizontal slices. These can be captured electronically and viewed sequentially allowing the three dimensional nature of the film to be studied (Wilson, 1990; Litchman, 1994).

A second approach for estimating microalgal abundance is to use chlorophyll, which is present in all plant cells, to provide an index of standing stock. The amount of chlorophyll present can be assessed directly by fluorometry, or by extraction in solvents followed by spectrophotometric analyses (H.M.S.O., 1983) or High Pressure Liquid Chromatography (HPLC: Mantoura and Llewellyn, 1983; Pinckney *et al.*, 1994a). HPLC is very accurate, but is time consuming and is therefore of limited use for broad scale ecological studies (Pinckney *et al.*, 1994a). Fluorometry is frequently used to estimate the abundance of phytoplankton but there are no accounts of this technique being used to quantify epilithic algae. However, this approach would be limited as cells would first have to be removed from the surface of the substratum.

Chlorophyll extractions followed by spectrophotometry have been extensively used to estimate microalgal abundance. However, a range of methods are available for this and estimates can vary considerably depending on the technique adopted (e.g. Hill and Hawkins, 1990). There is no single common method between researchers and hence there is a clear need for a standardised protocol.

1.7 AIMS OF THIS THESIS

The overall aims of this thesis were to explore the little known role of microalgal films in the ecology of rocky shores. Although some work had been done in the British Isles (Aleem, 1950; Edyvean *et al.*, 1985; Patterson *et al.*, 1986; Hill and Hawkins, 1990; Hill and Hawkins, 1991) it was clear at the outset that much remained unknown. In part this was caused by methodological problems. A good start had been given by MacLulich (1986) and Hill and Hawkins (1990), but early on in the present study it was clear that certain technical problems still needed to be resolved.

Section II is an account of the methods development which was undertaken as a necessary prelude to the rest of the study. Chapter 3 within this describes work to develop a standard method of chlorophyll extraction from rock chips. Additional technical development using confocal microscopy was conducted as a collaborative study with colleagues from the Department of Foetal and Infant pathology at Liverpool University. This work is presented at the end of the thesis (Section IV).

Grazing by limpets is a major factor influencing the biomass of both microalgae and macroalgae (Southward, 1964; Hawkins, 1981; Hartnoll and Hawkins, 1985; Hill, 1990). Measuring grazing activity at the population level is difficult despite recent technical innovations (Chelazzi *et al.*, 1994b). Therefore an indirect method of recording grazing activity was developed (Chapter 4).

The techniques developed in Section II have been applied to quantifying variations in microalgal biomass and their causes. Previous work on the Isle of Man had studied only one shore level on a moderately wave exposed shore. Chapter 5 widens the perspective by comparing microalgal standing stock at several tidal heights on shores of differing exposure at different times of the year. Causes of the patterns observed, particularly the

interaction of grazing, insolation and desiccation stresses in the lower eulittoral zone are explored experimentally in Chapter 6.

The importance of intertidal biofilms to subsequent colonisation and succession by macrobiota was examined with the barnacle, *Semibalanus balanoides* (L.), which is the dominant occupier of space on moderately wave exposed Manx shores. Here, settlement of barnacle cyprids was compared on films of differing ages and on films from a series of shore levels within the range that is normally populated by adult barnacles (Chapter 7).

In the discussion (Chapter 8), after a consideration of the limitations of the work, three themes are explored. These are: a comparison between microalgal standing stock and microalgal productivity, restrictions of experimental design in manipulative field ecology, and the importance of 'bottom-up' and 'top-down' processes in regulating microalgal community structure on rocky shores.

Methods developed in section II were also used in a collaborative project undertaken with other rocky shore ecologists at Port Erin Marine Laboratory. In this study biodiversity in the intertidal was compared at a range of spatial scales from microbiota to macrobiota. This work has recently been published and a copy of the manuscript is included at the end of the thesis (Section IV).

CHAPTER TWO

Study Sites

2.1 STUDY SITES

Study sites and sampling methods are described in detail at the beginning of each chapter. However, a résumé of these may be helpful at this stage.

All work was conducted on rocky shores in the south of the Isle of Man (4°W, 54°N, Figure 1). These comprised gently sloping planes of carboniferous limestone (Ford, 1993).

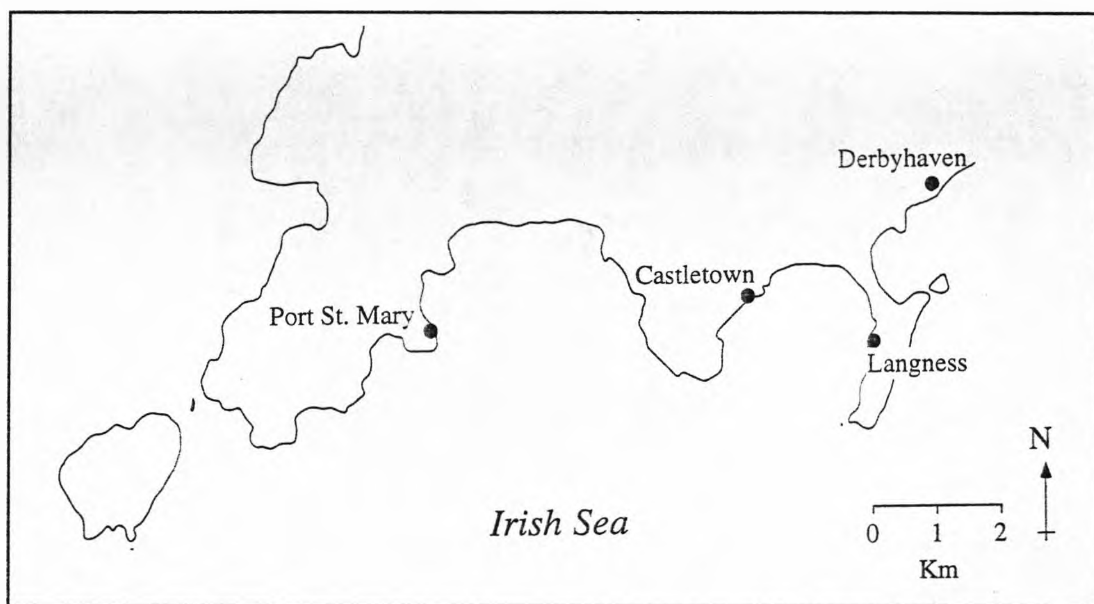


Figure 1) Map showing four study sites in of the south of the Isle of Man.

Seasonal monitoring of microalgal abundance, and experimental work was mostly conducted on a moderately wave exposed shore at Port St. Mary. For comparisons between shores an additional moderately wave exposed site was chosen at Derbyhaven, and sheltered shores were selected at Castletown and Langness. Wave exposed shores were dominated by a mosaic of fucoid algae and barnacles interspersed with areas of open rock (Figures 2 & 3). Sheltered shores were dominated by a dense canopy of *Ascophyllum nodosum* (Linnaeus), beneath which were patches of turf forming algae, coralline algae and open rock (Figures 4 & 5). The topography and biota of these shores has been described extensively (Southward, 1951; 1953; Bruce *et al.*, 1963; Hawkins, 1979; Hill, 1990).

Figure 2) View of the moderately wave exposed shore at Port St Mary looking east towards Castletown.

Figure 3) Typical area of upper shore at Port St Mary showing open rock with patches of macroalgae together with limpets and barnacles. bar = 10cm.

Figure 4) View of the sheltered shore, with dense macroalgal cover of *Ascophyllum nodosum* (L.), at Langness looking west towards Castletown.

Figure 5) Area of open rock with limpets and littorinids beneath the canopy of *Ascophyllum nodosum* (L.) at Langness. bar = 2cm.

SECTION II

Methods Development

CHAPTER THREE

Problems in Extraction and Spectrophotometric Determination of Chlorophyll from Epilithic Microalgae

3.1 ABSTRACT

A variety of techniques are available to extract chlorophyll from epilithic microalgae using solvents. The relative efficiency of each method has not been compared and there is no recognised standard procedure. In this chapter methods of chlorophyll extraction are reviewed together with procedures for sample collection, storage and preparation. Various ways for preparing and storing samples were compared experimentally. The relative efficiency of eight extraction techniques was determined, and methods used to correct for the presence of degradation products or chlorophylls b and c were examined.

Extraction of chlorophyll was incomplete unless microalgae were fully hydrated. This factor was highly significant for all the solvents tested, with up to three times more pigment being extracted from hydrated samples than from dry ones. Extraction from fresh material was preferable since storage reduced estimates by up to 25%.

Methanol was the most efficient solvent, extracting over 96% of the total chlorophyll; hot ethanol extracted 86%, whilst cold acetone was of little use, extracting less than 50%. Consequently, extractions in cold 95% methanol are recommended. This method required minimal monitoring and gave a chlorophyll extract which was stable for up to 24 hours.

Centrifuging samples to remove suspended material did not alter estimates and was not necessary as a standard procedural step. Correcting chlorophyll a values for the presence of degradation products or chlorophylls b and c produced spurious results with both epilithic algae from the shore and standard solutions of chlorophyll a. Consequently spectrophotometry is not recommended for assessing the proportions of these pigments.

Rugose rock surfaces released more chlorophyll than smooth ones. A simple method for quantifying surface rugosity was not available. However, detailed analyses showed surface area could be underestimated by over 50%, with proportional consequences for estimates of standing stock, if this factor was not quantified.

Based on these observations, a standard method for chlorophyll extractions from epilithic microalgae is proposed using 95% methanol at room temperature. This technique requires around 25% less operator participation than previously preferred methods.

3.2 INTRODUCTION

Microbial films form on hard substrata immersed in seawater. In shallow water they comprise mainly of microalgae such, as diatoms and cyanobacteria, and play a key role in rocky shore ecology (Underwood, 1979; Hawkins and Hartnoll, 1983; Underwood, 1984c). They are the site of attachment of algal propagules and larvae (Crisp and Meadows, 1963; Wahl, 1989; Chapman, 1995) and can influence both settlement (Crisp, 1974; Huang and Boney, 1984; Huang and Boney, 1985a; Rodriguez *et al.*, 1993) and survival (Underwood, 1979; Hawkins and Hartnoll, 1983; Lubchenco, 1983; Amsler and Neushul, 1990). These algae provide a resource for grazers (Steneck and Watling, 1982; Hawkins *et al.*, 1989) and are a major source of primary production in many systems (Gilbert, 1991; Bustamante *et al.*, 1995). Understanding the ecology of microbial films is beset with difficulties (MacLulich, 1986; Hill and Hawkins, 1990; Hall, 1992). In this Chapter I consider problems in quantifying the standing crop of microalgae within these films using chlorophyll extracted from algal cells as an index. My aim was to establish a robust and easy standard method.

Chlorophyll a has long been used as an index of microalgal standing stock in planktonic (Richards and Thompson, 1952; Parsons and Strickland, 1963) and benthic systems, both in soft sediments (e.g. Eaton and Moss, 1966; Admiraal, 1977; Admiraal and Peletier, 1980; H.M.S.O., 1983) and on hard substrata (e.g. Strain and Manning, 1942; Castenholz, 1963; Marker, 1972; H.M.S.O., 1984; Bustamante *et al.*, 1995). In this Chapter only epilithic microalgae were considered. Sample collection and solvent extraction techniques are reviewed and various common methods are compared. The study focuses on extraction media, extraction protocol, storage and state of sample hydration - particularly important in intertidal studies. After discussion of the advantages and disadvantages of various techniques a standard method is proposed

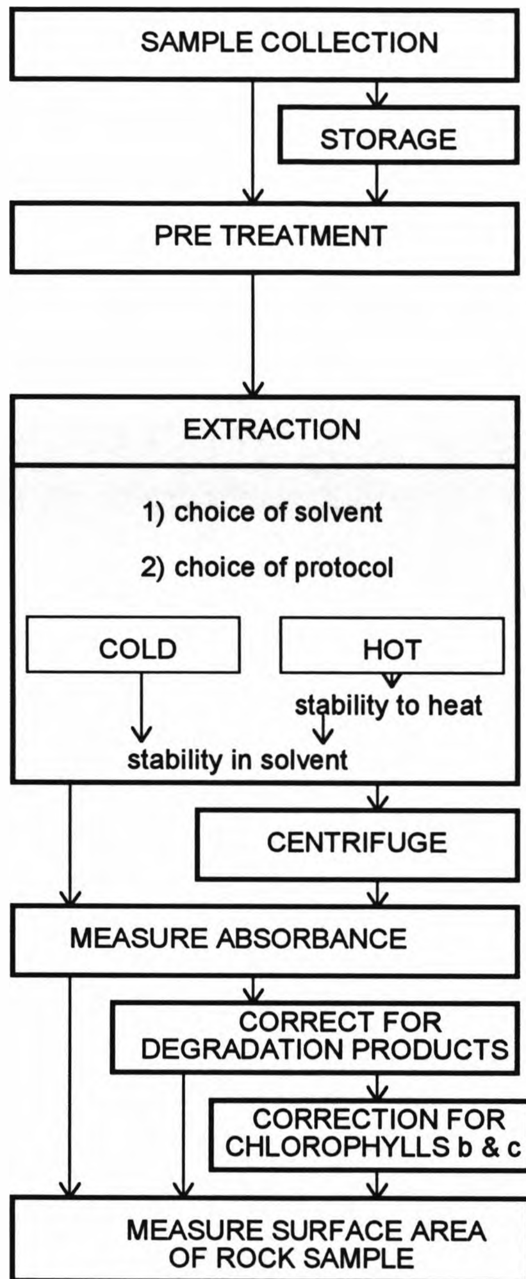
3.3 REVIEW OF METHODS

Obtaining estimates of chlorophyll concentration from epilithic microalgae involves several steps (Figure 1) each of which has the potential to increase analytical error. The importance of each step has been reviewed.

3.3.1 Sample Collection

Large numbers of samples may be needed in order to overcome the natural spatial variability of microalgal communities, and an efficient method for sample collection is imperative (MacLulich, 1986; Hill and Hawkins, 1990). Microalgae have been removed from rock surfaces by brushing (Castenholz, 1963; Nicotri, 1977; MacLulich, 1986), and scraping (H.M.S.O., 1984; Underwood, 1984c; MacLulich, 1986). Alternatively, rock fragments have been chiselled from the shore, with the algae intact (Hill and Hawkins, 1990; Dye and White, 1991). Chiselling is the most efficient technique on harder rocks since it causes minimal damage to constituents of the film and ensures endolithic algae are included in the sample (MacLulich, 1986; Hill and Hawkins, 1990; Takada, 1993)

Steps in the extraction of chlorophyll from epilithic microalgae



estimate of chlorophyll concentration

Summary and conclusions of work from this study

Not examined here, see Hill & Hawkins (1990). Ensure samples are of consistent rugosity unless profilometer available to measure surface relief.

Avoid storage if possible. Hydrated samples can be stored for 1wk. at 4°c. Freezing not recommended.

Samples must be fully hydrated before extraction.

Methanol is superior to ethanol, acetone or chloroform : methanol mixture

Equally efficient. Hot extractions are faster. Cold extractions, require less monitoring and may permit more samples to be processed.

chlorophyll more stable in 95% methanol than in 100% methanol

Not necessary if A750 < 0.005 per cm.

Problematic with methanol. Use HPLC.

Problematic with methanol. Use HPLC.

Measure area (2d.) with video / image analysis system or use profilometer to estimate 3d.area

Figure 1) Steps in the extraction of chlorophyll from epilithic microalgae, and a summary of conclusions and recommendations from this study.

3.3.2 Pre-treatment

The state of hydration of rock samples from the intertidal zone varies with the tidal cycle. Depending on weather conditions whilst the tide is out, biofilms may remain hydrated or dry out. Castenholz (1961) and Nusch (1980) reported that extraction was incomplete unless algal samples were moist before processing. In contrast, Dye and White (1991) dried samples prior to extraction, whilst other researchers do not refer to the state of hydration and apparently did not standardise this factor (e.g. Underwood, 1984c; Hill and Hawkins, 1990; Hill and Hawkins, 1991). The effects of state of sample hydration on chlorophyll extraction clearly requires examination.

3.3.3 Storage

Appropriate methods for storing microalgae without degradation of chlorophyll permit sampling programmes to proceed uninterrupted by processing, and facilitate collection from remote locations. Dye and White (1991) dried samples, ground them in chloroform : methanol (2 : 1) and stored the extracts obtained for several days before measuring their absorbance. This method relies on a slow extraction and minimal degradation of chlorophyll in the solvent. Alternatively, Castenholz (1961) found freezing to be an acceptable storage method. Hydrated planktonic algae have also been successfully stored frozen (Holm-Hansen and Riemann, 1978; Marker *et al.*, 1980; Nusch, 1980), However, chlorophyll estimates were reduced if samples were dried prior to freezing (Sand-Jensen, 1976). In this Chapter, the stability of chlorophyll in methanol was examined, and methods for storing epilithic algae, prior to extraction were compared.

3.3.4 Chlorophyll Extraction

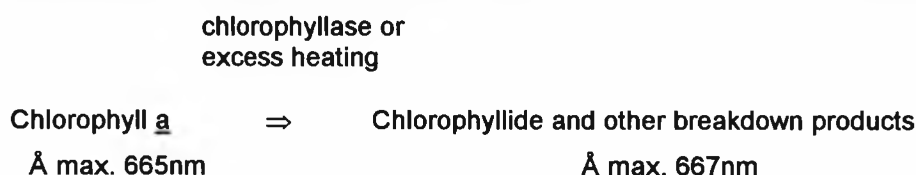
Chlorophyll is extracted from the microalgae by immersing samples in acetone (Nicotri, 1977; H.M.S.O., 1983; Underwood, 1984c), alcohols (H.M.S.O., 1983; Hill and Hawkins, 1990; Bustamante *et al.*, 1995) or mixtures of solvents (Dye and White, 1991). Acetone is considered satisfactory for extractions from diatoms, whilst alcohols are superior for extractions from cyanobacteria (see Marker *et al.*, 1980). Similarly, for mixed communities of diatoms and cyanobacteria, Hill & Hawkins (1991) found methanol gave a better extraction than acetone. In addition to the range of solvents used, a variety of extraction procedures are also available. Some researchers favour rapid extractions in hot alcohol (Hill and Hawkins, 1990) others prefer longer extraction periods at room temperature (Castenholz, 1963; Nicotri, 1977; Dye and White, 1991) or under refrigerated conditions (e.g. HMSO, 1983). When considering which extraction to use there are various operational factors which have consequences for the accuracy and precision of the final chlorophyll estimate (Table 1). In this Chapter various commonly used solvents and extraction methods (Appendix 1) are compared.

Table 1) Important considerations in the choice of precise, accurate protocols for extraction of chlorophyll from epilithic microalgae.

Consideration	**benefit**
completeness of extraction	gives reliable estimate with minimal variation
speed / simplicity	maximises possible replication
low cost of solvents	maximises possible replication
non-hazardous solvent	requires minimal laboratory facilities
minimum interaction with chlorophyll	breakdown of chlorophyll minimised
amenable to measurement of accessory pigments and degradation products	permits estimation of true chlorophyll a content

Heating may assist extraction and help to minimise chlorophyll degradation by denaturing chlorophyllases which are released from the algae, together with chlorophyll, during processing (Bacon and Holden, 1967; Talling, 1969; Nusch, 1980). However, excess heating will increase chlorophyll degradation (see Equation 1). Long extraction or cooling periods could also increase the proportions of degraded chlorophyll (see Nusch, 1980). Optimal times for heating and cooling samples in order to give maximum extraction and minimal chlorophyll degradation were determined.

Equation 1) Breakdown of chlorophyll a by chlorophyllase or excess heating (Å = Absorbance).



3.3.5 Centrifugation

Some protocols suggest centrifuging the chlorophyll/solvent extract to remove suspended particles which would otherwise interfere with absorbance readings (e.g. H.M.S.O., 1983). Unlike extractions from plankton or soft sediments, fine particles are probably not abundant in extracts from hard substrata and so the effects of centrifugation were examined in order to determine whether this step was advantageous.

3.3.6 Spectrophotometric Determination

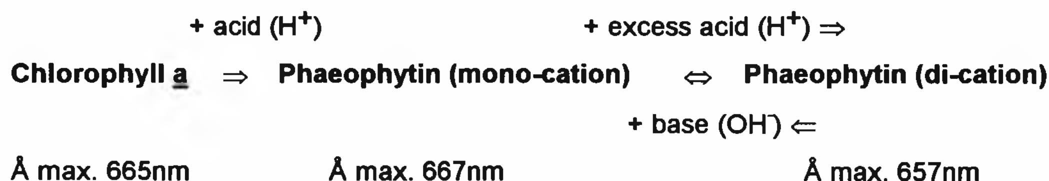
Chlorophyll a concentrations can be quickly determined by measuring absorbance (Å) at the maxima for chlorophyll a, 665nm, using a spectrophotometer, then measuring for background absorbance at 750nm and subtracting this from the reading at 665nm (e.g. H.M.S.O., 1983; Marker, 1994, see Appendix 2). However, this procedure takes no account of chlorophyll degradation products, such as phaeophytin, which may be present as a result of natural chlorophyll a breakdown by chlorophyllase

enzymes in the algae (Equation 1). These pigments have absorbance maxima at 667nm and will increase the estimates of chlorophyll a. Degradation products may be abundant in samples from plankton (16 - 60% Marker *et al.*, 1980) or soft sediments and cause chlorophyll a estimates to be considerably overstated (80 -100%, see Nusch, 1980; H.M.S.O., 1983). In contrast, degradation products are thought to be minimal in biofilms (H.M.S.O., 1983) and correction may not be worthwhile.

Chlorophyll degradation can also occur during extraction. This is undesirable since it will lead to underestimation of the true chlorophyll content of a sample. Chlorophyll breakdown is affected by the proportion of water present (Nusch, 1980; Jensen and Knutsen, 1993), extraction duration and extraction temperature (Equation 1, See Nusch, 1980). The amount of chlorophyll a degradation which occurred during processing was compared between different extraction methods.

The proportion of degraded chlorophyll (degradation products) can be determined qualitatively by measuring absorbance at 410nm and 430nm (H.M.S.O., 1983, see Appendix 2) or, more accurately, by acidifying samples to convert all the chlorophyll a to phaeophytin and then measuring absorbance again at 665 and 750nm (H.M.S.O., 1983, see Appendix 2). However, if the pH of the solution falls below 2.6, phaeophytin will become ionised to the di-cation leading to overestimation of chlorophyll (Equation 2). Ionisation is easiest to control in acetone and hardest to control in alcohols (Usecheva, 1971; Moed and Hallegraeff, 1978). Addition of a small quantity of distilled water (~ 10%) to the solvent helps to maintain pH and prevent the solution becoming too acidic (Moed and Hallegraeff, 1978).

Equation 2) Breakdown of chlorophyll a to phaeophytin and the effect of pH on the phaeophytin monocation - dication equilibrium (Å = Absorbance).



Additional inaccuracies will occur if chlorophylls b and c are present as these will increase absorbance readings at 665nm. Their presence can be detected by measuring absorbance at 630 and 645nm (H.M.S.O., 1983). Unfortunately, the correction procedure requires high quality instrumentation (Jeffrey and Humphrey, 1975) and is susceptible to error (Marker *et al.*, 1980). In addition, equations for calculation of chlorophylls b and c are not valid if the extractant contains more than five percent degradation products (Marker, 1994).

For diatoms, 50 to 60% of the total chlorophyll may be present as chlorophyll c (Jeffrey, 1976), whilst for cyanobacteria up to 50% may be bacterio-chlorophyll (Stahl *et al.*, 1984). However, there are no accounts of any research actually measuring either the proportions of degradation products or chlorophylls b and c in solvent extracts from epilithic biofilms, and assumptions about their relative abundance need to be tested. Estimates of the proportions of chlorophyll degradation products and chlorophylls b and c were examined and the reliability of these estimates was determined using standard chlorophyll solutions.

3.3.7 Measurement of Rock Area Sampled

In order to convert chlorophyll estimates to values per unit area, the surface area of the rock sample must be determined. Methods such as drawing around samples on to graph paper by hand or videoing and measuring with image analysis software (Dye and White, 1991) have been used. However, these techniques measure the rock surface in two dimensions (X and Y). For

rugose samples this may considerably underestimate total surface area. Hill and Hawkins (1990) incorporated rugosity (Z dimension) by pressing aluminium foil to the surface relief of samples then flattening the foil and measuring its total area (A. Hill, pers. comm.). These methods for determining surface area were compared.

3.4 GENERAL METHODS

3.4.1 Sample Collection

Rock chips, together with associated microalgae, were collected from the shore at mid tide level at Kallow Point, Port St Mary, Isle of Man (4°44'12W, 54°4'0N, see Southward, 1951; Southward, 1953; Hawkins, 1979; Hawkins, 1983, for site description). Rock chips were chiselled from areas of 'bare' rock which had relatively smooth surface topography and were not swept by macroalgae. Care was taken to ensure macroalgal germlings, encrusting macroalgae or barnacles, which would have increased chlorophyll estimates, were not present on the samples (Underwood, 1984c, R. C. T. unpublished data for barnacles).

Sequential mean estimates indicated that a total area of rock surface in excess of 20 cm^2 was required to overcome natural spatial variability (see Hill and Hawkins, 1990). This was achieved by collecting a minimum of ten 2cm x 2cm (approx.) rock chips for each experimental treatment. Where several similar sets of samples were required (for example to compare extraction between several solvents), between sample variation was minimised by either prising a small slab of rock from the shore (~ 30cm x 30cm) and breaking this into smaller pieces, or by chiselling small samples from adjacent positions on the shore. Rock chips were then placed in separate plastic bags and kept in the dark at 10°C. All samples, with the exception of those used to compare storage techniques, were processed within 48 hours of collection.

3.4.2 Laboratory Processing

Work was conducted in subdued light as a standard precaution during chlorophyll analyses (H.M.S.O., 1983; Marker, 1994). Where rehydration was required as a pre-treatment this was achieved by immersing samples in filtered (0.22 µm) sea water for a minimum of 30 minutes. Conversely, where drying was required, rock chips were exposed to air at room temperature overnight.

Extractions were carried out by placing each rock chip into a separate 60ml screw topped glass jar and adding around 20-25ml of solvent. Jar lids were then tightened to minimise evaporation. The exact volume of solvent was not critical so long as it was sufficient to ensure the algae were completely immersed and to produce a final concentration of chlorophyll within the tolerances of the spectrophotometer (Å665nm within the range 0.05 to 0.700, H.M.S.O., 1983). Solvents were Analar® grade, and were diluted with distilled water where required (see Appendix 1 for details). They were always handled in a fume cupboard or well ventilated area.

Where heating was required as part of the extraction, this was carried out by warming solutions from room temperature in a water bath situated in a fume cupboard. During heating, jar lids were loosened. Depending upon the protocol followed, cold extractions were either carried out in a refrigerator (4°C) or at room temperature. The volume of solvent, remaining after extraction was determined by subtracting the weight of the jar and rock sample prior to extraction, from the weight of jar, rock sample and solvent at the end of the extraction, and multiplying this value by the solvent density (g cm^{-3}).

When centrifuging was required, approximately 12ml of solvent/chlorophyll mixture was removed from each jar, transferred to a centrifuge tube using a plastic syringe and centrifuged for 7 minutes at 3500 rpm (H.M.S.O., 1983). The supernatant was transferred to glass optical cells (1cm or 4cm path

length) and its absorbance measured on a Phillips PU8670 spectrophotometer. Wavelengths used were 665nm for chlorophyll a, 750nm to correct for interference, 630 and 645nm for chlorophylls b and c, 410 and 430nm for rapid determination of chlorophyll degradation products. A more precise determination of degradation products was made by acidifying samples and measuring absorbance again at 665 and 750nm (H.M.S.O., 1983, see Appendix 2). Rock samples were removed from the solvent, washed, allowed to dry and their surface area measured using a video and image analysis software.

3.4.3 Statistical Analyses

Comparisons between sets of samples subjected to two different extraction methods were made using *t*-tests. Comparisons of a single chlorophyll extract before and after a treatment were made using paired sample *t*-tests. Comparisons between several sets of samples where made using ANOVA. Cochran's test for homogeneity of variance was performed to check for homoscedasticity (Winer *et al.*, 1971; Underwood, 1981b) and data were transformed where desirable. Plots of residuals were examined after each ANOVA to check that error terms were normally distributed. Coefficients of variation were calculated to allow comparison of precision between methods. Data were compared using two-tailed tests so that differences would be apparent irrespective of theoretical expectations as to their direction. Statistical power varies between these methods and so comparisons of levels of significance between data tested in different ways should be avoided. Instead, it may be more appropriate to consider the extent to which results were modified by a particular treatment rather than the absolute level of significance for the difference.

3.5 EXPERIMENTAL EVALUATIONS

3.5.1 Pre-treatment: State of Sample Hydration

3.5.1.1 Methods

The effects of microalgal hydration on extraction efficiency was examined during a mid-day low tide in summer 1994 when weather conditions were dry and sunny. An area of shore was selected which had just become uncovered by the ebb tide and the rock surface was still moist. Ten rock chips were chiselled from the shore and each was split into two to give a pair of samples. One member of each pair was repositioned on the shore in the place it had been in prior to chiselling whilst the other was sealed in a plastic bag and stored at 10°C. Samples on the shore were collected after they had been exposed to the air for three hours. All rock chips were then processed in hot 100% methanol (Appendix 1) and absorbance measured at 665 and 750nm.

The experiment was repeated with different solvents (95% methanol, chloroform : methanol 2 : 1, and 90% acetone), using samples which were either allowed to dry or were hydrated in filtered seawater (22µm pore size). In each case ten samples were processed dry and ten were processed hydrated.

Hydrated samples yielded at least three times more chlorophyll than dried ones (see results). To determine whether this difference was caused by an incomplete extraction of chlorophyll from the dried microalgae, a further experiment was conducted. Five of the rock chips which had initially been processed dry and five which had initially been processed hydrated were selected at random from both 100% methanol and 90% acetone extractions (above). These samples were all rehydrated and extracted for a second time using fresh solvent.

An effective but easily achievable standard method for hydrating microalgae, prior to chlorophyll extraction, was then determined. Three sets of 15 samples were collected, one set was allowed to dry and two sets were hydrated. One hydrated set was then lightly blotted with tissue to remove excess water whilst the other set was left 'wet'. All samples were processed in hot 100% methanol (Appendix 1).

To determine the time required for complete rehydration a further two sets of samples (n = 15) were collected and left to dry. One set was then rehydrated for 12 hours and the other rehydrated for 30 minutes. Samples were removed from the seawater, blotted and processed in hot 100% methanol (Appendix 1).

An equation to convert chlorophyll estimates obtained from dry material to corresponding values obtained from wet material was calculated using model I regression. During January 1995, forty five rock chips were collected from positions on the shore which were known to have differing microalgal abundance. Each chip was split into two giving a pair of samples. One sample from each pair was processed fully hydrated whilst the other was processed dry. The experiment was repeated during March 1995 to confirm the relationship between the two methods was consistent between sampling dates. All data were log transformed to achieve homogeneity of variance prior to regression analysis.

3.5.1.2 *Results*

Samples collected shortly after they had been uncovered by the tide and processed whilst still wet gave significantly greater estimates of chlorophyll than those collected dry three hours later. The same result was obtained with each of the extraction solvents examined ($t_{0.05\,(2)\,18} = 2.10$, $P < 0.001$ in all cases, Figure 2).

Additional chlorophyll was released from samples after hydrating and extracting for a second time; those which were initially processed dry yielded more chlorophyll than those which were initially hydrated. This difference was significant with acetone extractions, and just short of significance with methanol extractions (Figure 3).

Hydrated and dry samples used to determine the optimal hydration method followed the same pattern: those that had been dried and then rehydrated gave significantly greater chlorophyll estimates (one-way ANOVA, $F_{2,27}$ = 74.43, $P < 0.001$ for both wet samples and blotted samples) than those which were left dry. Wet samples gave similar estimates (mean ± variance, 13.34 ± 31.82) to blotted samples (mean ± variance, 10.54 ± 4.52). However, the variance of wet samples was greater than that of blotted samples.

Chlorophyll a estimates were not significantly different between samples hydrated for 30 min or for 14 hrs. ($t_{0.05(2),16}$ = 0.48 n.s.; mean ± 1SE, 30 min. = 8.81 ± 0.67; 14 hrs. = 9.36 ± 0.9). Rehydration for a minimum of 30 minutes, followed by blotting was adopted as a pre-treatment for all future determinations.

Regression analysis gave a significant correlation between hydrated and dry extractions. The results for samples collected in March were more variable than those for January, but the regressions were not significantly different ($t_{0.05(2),84}$ = 0.49 n.s.). The analysis was repeated using combined data from both sampling dates (Table 2) and the regression equation obtained was used to convert chlorophyll estimates from dry extractions to values comparable with wet extractions (e.g. Chapter 5).

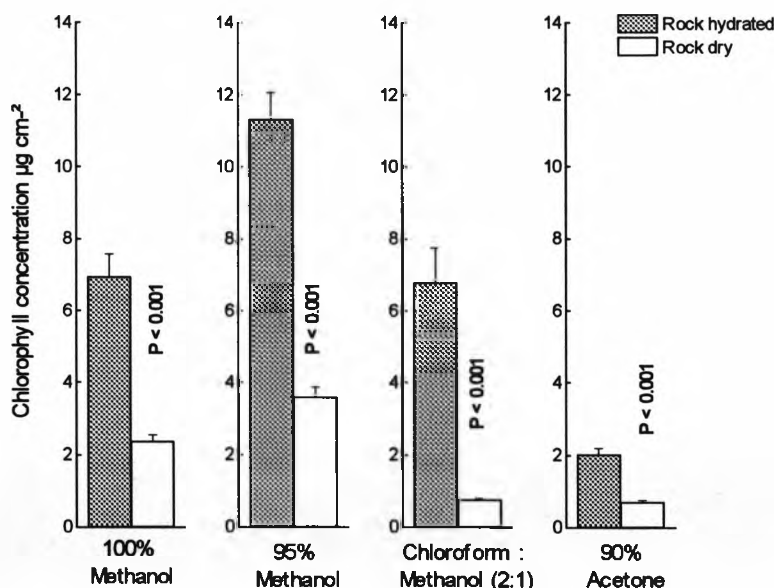


Figure 2) Estimates of chlorophyll a concentration from hydrated and dry intertidal rock samples extracted in various solvents (n = 10 in each case, bars = 1 SE). The significance of differences are given. Comparisons between solvents should not be made as sampling dates varied.

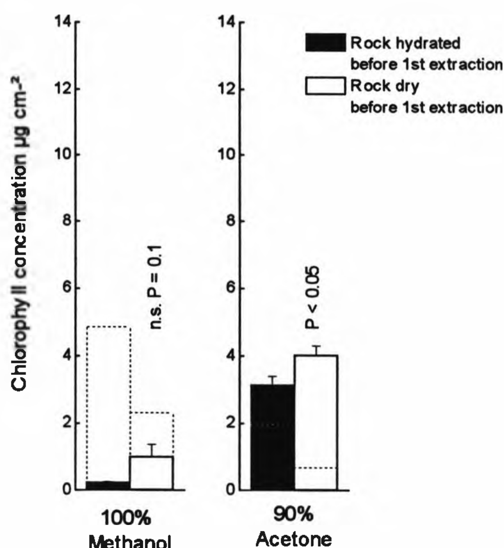


Figure 3 Additional chlorophyll a released after rehydrating intertidal rock samples and extracting for a second time in either methanol or acetone. Chlorophyll already released during the first extraction, in which samples were either hydrated or dry, is shown (dotted line). The significance of any differences between chlorophyll released by hydrated and Dry samples during the second extraction is given (bars = 1SE).

Table 2) Comparison of regression equations for relationship between estimates of chlorophyll extracted from samples processed using hydrated (wet) or dry extraction methods on different dates.

	Comparison of regression analyses from hydrated vs. dry extraction											
source of variation	January data				March data				Combined data			
	df	MS	*F*	*P*	df	MS	*F*	*P*	df	MS	*F*	*P*
Regression	1	0.715	59.39	***	1	0.547	14.10	***	1	0.873	39.30	***
Error	40	0.012			44	0.039			85			
Cochran's test	C_{crit}, P0.01 = 0.71; C = 0.66				C_{crit}, P0.01 = 0.71; C = 0.56				C_{crit}, P0.01 = 0.61; C = 0.58			
Regression equation	Log wet = 0.56+ 0.53 Log dry				Log wet = 0.55+ 0.62 Log dry				Log wet = 0.61+ 0.48 Log dry			
r^2	0.59				0.23				0. 31			

3.5.2 Sample Storage

3.5.2.1 *Method*

Various methods of sample storage were examined. Seven sets of 15 rock chips were collected. Four sets were hydrated for 30 minutes whilst the other three were allowed to dry. One hydrated set was extracted immediately, in 100% hot methanol (Appendix 1), as a control. One set was stored hydrated and another stored dry in each of the following ways: refrigerator (4°c) for one week, freezer (-14°c) for six weeks, and for 40 weeks. After storage, all samples were rehydrated, and then extracted in hot 100% methanol. Chlorophyll estimates from stored material were compared to those from the control set using Dunnett's Test (Zar, 1984).

3.5.2.2 *Results*

Chlorophyll values calculated from samples in each of the six types of storage method gave lower, but not significantly different, estimates than those obtained from fresh ones (one-way ANOVA, $F_{6,96}$ = 1.80, n.s.). Estimates from samples stored wet at 4°C for one week were closest to those obtained from fresh samples (9% reduction). Samples stored in the freezer the gave lowest chlorophyll estimates (10 - 25% reduction) with considerably increased coefficients of variation in some cases (Figure 4).

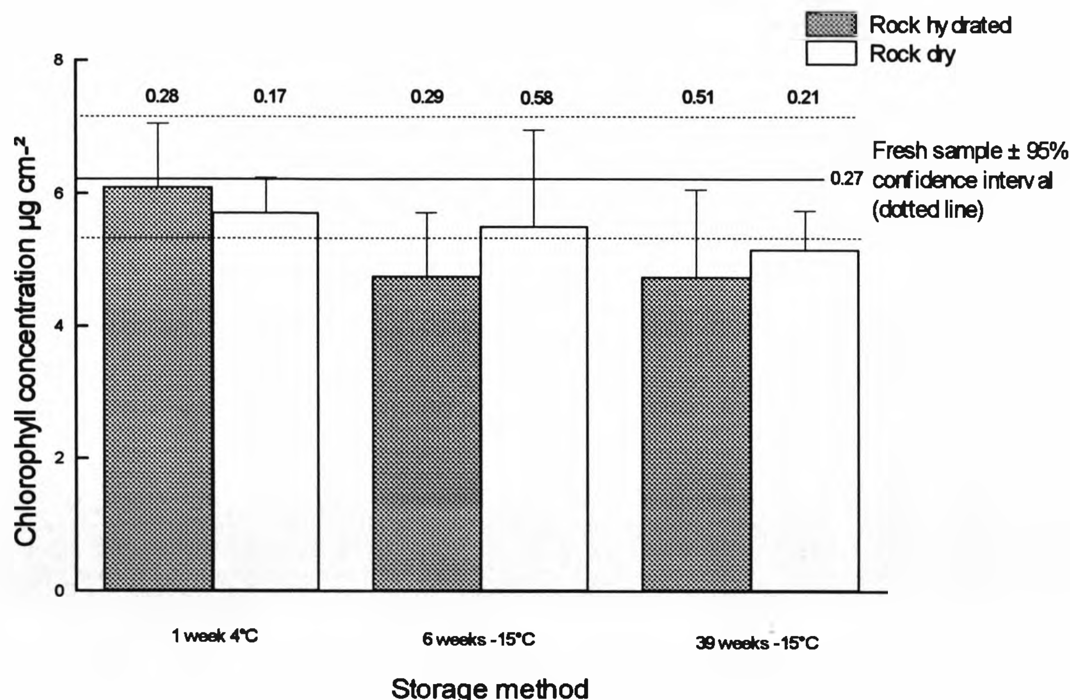


Figure 4) Comparison of chlorophyll estimates between freshly processed rock samples and samples which were stored either hydrated or dry in different conditions (bars = 1SE). The coefficient of variation is given above each estimate.

3.5.3 Choice of Extraction Solvent and Protocol

3.5.3.1 *Methods*

Eight sets of 15 samples were collected, each set was hydrated, blotted dry and then processed using a different extraction method. (all methods except cold 95% methanol, Appendix 1). The absorbance of each sample was recorded at 665 and 750nm.

To determine the completeness of this initial extraction, samples (n = 6) from hot 95% methanol, hot 100% methanol, cold 100% methanol, hot 90% ethanol and cold 90% acetone were processed again for a second, and third time in a fresh portion of solvent. Between extractions, samples were removed from the solvent, rinsed in filtered seawater and rehydrated. The amount of chlorophyll released during the first extraction was then determined as a percentage of the total released during all three extractions. This gave a measure of efficiency of the initial extraction.

3.5.3.2 *Results*

Chlorophyll was released from all samples during the first extraction. However, there were considerable differences in the amount extracted between each of the solvents (Figure 5). Extractions in methanol yielded the greatest chlorophyll concentration, hot 95% methanol gave a lower coefficient of variation than the other methanol methods. Hot and cold acetone both gave very poor extractions yielding less than half the amount of chlorophyll released by methanol.

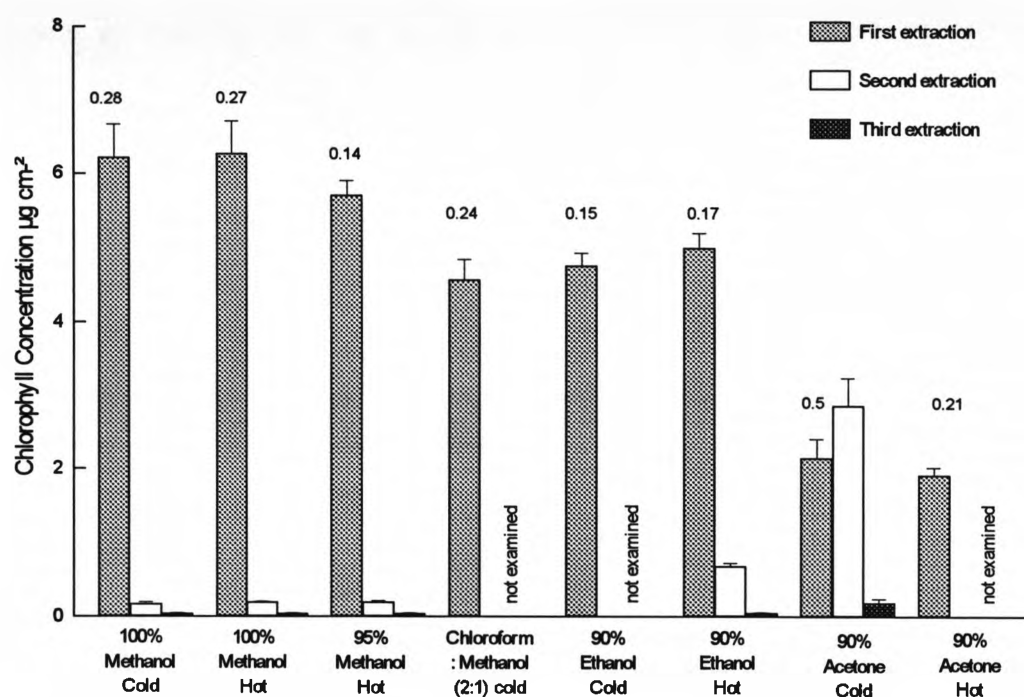


Figure 5) Estimates of chlorophyll concentration extracted from the surface of intertidal rock using a variety of methods. Each sample (n = 15 for each solvent) was extracted three times in a fresh portion of solvent, as guide to the efficiency of each method (bars = 1 SE). The coefficient of variation for the initial extractions is shown above the columns.

Additional chlorophyll was released during subsequent extractions (Figure 5). Solvents which had given high chlorophyll concentrations during the first extraction yielded virtually no additional chlorophyll during the second extraction, indicating that most of the chlorophyll present had already been extracted. Conversely, solvents which had given lower chlorophyll

concentrations in the first extraction yielded appreciable quantities in the second extraction. Surprisingly, 90% acetone extracted more chlorophyll during the second extraction than during the first. The third extraction yielded little chlorophyll in any of the solvents.

The efficiency of the first extraction (amount of chlorophyll extracted initially as a percentage of the total extracted in all three extractions) varied significantly between methods (one-way ANOVA, $F_{(4,29)} = 2.76$, $P < 0.001$; Table 3). Methanol extracted over 96% of the available chlorophyll during the first extraction. This was significantly more than with either ethanol (88%) or acetone (42%). Hot and cold extractions in methanol yielded similar amounts of chlorophyll. Cold extractions took slightly longer to complete, but required less monitoring.

Table 3) Total chlorophyll released from epilithic microalgae during three sequential extractions in fresh portions of solvent. The efficiency of the first extraction is expressed as a percentage of the total extraction and the direction of any significant differences are shown.

Solvent	Total extraction µg cm^{-2} (mean ± SE)	First extraction (%)	Direction of significant differences Tukey's test for pairwise comparisons
Cold methanol 100% (cm 100)	5.65 (±0.29)	96	**cm 100** > he 90, > ca 90.
Hot methanol 100% (hm 100)	6.33 (±0.72)	97	**hm 100** > he 90, > ca 90.
Hot methanol 95% (hm 95)	5.34 (±0.32)	98	**hm 95** > he 90, > ca 90.
Hot ethanol 90% (he 90)	5.64 (±0.38)	88	cm 100, hm 100, hm 95, < **he 90** > ca 90
Cold Acetone 90%(ca 90)	5.72 (±1.41)	42	cm 100, hm 100, hm 95, **< ca 90**

Cochran's test $C = 0.289$
C_{crit}, P0.05 = 0.507
$P < 0.001$, $F_{(4,29)} = 2.73$; one way ANOVA between solvents (arcsine transformed data)

3.5.4 Extraction Efficiency and Stability of Chlorophyll in Hot and Cold Methanol

3.5.4.1 *Methods*

The duration of heating required to give maximum extraction with minimal degradation was determined for both 100% and 95% methanol. For this experiment it was necessary to remove small (< 10cm³) portions of solvent during the extraction, and so a large volume of solvent and a large area of rock surface were used. Several rock chips (combined surface area ~ 15cm²) were put into each of eighteen (250 ml) screw top containers, 100 ml of 100% methanol was added to nine of these and 100ml of 95% methanol added to the other nine. Chlorophyll was then extracted by bringing samples to the boil in a water bath and boiling for 40 minutes. During the extraction portions of solvent were removed from each container at intervals (see Figure 6 for sampling times) and their absorbance measured at 665 and 750nm. The proportion of degradation products present (see Appendix 2) was also measured on three occasions (see Table 4 for sampling times).

After heating, samples are usually allowed to cool prior to spectrophotometric measurement (H.M.S.O., 1983). The optimum time for cooling and the optimum total extraction duration for cold extractions were examined in a similar manner to that for the optimum heating period experiment (above). Once again 100% and 95% methanol were used. The objective was to determine a time window for maximum extraction prior to any degradation or deterioration which might take place thereafter. Several rock samples (combined surface area ~15cm²) were put into each of 36 separate 250ml screw top containers, 100 ml of 100% methanol was added to 18 of these and 100ml of 95% methanol added to the other 18. Nine containers from each solvent concentration were then heated until the solvent started to boil. The remaining nine from each solvent concentration were not heated (cold extraction). All containers were then kept in the dark at room temperature (20°c) and small amounts of solvent (< 10 cm³) were removed from each at intervals over the next four days (see Figure 6 for

sampling times). On removal, the absorbance of the solvent was measured at 665 and 750nm. The concentration of degradation products (Appendix 2) was measured on four occasions.

For both these experiments, each jar was wiped dry and weighed before and after removal of solvent. Changes in weight were used to correct for increases in chlorophyll concentration caused by evaporation of solvent. It was hoped that by using a large volume of solvent and removing small portions for analysis the influence of any changes caused by the removal of solvent would be minimal.

3.5.4.2 *Results*

Similar amounts of chlorophyll were extracted by heating microalgae in either 100% or 95% methanol (Figure 6). Ninety-five percent methanol gave slightly greater variation between samples. This was mostly caused by two samples which released unusually large amounts of chlorophyll.

The extraction could be divided into three stages (Figure 6): an extraction phase (lasting 70 minutes) between addition of the solvent and the solution reaching boiling point (65°C) with most of the extraction being complete by the first sampling occasion after 37 minutes (approx. 45°C); a stable phase immediately after boiling where estimates remained constant; and then a distortion phase where estimates became spuriously elevated. The stable phase was longer lasting with 95% methanol (25 min.) than with 100% methanol (7 min.), suggesting that the presence of water may have helped to delay the changes which were responsible for the subsequent elevated readings. The degree of degradation of chlorophyll a remained relatively constant throughout (Table 4) and so, unless further breakdown of phaeopigments occurred (Equation 2), it seems unlikely that elevated estimates in the deterioration phase were caused by degradation. However, the determination of the proportions of degradation products was problematic (see later).

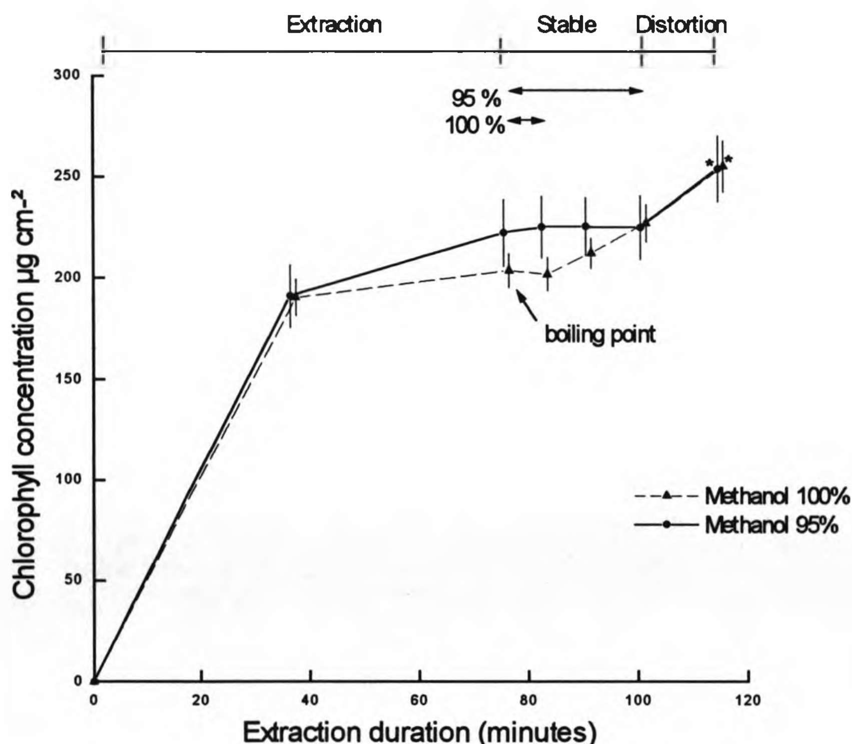


Figure 6) Estimates of chlorophyll a concentration obtained from epilithic microalgae by heating in either 100% or 95% methanol for various periods (bars = 1SE). Three phases were observed: extraction, stable and distortion (see text). The duration of the constant phase is indicated by arrows for each solvent. Values marked * are probably not chlorophyll a and may indicate formation of a chlorophyllide which has a similar absorbance to chlorophyll a.

Table 4) Degree of degradation of chlorophyll in 100% and 95% methanol at boiling point and on continued heating thereafter. Values around 1.6 indicate no degradation, 1 indicates complete degradation.

Solvent	Total extraction duration (minutes)		
	76 (boiling point)	91	115
100% Methanol	1.62	1.64	1.61
95% Methanol	1.52	1.46	1.6

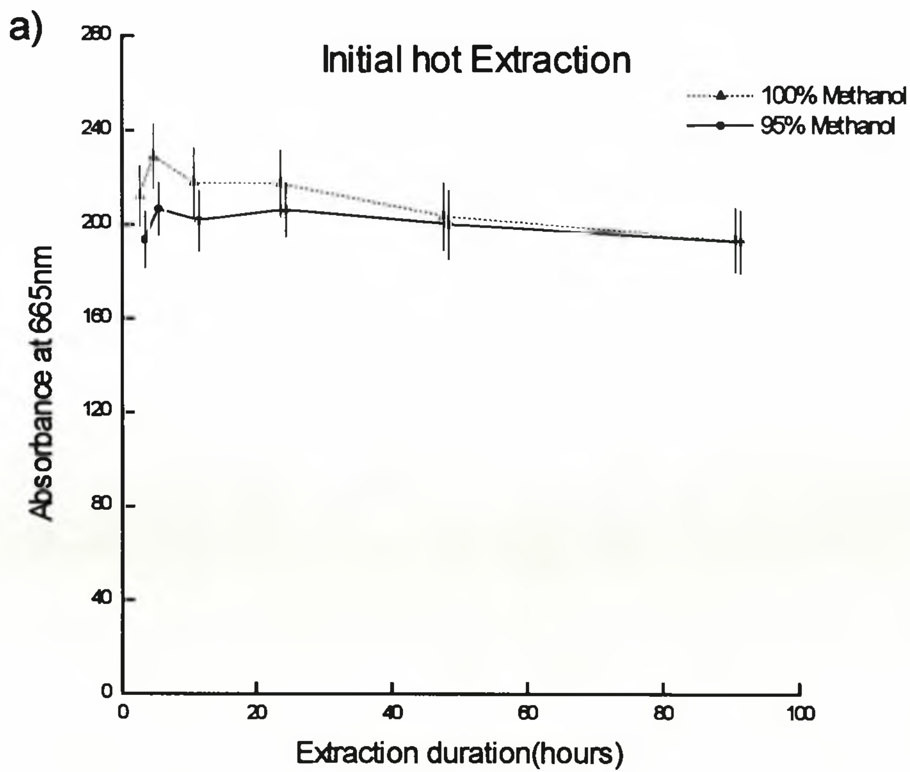
Hot extractions in both 100% and 95% methanol, gave greatest absorbance (maximum extraction) when first measured three hours after the addition of solvent (Figure 7a). For cold extractions in both 100% and 95% methanol, extraction was maximal after five hours (Figure 7b). At this time, absorbance was similar to that in hot extractions. The stability of these solutions was

considered using an arbitrary level of five percent for the maximum tolerable discrepancy of subsequent estimates (Table 5). Estimates from cold extractions remained stable for at least 19hrs. (readings taken 24h. after addition of solvent) whilst estimates from hot extractions were stable for less than 8h. (readings taken 11h. after addition of solvent).

Pure methanol (100%) gave slightly greater (plus 5 to 10%) initial extraction than 95% methanol. However, estimates from 100% methanol decreased more rapidly than those from 95% methanol. (Figure 7, Table 5). This suggests that the presence of water helped to retard chlorophyll deterioration. For all methods the degree of degradation increased slightly during the four days, indicating that the deterioration (decrease in absorbance) was at least partly caused by breakdown of chlorophyll a into phaeophytin (Table 5).

Table 5) Degree of degradation (values around 1.6 indicate no degradation, 1 indicates complete degradation) and percentage decrease (bracketed) in chlorophyll a estimates at various times after hot and cold extractions in 100% and 95% methanol. Arrowed lines indicate period during which values were within 5% of the maximum estimate (nm = not measured, < max = maximum extraction not reached).

Solvent	Hours in solvent					
	3	5	11	24	48	91
100% hot methanol	nm (0)	1.64 (-3)	nm (-10)	1.54 (-13)	1.69 (-15)	1.47 (-23)
95% hot methanol	nm (0)	1.63 (-1)	nm (-7)	1.54 (-10)	1.59 (-12)	1.54 (-15)
100% cold methanol	< max.	1.58 (0)	nm (-5)	1.53 (-5)	1.62 (-11)	1.55 (-15)
95% cold methanol	< max.	1.6 (0)	nm (-3)	1.67(0)	1.62 (-3)	1.55 (-7)



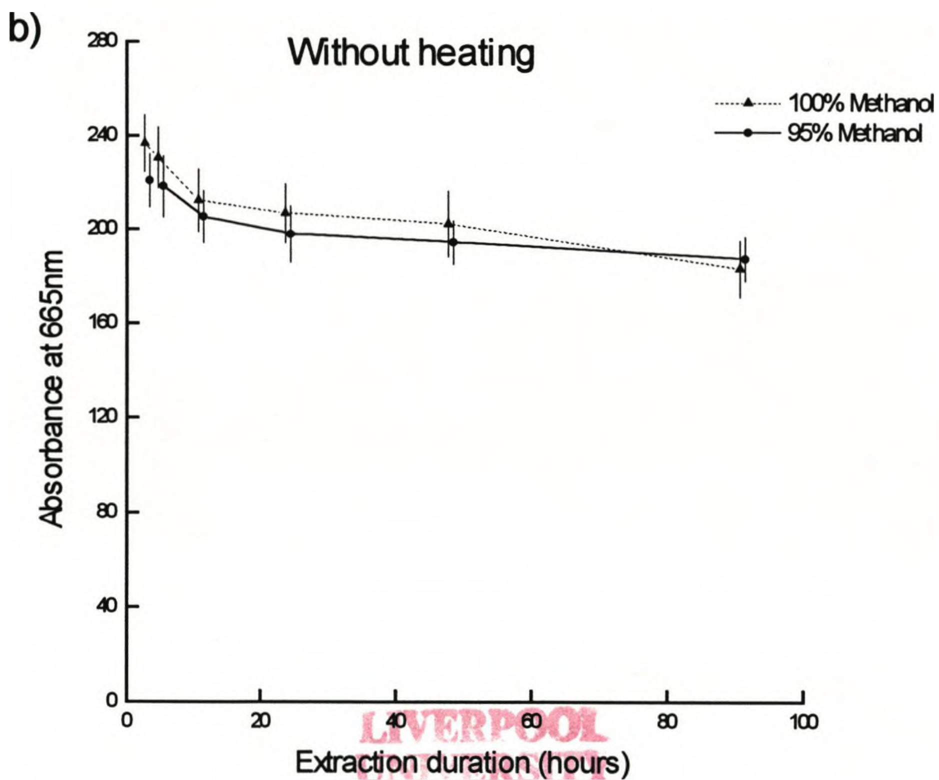


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Figure 7) Absorbance of chlorophyll extracted from epilithic microalgae over extended time periods, after an initial hot extraction (a) and without heating (b), in 100% methanol and 95% methanol (bars = 1SE).

3.5.5 Centrifugation

3.5.5.1 *Methods*

The importance of centrifugation (recommended in H.M.S.O., 1983) as a step in the extraction was examined. Fifteen rock samples were collected and processed using hot 100% methanol (Appendix 1). Sample jars were left to cool for 3 hours and then $12cm^3$ of solvent was removed using a small syringe. Care was taken not to disturb any small fragments of rock that had settled on the bottom of the jars. The absorbance of these samples was measured at 665 and 750nm. Samples were then centrifuged at 3500rpm for 7 min. (H.M.S.O., 1983) and absorbance measured again. Differences between absorbances at Å665, Å750 and Ånet before and after centrifugation were compared using a two tailed t-test for paired samples.

3.5.5.2 *Results*

Before centrifuging, readings at 750nm were within the tolerances of the method (Å750 <0.005 per cm path length of optical cell, H.M.S.O., 1983) suggesting that interference was slight. Absorbances at both 665nm and 750nm were not significantly different after centrifuging (665nm, ($t_{0.05(2),22}$ = 1.35 n.s.; 750nm, $t_{0.05(2),22}$ = 1.71). Combining these results to give the net concentrations, gave a significant ($t_{0.05(2),22}$ = 2.55, P <0.05) but tiny (1%) increase in chlorophyll estimates after centrifuging. Calculating net absorbance combined the changes in Å665 and Å750 in an additive way giving this difference. However, the result cannot have been caused by removal of particulate matter from solution since this would have reduced the absorbance. The difference in Ånet was most probably the result of evaporation during centrifugation.

3.5.6 Correction for Degradation Products and for Chlorophylls b and c

The relative proportions of chlorophyll degradation products and chlorophylls b and c present in epilithic microalgae were examined using rock samples from the shore. Further comparisons were made using standard solutions of pure chlorophyll a to confirm the accuracy of the results from field samples,

and to determine whether any deterioration of chlorophyll occurred during processing.

3.5.6.1 *Methods: Field Sample*

Five sets of 15 five rock chips were collected. Each set was processed in one of the four more efficient solvents from earlier experiments (100% methanol cold, 100% methanol hot, 95% methanol hot, 90% ethanol hot) and also in 90% cold acetone as a comparison since correction methods are well established for this solvent (Lorenzen, 1967; H.M.S.O., 1983). Absorbance was recorded at 665, 750, 630, and 645nm to measure for chlorophylls a, b and c, at 410 and 430nm as a rapid estimate of degradation products and then once again at 665 and 750nm after acidification to determine the proportion of degradation products more precisely (H.M.S.O., 1983, see Appendices 1 and 2). Acidification was achieved by adding small quantities of dilute hydrochloric acid to measured portions of the solvent/chlorophyll mixture using a Gilson pipette (Marker and Jinks, 1982; H.M.S.O., 1983). With ethanol extraction, protocols for determination of chlorophylls b and c were not available and no correction was made. Chlorophyll a estimates obtained before and after correction were compared using two tailed t-tests for paired samples.

With methanol an additional neutralisation step was carried out in order to minimise changes in the absorbance maxima of the solution caused by acidification (Equation 2). This was achieved by adding small quantities of dimethylaniline until the pH of the solution increased to between 2.6 to 2.8. (Moed and Hallegraeff, 1978). The effectiveness of neutralisation was assessed by measuring the pH and the absorbance maxima of these solutions before and after addition of acid and after subsequent addition of base.

3.5.6.2 *Methods: Standard Chlorophyll*

Three methanol extraction techniques were examined using known chlorophyll standards to establish whether any degradation occurred during extraction. Standard solutions of pure chlorophyll a (from the cyanobacterium *Anacystis nidulans*, Sigma C-6144) were prepared to give final concentrations of $1mgl^{-1}$, $0.5mgl^{-1}$ and $0.25mgl^{-1}$ chlorophyll in both 100% and 95% methanol. Three replicate 25ml portions of each solution were pipetted into separate 60ml jars. The absorbance of these samples was read immediately at 665, 750, 645* and 630*, at 410* and 430*nm for a rapid estimate of degradation products (*$1mgl^{-1}$ samples only), and then again at 665 and 750nm after acidification (see Appendix 2). Three replicate portions of each solution were then processed using each of the five different extraction methods (Figure 8, Appendix 1). The absorbance of the solutions obtained was recorded at the same wavelengths used initially.

3.5.6.3 *Results: Field Sample*

Different extraction methods gave differing chlorophyll a estimates (Figure 8). Where extractions were over 24 hours (cold), correction for degradation products reduced estimates of chlorophyll a. These differences were highly significant. The correction also significantly reduced estimates made with hot ethanol. With hot methanol, estimates increased significantly after correction. Theoretically, correction cannot increase chlorophyll estimates, however, the increases were small (<10%), and may have been caused by a shift in the absorbance maxima of the chlorophyll solutions after acidification (Equation 2).

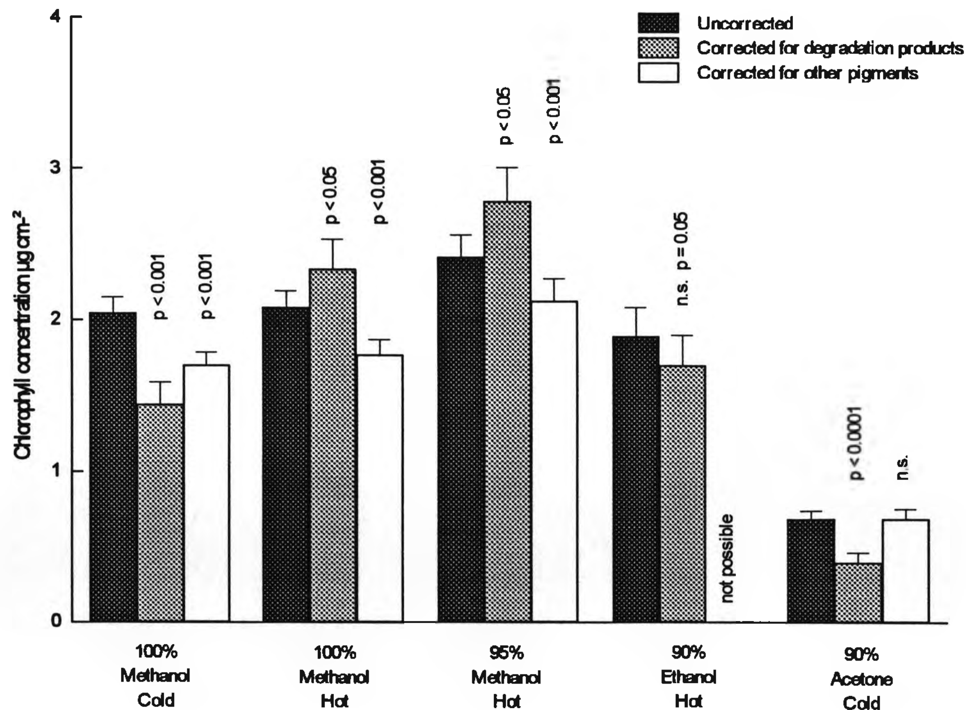


Figure 8) Estimates of chlorophyll concentration extracted from epilithic microalgae using a variety of extraction methods (n = 15 in each case). Uncorrected chlorophyll a estimates and estimates corrected for presence of either degradation products or other pigments are shown (bars = 1SE). The significance of differences between corrected and uncorrected estimates is given (n.s. = not significant).

Correction for chlorophylls b and c reduced the original chlorophyll a estimates (not corrected for degradation products) for each solvent. These differences were highly significant ($P < 0.001$ in all cases except for extractions in acetone), suggesting that chlorophylls b and c were important constituents of the microflora (Figure 8). However, these corrections were not valid for cold methanol and acetone since degradation products were also present (Marker, 1994) and must be viewed with caution for extractions in hot methanol since it is not certain whether degradation products were present (because of the spurious results obtained - see above).

Acidification of chlorophyll in methanol reduced the wavelength of the absorbance maxima for the mixture. The reduction was much greater for 100% methanol (Figure 9a) than for 95% methanol (Figure 9b). Addition of the base to either of these solvent/acid mixtures restored the absorbance

maxima to approximately 665nm. However, because of the larger shift in Åmax. for 100% methanol, there was a risk of underestimating absorbance if the pH of the solution was not adjusted correctly by addition of base.

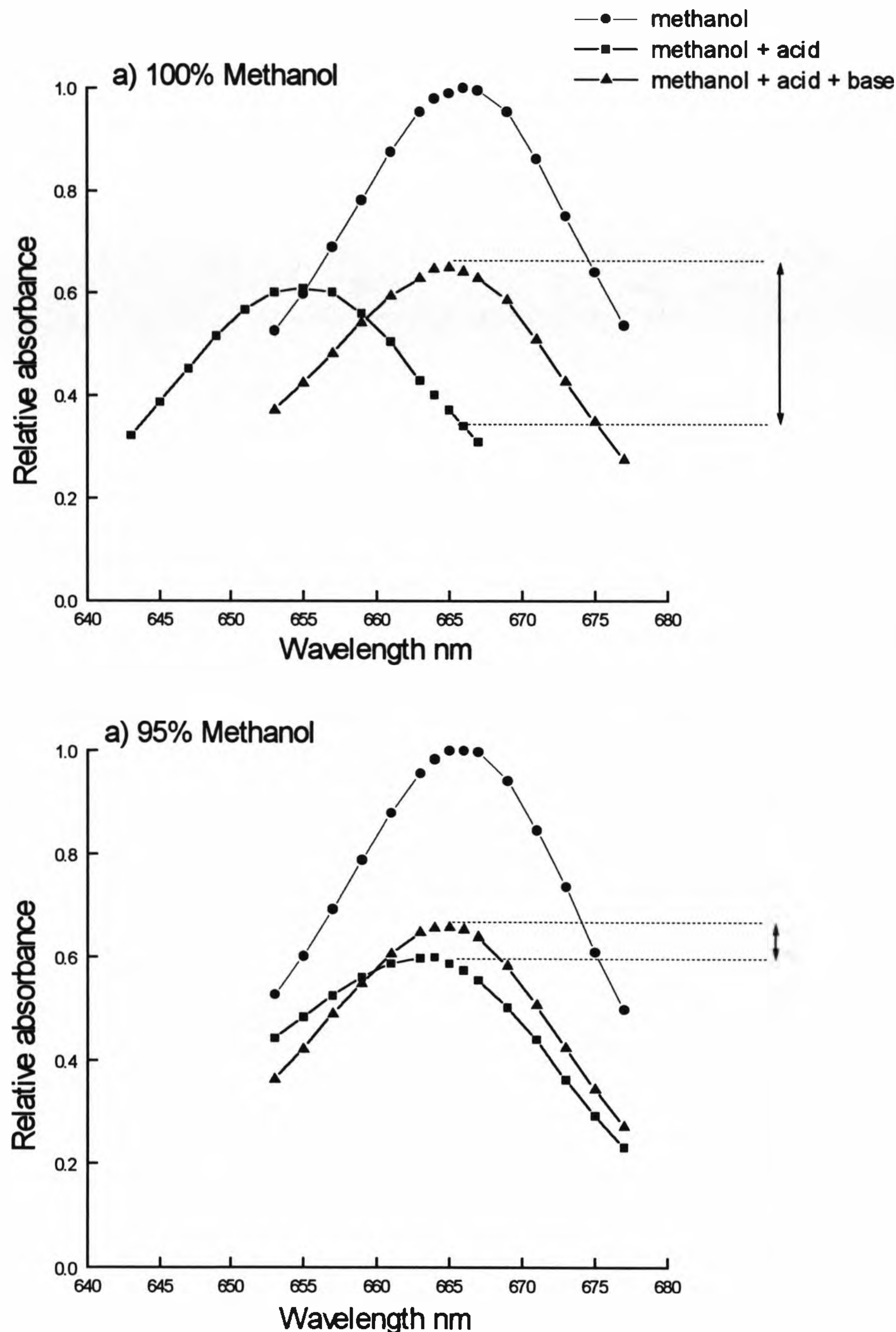


Figure 9) Absorbance (expressed as a proportion of the maximum absorbance) of chlorophyll in solvent, in solvent plus acid (0.3M HCl), and in solvent plus acid plus base (0.3 M dimethylaniline) for 100% and 95% methanol. Arrowed lines show maximum underestimation of Å665 if pH is not adequately restored by addition of base.

3.5.6.4 *Results: Standard Solutions*

With standard solutions of 100% and 95% methanol, uncorrected estimates were similar to the actual concentration of the chlorophyll standard prior to processing. Estimates obtained from the solvent/chlorophyll mixtures changed during processing, and some of these differences were significant (Table 6). Absorbance of extracts processed in either hot or cold 100% methanol tended to decrease (10% on average). Whilst those in 95% methanol increased (11% on average).

Table 6) Estimates (mean ± SE) of chlorophyll concentrations (μg l^{-1}) obtained from standard solutions before and after processing and correction for either degradation products or chlorophylls b and c. The percentage change (bracketed) and the significance of differences between initial estimates and those made after processing are shown.

	Concentration of standard						
	1.0 μg l^{-1} Uncorrected	Corrected for degradation products	Corrected for chlorophylls b & c	**0.5 μg l^{-1}** Uncorrected	Corrected for degradation products	**0.25 μg l^{-1}** Uncorrected	Corrected for degradation products
Estimate in 100 % Methanol							
Before extraction	1.0 ± 0.01	1.38± 0.05	0.75 ± 0.04	0.51 ± 0.01	0.71 ± 0.03	0.24 ± 0.01	0.29 ± 0.02
Hot extraction	0.87± 0.02 $P < 0.05$ (-13%)	0.97 ± 0.02 $P < 0.05$ (-30%)	0.75 ± 0.03 n.s. no change	0.42 ± 0.01 $P < 0.05$ (-18%)	0.51 ± 0.07 n.s. (-28%)	0.23 ± 0.02 n.s. (-4%)	0.25 ± 0.01 n.s. (-14%)
Cold extraction	0.89 ± 0.01 $P < 0.05$ (-11%)	1.15 ± 0.04 n.s. (-17%)	0.77 ± 0.02 n.s. (+3%)	0.44 ± 0.01 $P < 0.05$ (-14%)	0.52 ± 0.04 n.s. (-27%)	0.23 ± 0.01 n.s. (-4%)	0.38 ± 0.03 n.s. (+24%)
Estimate in 95% Methanol							
Initial	0.95 ± 0.02	1.05 ± 0.04	0.65 ± 0.02	0.48 ± 0.01	0.57 ± 0.02	0.25 ± 0.01	0.29 ± 0.02
Hot extraction	1.03 ± 0.01 n.s. (+8%)	1.08 ± 0.04 n.s. (+3%)	0.89 ± 0.02 $P < 0.01$ (+27%)	0.5 ± 0.03 n.s. (+4%)	0.57 ± 0.04 n.s. no change	0.3 ± 0.02 $P < 0.05$ (+17%)	0.41 ± 0.03 n.s. (+30%)

Correction for degradation products increased chlorophyll a estimates. Some of these differences were significant (Figure 10). However, the standard solution used did not contain degradation products. Some may have been formed during processing (Equation 1), but if so these would have reduced the chlorophyll a estimates. The elevated estimates could have been caused by inadequate neutralisation of the acidified extract (Equation 2). This is possible as some researchers consider the pH range (2.6 - 2.8)

recommended by Moed and Hallegraeff (1978) to be too low, resulting in ionisation of the phaeophytin molecule or inadequate neutralisation of the solution. A range of pH 5 - 6 may be more appropriate (A. Marker pers. comm.). Correcting for chlorophylls b and c reduced chlorophyll a estimates of the standard solutions in similar proportions to those observed with extracts from field samples. These differences were significant in all cases ($P < 0.01$, Figure 10).

3.5.6.5 *Results: Rapid Determination*

Qualitative estimates of the proportion of degradation products present were similar for field samples and standard solutions (Table 7), the proportion of degradation products being less than 50% in all cases. This value may be correct, but the usefulness of the test could not be established without an accurate quantitative method for comparison.

Table 7) Qualitative estimates of the degree of chlorophyll degradation for field samples and standard chlorophyll solutions in a variety of solvents. Ratio of Å430 / Å410 > 1, degradation probably < 50%; Ratio of Å430 / Å410 < 1 , degradation probably > 50%.

Solvent	Ratio Å430/Å410 mean ± SE	No. of samples with degradation < 50%	No. of samples with degradation > 50%
Field samples			
100% Methanol cold	1.39 ± 0.09	15	0
100% Methanol hot	1.30 ± 0.01	15	0
95% Methanol hot	1.24 ± 0.01	15	0
90% Ethanol hot	1.26 ± 0.04	15	0
90% Acetone cold	1.19 ± 0.01	15	0
Standard samples			
100% Methanol initially	1.09 ± 0.02	3	0
100% Methanol cold 24h.	1.25 ± 0.01	3	0
100% Methanol hot	1.13 ± 0.06	3	0
95% Methanol initially	1.08 ± 0.01	3	0
95% Methanol hot	1.12 ± 0.01	3	0

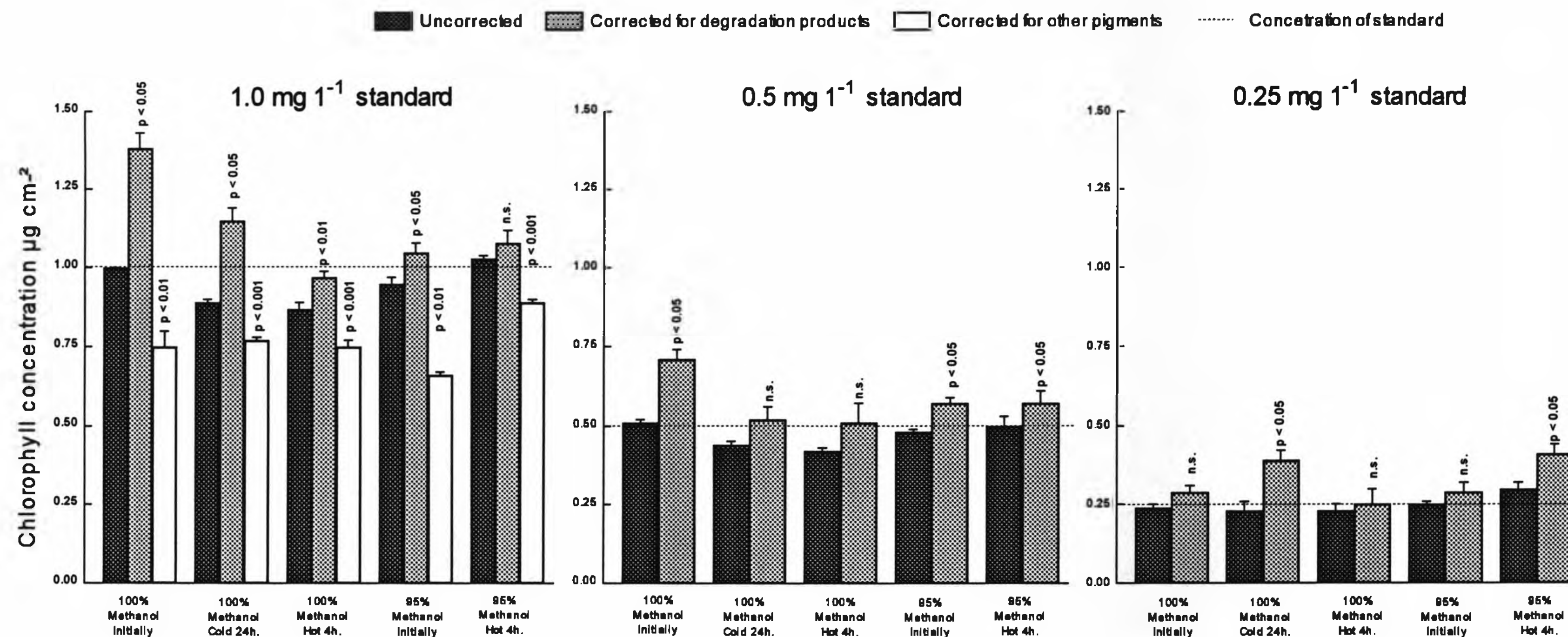


Figure 10) Estimates of chlorophyll concentration obtained from 1.0, 0.5 and 0.25 mg l^{-1} standard solutions of chlorophyll in 100% and 95% methanol (n = 3 in each case). Estimates were made after processing using five different methods (uncorrected). The effect of correction for degradation products and presence of other pigments (both of which were absent from the standards) is shown and the significance of these differences from uncorrected values is given (bars = 1 SE).

3.5.7 Measurement of Sample Surface Area

3.5.7.1 *Methods*

An initial experiment was conducted to determine whether there was a correlation between the micro-topography of a rock surface and the abundance of microalgae per mm^2 of two dimensional (2d.) surface area. Thirteen rock chips were selected from a batch of samples which had recently been extracted in methanol. These were chosen to represent a range of differing rugosities. The chips were examined by four people and ranked subjectively from smoothest (rank 1) to roughest (rank 13). Chlorophyll estimates for the same samples (based on areas measured in two dimensions using a video camera and image analysis system) were also ranked from the sample with the least chlorophyll to the sample with the most chlorophyll.

Three alternative methods for measuring surface area were compared. The simplest of these was to draw around samples on to graph paper and then count the squares. This measured area in 2d. and required minimal equipment. Area was also measured in 2d. using a video camera and image analysis software. The third method attempted to quantify surface area in three dimensions (3d.) using aluminium foil pressed into the surface relief of a sample. After flattening, the total area of the foil was measured using the video camera and image analysis software. In each case care was taken not to include any areas which had splintered and lost the original top surface together with associated microalgae.

The reproducibility of each technique was determined by comparing the coefficient of variation between observers. Statistical comparison between estimates were not undertaken since the 'actual' area of samples was unknown. The objective was simply to determine a rapid, easily reproducible, method for quantifying surface area. Correlation between additional surface area measured using the tinfoil method and rank surface rugosity (visual estimate) were tested (one tailed Spearman's Rank Correlation).

Two additional rocks, one smooth and one rugose, were also measured using the 2d. and 3d. (foil) image analysis methods. The topography of a 1mm x 5mm area on the surface of each of these samples was then examined in detail using a Rodenstock non-contact profilometer (courtesy of Shell Research). This apparatus scans surfaces at a selected resolution and calculates the 'stretched' or total surface area. The resolution chosen (*c.* 10µm) appropriated to the scale of a single diatom, or cyanobacterial filament.

3.5.7.2 *Results*

There was a positive correlation between rank rugosity and rank chlorophyll concentration per mm^2 (P <0.05; one tailed Spearman's Rank Correlation), indicating that small scale variations in surface relief increased microalgal colonisation.

Two dimensional image analyses of rock surfaces was the fastest technique for estimating surface area. This method gave the most conservative estimate of area with the lowest coefficient of variation between researchers. Drawing around samples increased area estimates by an average of eight percent and had the greatest coefficient of variation. Measuring in 3d. using tinfoil increased area estimates by 13%, but took twice as long as the 2d. image analysis technique (Table 8).

The ranked increases in surface area obtained using the tinfoil method correlated with ranked rugosity estimates (P<0.01) indicating that the method was measuring some of the additional area contributed by surface relief. However, with a very rugose sample this method was only able to detect around one third of the increase in area measured using the profilometer (Table 9).

Table 8) Estimates of rock chip surface area made in two dimensions (2d.) by drawing around samples or using image analysis software, and in three dimensions (3d.) using foil and image analysis. Individual estimates from researchers are shown, together with a mean estimate and the coefficient of variation.

Method	Time per sample (min.)	n.	Mean area estimate (mm) obtained by each researcher (1-4)				Overall mean (mm ± SE)	Coefficient of variation
			1	2	3	4		
Drawing 2d.	3	13	475	557	524	575	533 ± 22	0.08
Image analysis 2d.	2	13	487	503	482	475	487± 6	0.02
Foil / image analyses 3d.	4	13	545	582	511	557	549 ± 15	0.05

Table 9) Comparison of estimates of rock surface area made using one two dimensional (2d.) method and two three dimensional (3d.) methods.

Sample	Area (mm) image analysis 2d.	Area (mm) foil / image analysis 3d.	% inc. using foil / image analysis 3d.	% inc. using profilometer 3d.
A	307	327	6	7
B	733	888	18	57

3.6 DISCUSSION

3.6.1 Pre-treatment: State of Sample Hydration

Hydration of epilithic microalgae prior to processing was crucial for complete chlorophyll extraction. In some instances dry algae released less than 34% of the chlorophyll released by hydrated algae. Samples that were initially processed dry then rehydrated and processed again, released considerable additional chlorophyll. The difference between extractions from hydrated and dry material seems to have resulted from an inefficiency of solvents to extract chlorophyll from dry microalgae.

The importance of hydration for extractions from epilithic and planktonic algae has been recognised for some time (Castenholz, 1963; Nusch, 1980). Hydration has not been problematic in phytoplankton studies as the algae were normally filtered from seawater and the filter papers obtained immersed in alcohol whilst fully hydrated (Sand-Jensen, 1976; Holm-Hansen and Riemann, 1978; H.M.S.O., 1983). However, reduced extraction has been reported for filter papers which had previously been dried and frozen (Sand-Jensen, 1976), but not from those frozen hydrated. The reduced extraction from dried papers may also have been caused by dehydration of the algae.

The state of hydration may have afffected results obtained from intertidal epilithic algae. This is most likely for samples collected during warm weather and may have exaggerated the seasonal trends observed by Underwood (1984c), Hill and Hawkins (1991), Dye and White (1991) and Bustamante (1994), who all recorded reduced chlorophyll concentrations during the summer. State of sample hydration should be standardised in future work.

3.6.2 Sample storage

Stored samples consistently released less chlorophyll than fresh samples. This effect was considerable with frozen storage where estimates were reduced by up to 25%. However, there was substantial between sample variation and the experiment needs to be repeated with larger sample sizes in order to confirm the significance of these effects. Other researchers have obtained conflicting results after storing samples frozen (see Marker *et al.*, 1980; Nusch, 1980). Processing fresh material is recommend wherever possible, but if necessary, samples can be stored in the dark at 4°C for a maximum of one week.

3.6.3 Choice of Extraction Solvent and Protocol

Efficient extraction, safety, cost and amenability to correction are all important considerations in the choice of an extraction solvent (Table 1). The relative merits of the solvents examined in this study are outlined in Table 10.

Table 10) Considerations when choosing solvents for extraction of chlorophyll from intertidal epilithic microalgae. Safety values are occupational exposure standards (OES) for long-term exposure (8 hour) set by the United Kingdom Health and Safety Executive (HMSO, 1993). Costs are for purchase of small volumes (~ 2.5l) and are in pounds (sterling) excluding taxes and duty.

Solvent	Safety (OES ppm)	Cost £/litre (1996 prices)	Completeness of extraction from intertidal microalgae %	Amenable to Chlorophyll a determination	Amenable to degradation products determination	Amenable to Chlorophyll b & c determination
Acetone	750	4.2	42	Yes	Yes	Yes
Ethanol	1000	20.0	88	Yes	problematic	problematic
Chloroform: Methanol	2	5.6	not tested	(constants not known)	(constants not known)	(constants not known)
Methanol	200	3.6	97	Yes	problematic	problematic

Methanol has been recognised as an excellent extraction solvent for some years (Marker, 1972; Holm-Hansen and Riemann, 1978). This study confirms its efficiency, with all three methanol methods extracting over 96% of the available chlorophyll. Methanol is least expensive, but is more hazardous than either acetone or ethanol. Acetone and chloroform : methanol both have considerable. For acetone, established procedures are available to correct chlorophyll a estimates for the presence of other pigments (H.M.S.O., 1983), but this solvent has very poor extraction efficiency. Chloroform : methanol is very toxic and there is uncertainty about chlorophyll determinations using solvent mixtures (Marker *et al.*, 1980; Nusch, 1980).

Ethanol was efficient, extracting 86% of the available chlorophyll, but was the most costly solvent. For extractions from plankton ethanol has recently been recommended in preference to methanol and acetone (Marker, 1994). This choice was made in the interests of minimising health risks and standardising with methods used elsewhere in Europe. However, the efficiency of ethanol for extracting chlorophyll from cyanobacteria is poor, and so for samples containing substantial proportions of cyanobacteria, methanol is still advocated (Marker, 1994). Cyanobacteria were abundant at the site used in this investigation (Chapter 5) and are important constituents of both marine and freshwater epilithic biofilms world-wide (Fogg *et al.*, 1973). Therefore, provided that appropriate safety requirements can be maintained, methanol is recommend as the standard solvent for chlorophyll extractions from epilithic microalgae.

3.6.4 Extraction Efficiency and Stability of Chlorophyll in Hot and Cold Methanol

Hot extractions did not result in chlorophyll a degradation as has been previously suggested (Nusch, 1980, Equation 1). However, spuriously elevated chlorophyll estimates were obtained when solutions were boiled for more than a few minutes. This problem was less critical for 95% than for 100% methanol, but could only be avoided by monitoring solutions closely and removing them from the water bath shortly after they began to boil. Cold extractions took a couple of hours longer to complete than hot ones, but were just as efficient and did not give elevated readings. Once extraction was complete, chlorophyll solutions obtained by heating remained stable for a few hours whilst those from cold methods remained stable for 19 hours in 100% methanol and 48 hours in 95% methanol.

Cold extractions are recommended, since these require less monitoring, are safer (less toxic vapour) and have a much longer operating window during which absorbance readings can be made. For example, with cold methanol it

was possible to leave large batches of samples to extract overnight and then measure absorbance the following day.

Marker (1994) claimed 100% methanol was superior to methanol with a small water content, whilst Moed and Hallegraeff (1978) and Nusch (1980) suggest that a small proportion of water will facilitate extraction and reduce variation caused by water introduced with the sample. This point will be especially relevant to extractions from hydrated material which are recommended here. Extractions were equally efficient in either 100% or 95% methanol. Chlorophyll was more stable, both during heating, and after extraction, in 95% methanol.

This study and that of Moed and Hallegraeff (1978) also showed that pH was easier to control during correction for degradation products with 95% methanol than with 100% methanol. Therefore, 95% methanol is recommended in preference to pure solvent. However, comparisons between estimates of chlorophyll concentrations made here suggest chlorophyll a estimates may be slightly understated (approximately 5%) in 95% methanol. Possibly this occurred because the specific absorption coefficient for 100% methanol was used in place of a coefficient for 95% methanol (which was not available).

3.6.5 Centrifuging

Centrifuging samples (H.M.S.O., 1983) was time consuming and was not advantageous. Interference from suspended particles was minimised by rinsing samples in filtered sea water to remove loose debris prior to extraction and by leaving samples to settle after extraction, then removing the mid portion of the extractant for spectrophotometric determination. If the volume of extractant is large compared to the portion used for the determination, checks should be made to ensure that chlorophyll has not become stratified in the container (Chen and Hara, 1994). Provided these precautions were followed interference from suspended material was well

within the recommended tolerances of the spectrophotometer and centrifuging was unnecessary. However, the chlorophyll measured here was extracted directly from microflora on the surface of limestone and different results might be obtained with more friable substrata.

3.6.6 Correction for Degradation Products and for Chlorophylls b and c

Corrections to chlorophyll a estimates for either the presence of degradation products or chlorophylls b and c were inconsistent. For epilithic algae from the shore, adjustments for degradation products reduced estimates of chlorophyll a in some solvents but increased them in others. Correction for chlorophylls b and c reduced estimates suggesting that these pigments were present. However, this determination is not possible on theoretical grounds if degradation products are also present (> 5%, see Marker, 1994) and therefore must be regarded with caution.

Inconsistencies with corrections for degradation products, obtained with field samples, were reinforced by determinations made from standard solutions. Standards were made up from undegraded chlorophyll a they did not contain chlorophyll b and were believed to contain negligible chlorophyll c (Sigma pers. comm.), yet chlorophyll estimates increased in all samples after correction for degradation products. The result could not have been caused by the formation of degradation products during processing, as this would have reduced the chlorophyll a values. Corrections for chlorophylls b and c reduced estimates of chlorophyll a in a similar manner to the results from the field samples.

These results suggest inadequacies in either the protocol, spectrophotometer precision or the formulae for calculating the proportions of these pigments. Determinations for chlorophylls b and c do require high quality instrumentation and are susceptible to error (Jeffrey and Humphrey, 1975). However, the equipment used here is also regularly used to estimate

degradation products in chlorophyll extracted from plankton samples with acetone. Also, shortly after this work was completed, the spectrophotometer was checked by a service engineer and was reading correctly.

I suspect that discrepancies may have arisen because of complications in the degradation product determination using methanol although this procedure has been advocated for some years (H.M.S.O., 1983). Variations may also have arisen with pigment extracted from cyanobacteria which were present on samples from the shore and were the origin of the chlorophyll a used in the standard. Unless these difficulties can be resolved, high pressure liquid chromatography (HPLC) is suggested as an alternative method of calculating degradation products and chlorophylls b and c (see Mantoura and Llewellyn, 1983; Wright and Shearer, 1984; Zapata *et al.*, 1987).

3.6.7 Measuring Sample Surface Area

Quantifying surface area remains a problem. Using image analysis software to measure 2d. video images of samples was faster and less variable than drawing around samples by hand. The 3d. method using tinfoil was able to quantify some surface relief but took longer and was subject to greater variability than 2d. methods. Non-contact profilometry provided a more accurate measure of total surface area and increased 2d. measurements by over 50%. Unfortunately, the method was time consuming (1hr. per sample), and the equipment is also costly (£90,000). One possibility might be to have range of rock samples measured by profilometry and then use these as a reference for assessing others by eye. However, in such an assessment it is important that surface relief is considered at the scale experienced by a cyanobacterium or diatom (Thompson *et al.*, 1996). Even if total surface area could be measured, microalgal colonisation is unlikely to co-vary in a linear manner, and it may be more appropriate to adjust area measurements made in two dimensions in order to allow for rugosity. More work is required to resolve this. Meanwhile, this factor should be standardised by collecting

samples of similar rugosity. Alternatively, homogenous artificial substrata such as glass and slate could be used (Grzenda, 1960; Bustamante *et al.*, 1995) but, these surfaces may not accurately mimic the natural environment.

3.7 CONCLUSIONS

Natural spatial variation of intertidal microalgae is considerable and care must be taken to ensure sampling effort is adequate. A simplified standard method for estimating microalgal abundance using extracted chlorophyll a as an index is proposed (Appendix 3), but correction for degradation products, or chlorophylls b and c by spectrophotometry is not advocated. The revised technique requires around 25% less operator participation than methods which include hot extraction and centrifugation. This method has been extensively used for extractions from intertidal epilithic algae (Chapters 5 and 6), but could also be used for rock samples collected from subtidal or freshwater habitats.

CHAPTER FOUR

Assessment of Gastropod Grazing Intensity by Means of Radula Scrapes on Wax Surfaces

4.1 ABSTRACT

The feeding apparatus of many marine molluscan herbivores leaves distinctive marks on the surface of dental wax. Using this wax a method was developed to assess spatial and temporal patterns of gastropod grazing on rocky shores. Among the common gastropod grazers of intertidal habitats on the Isle of Man, distinctive rasping marks were made by limpets, top shells and larger littorinids. The technique for the field deployment of the wax surfaces is simple, and permits a realistic placement on the shore. This placement is achieved by casting the wax into small discs and setting them into pre-formed holes in the rock surface. By quantifying either the number of discs scraped or the area of the wax surface scraped, patterns of grazing intensity (defined as areal extent of the surface grazed in a given period) can be assessed over a variety of spatial and temporal scales. To illustrate this method and refine its use, grazing patterns of the limpet *Patella vulgata* (L.) were recorded for periods from 12 hr to 17 days. The optimal period of deployment depended on the specific habitat, but periods of between 1 to 14 days were appropriate on Manx shores. Regular arrays of discs also demonstrated that grazing intensity was spatially variable at a scale of 0.25 m, and that, grazing intensity increased throughout the late winter and spring. This method provides a cheap and direct measure of feeding intensity that is directly relevant to understanding the effect of grazing molluscs on algal communities.

4.2 INTRODUCTION

Many herbivorous molluscs feed by scraping the substratum with a specialised feeding appendage called a radula (see Steneck and Watling, 1982; Hawkins *et al.*, 1989; Norton *et al.*, 1990; Fretter and Graham, 1994, for reviews). Feeding activity by such grazers can have a considerable influence on the structure of intertidal communities by the removal of both microalgae and the propagules of macroalgae which have settled within the microalgal film (see reviews by Southward, 1964; Lubchenco and Gaines, 1981; Hawkins and Hartnoll, 1983; Jernakoff, 1985; and various papers in John *et al.*, 1992). In this manner, these grazers are capable of controlling the distribution, abundance, and biomass of both microalgae and macroalgae. These effects have been well demonstrated experimentally, by removing (e.g. Jones, 1948; Southward, 1964) or excluding the grazers (e.g. Hawkins, 1981; Johnson, 1992; Williams, 1993). Thus, although the general importance of these molluscs is well known, their role in determining the often patchy or ephemeral distribution of algae has been little explored. In particular, a more precise understanding of spatial and temporal patterns of grazing activity is needed (see comments by Chapman and Underwood, 1992).

Limpets are arguably some of the most important and well-studied herbivores found in rocky intertidal communities (Southward, 1964; Underwood, 1979; Underwood, 1980; Branch, 1981). Various methods have been used to monitor their foraging activity (see Hartnoll, 1986 for review). For homing limpets, a system of reed switches positioned adjacent to the home scar and magnets on the shell give a 'home' or 'away' signal to a data recorder (actography, e.g. Chelazzi *et al.*, 1990; Della Santina *et al.*, 1994). While this technique permits estimates of temporal activity, it provides little spatial information. Short term records of spatial distribution during foraging have been made by intermittent observations of groups of up to 50 limpets at low tide (Hawkins and Hartnoll, 1982; Little and Stirling, 1985; Little *et al.*,

1990) and at high tide by SCUBA diving (Hartnoll and Wright, 1977; Hawkins and Hartnoll, 1982), but these time-intensive approaches are difficult to sustain for long periods or at multiple sites. Other researchers have used light emitting diodes, mounted on the limpets, and photography (photomotography) to record the foraging routes of individual limpets over several weeks (Chelazzi *et al.*, 1990; Della Santina *et al.*, 1995), but unfortunately only with a few individuals (< 20) over small areas (< 3.5m^2). Alternatively, sonography, (e.g. Boyden and Zeldis, 1979; Kitting, 1979; Chelazzi *et al.*, 1994b) can directly assess grazing by recording the noise of the radula scraping against the rock. However, this approach only allows a few limpets to be monitored at one time and gives little information on the spatial distribution of foraging effort (but see Chelazzi *et al.*, 1994b for examples of studies using a combination of sonography and photomotography).

Thus, in general, the above techniques are either costly in terms of time or money, feasible for only small groups of limpets, or only provide limited information. While they might be appropriate for short term, spatially constrained behavioural studies, they are generally inadequate for ecological questions directed at the long term and broad scale effects of populations of grazers on algal assemblages. Consequently, researchers are often forced to use the abundance of grazers as a surrogate for overall grazing intensity (e.g. Castenholz, 1963; Southward and Southward, 1978; Underwood, 1984c). A simple, direct method for assessing grazing in the field would thus be of considerable advantage in both descriptive and experimental studies.

An ideal system for measuring grazing intensity would involve an inert substance that would record the scraping marks made by molluscs as they feed. Beeswax has been used in laboratory studies to examine the functional morphology of the radulae of various gastropods (Hickman and Morris, 1985). However, this material was unpalatable to the molluscs unless cured

in aerated seawater. Recently attempts have been made to record temporal patterns of grazing by limpets, using discs cut from dental wax and mounted on the shore (Johnson, 1989). Surfaces of dental wax are easily marked by scratching or scraping, yet remain unaffected by exposure to field conditions.

In this Chapter an inexpensive and versatile method of using dental wax to assess grazer intensity in the field is described. Examples are given that demonstrate its use and effectiveness in determining spatial and temporal pattern of grazing intensity. The frequency of scraped surfaces and the areal extent of the wax surface scraped is used to give an index of "grazing intensity" in a given period.

4.3 METHODS

4.3.1 Disc Preparation and Deployment

Discs were prepared by filling plastic rings with dental wax (Metrodent No. 1 - £6/kg; Metrodent Limited, Huddersfield, UK). The plastic rings were the base part of plastic screw covers (Plasplugs, Burton-on-Trent, Staffordshire, UK) which were an appropriate size (14 mm diameter) and had a small hole in the bottom that aided filling. The bases were placed upside down on either a glass plate which had previously been wiped with a trace of glycerol to prevent the wax from sticking or a glass-smooth sheet of silicon moulding rubber (Silastic RTV J, Dow Chemicals Corp., Midland, Michigan, USA) which could be peeled away from the discs after filling. Discs were then filled by introducing melted wax through the hole in the base with a warm Pasteur pipette (Figure 1a). For best results, the melted wax should be maintained at 95°C in a water bath; higher temperatures approach the flash point of the wax and can irreversibly change the colour and possibly the characteristics of the wax. After the wax had solidified (approximately 10 min), the discs were individually numbered and excess wax trimmed from the base and sides. The discs were then mounted on strips of self adhesive tape for storage or transport. After some practice, I was able to prepare discs quite

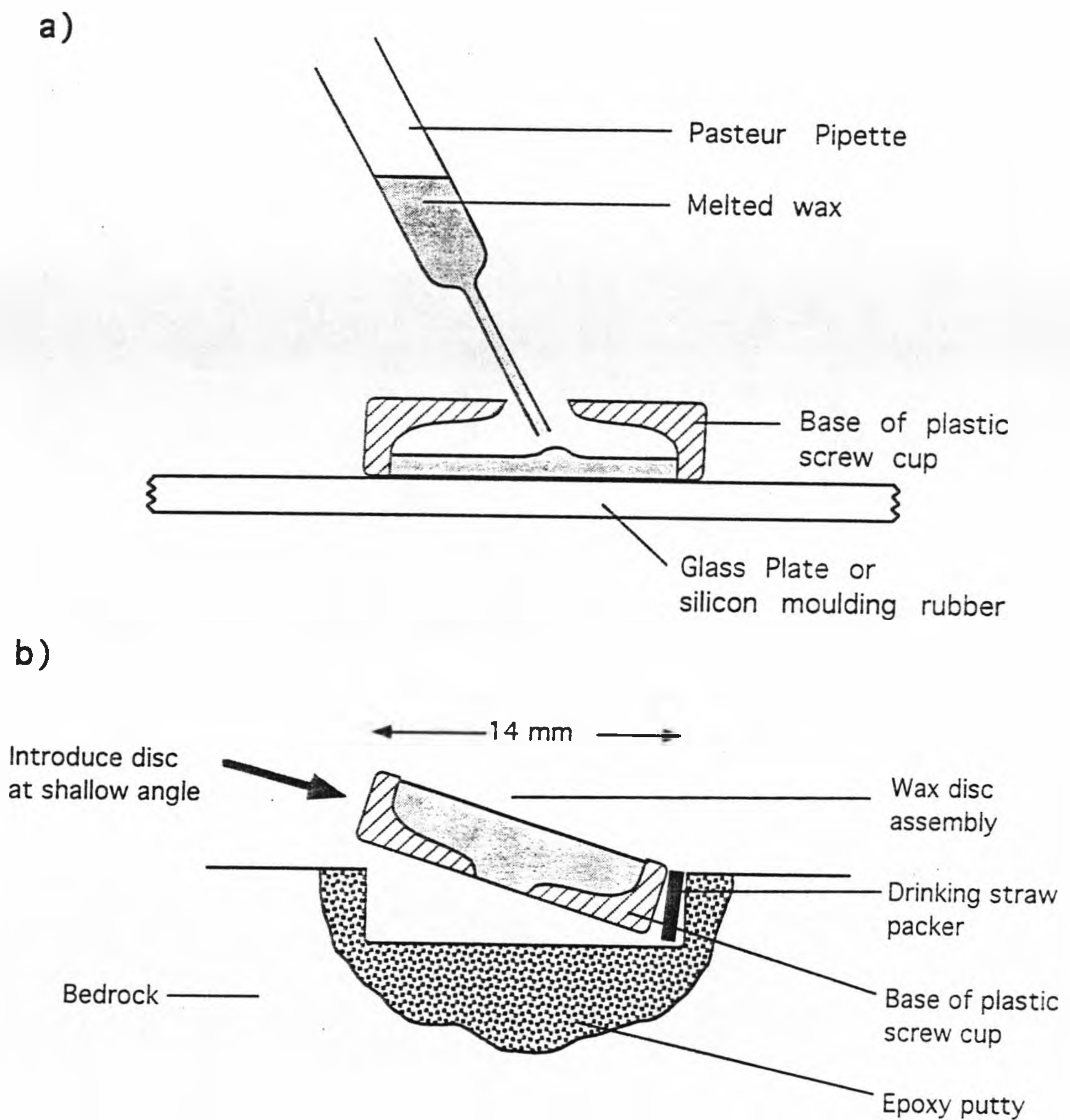


Figure 1) Cross section of plastic screw cover showing a) technique for filling with wax, b) cross section of disc assembly, and matching recess in the bedrock on the shore.

rapidly (approximately 200 per hour). Approximately 1000 bases could be filled per kilogram of the more inexpensive wax, making the total cost around £0.05 per disc. Each disc had a total surface area of 150 mm^2 of which approximately 70% was wax.

Using a technique similar to that for out-planting algal germlings (Brawley and Johnson, 1993), the discs were set into pre-formed holes in the intertidal bedrock. The holes were prepared by drilling a shallow hole (< 1 cm deep) slightly larger than the diameter of the disc. This initial hole was then filled with epoxy putty (Milliput® - The Milliput Company, Dolgellau, Mid Wales, U.K., or Sea Goin' Poxy Putty® - Permalite Plastics Corp., Newport Beach, California, USA). The final hole was created by either pushing in the end of a metal rod which had been machined to have the same diameter as the wax-filled discs or by embedding small discs made of a material (corn starch and Fix-All - Custom Building Products, Seal Beach, California, USA) that dissolved during the subsequent high tide leaving a hole slightly larger than the wax-filled discs. Wax-filled discs could then be set into the holes so that the surface of the wax was flush to the surrounding rock surface. The diameter of the discs and holes did vary slightly; a small screwdriver was used to lever tighter fitting discs into place, whilst looser fitting discs were held in place by plastics springs created by cutting short (2 mm) sections of a plastic drinking straw (Figure 1b). Unlike a previous method (Johnson, 1989), this design produced a smoother wax surface and permitted the wax surface to be deployed flush with the rock surface so that there was no interference with access of the grazers to the surface, therefore mimicking natural conditions as much as possible. This method of deployment permitted each disc to be retrieved and replaced in approximately one minute.

4.3.2 Recording the Grazing Marks of Various Molluscs

The usefulness of this technique to record the grazing of a variety of intertidal molluscs was examined in the laboratory. Five small aquaria were prepared, each containing a small microalgal covered boulder from the shore into which five wax discs had been fitted. Each tank was then stocked with a different species of intertidal mollusc: *Patella vulgata* (L.) - three individuals, *Littorina obtusata* (L.) - 16 individuals, *Gibbula* spp. (a mixed tank of six *Gibbula cineraria* (L.) and six *Gibbula umbilicalis* (da Costa), *Calliostoma zizyphinum* (L.) - 10 individuals, and *Littorina neglecta* (Bean) - approximately 100 individuals. The seawater in the tanks was changed every few days to minimise the accumulation of metabolic wastes. The discs were removed after two weeks, viewed with a binocular microscope, and the configuration of rasping marks drawn. In some cases methylene blue was gently wiped over the surface of the disc to help resolve finer scratch marks.

4.3.3 Period of Exposure and Scoring of Discs

Field trials of this technique were conducted in the intertidal at Port St. Mary and Derbyhaven on the Isle of Man, (4°W., 54°N.). Both shores were moderately wave exposed and the mid-shore zone (2.5 to 4.0m above L.A.T.) bore a patchy *Fucus-Semibalanus* mosaic where the dominant grazer was the homing limpet *Patella vulgata* (see Southward, 1953 for site description). The experiments were conducted in the spring and summer of 1991 and the summer of 1993 onwards.

In order to yield useful results, discs must be exposed to grazing for an appropriate period of time: long enough to have some of the discs scraped yet not so long that all the discs were scraped. To determine an appropriate period an initial study was conducted at Derbyhaven, several series of discs were set out: one for a 7-d period (13-20 Jul. 91), and another series for each sequential 1-d period during the same week. The two series of discs were set out in a 2 by 12 array of holes (25 cm spacing) with discs from each series in alternating holes, (i.e. a "checkerboard" pattern). The experiment

was conducted simultaneously near the top and bottom of the *Fucus-Semibalanus* zone. The numbers of discs grazed in each series was assessed in the laboratory. This experimental design also permitted a comparison of the additivity of the results of shorter periods of exposure (i.e. does the sum of the daily rates of grazing reflect the cumulative rate of the longer week period?).

Individual wax discs could be scored in two different ways: (1) the presence or absence of any grazing marks on the surface, i.e., scraped or not, or (2) the percentage of the surface area with grazing marks. Here, marks were scored which resembled those of the dominant molluscs on the shore, i.e., the limpets, (see Hawkins *et al.*, 1989) and all other scratches were ignored. Using an array of discs, and averaging either the number of discs scraped or the area each disc scraped, it was possible to obtain an index of foraging intensity. To compare these two methods of scoring, a series of 36 discs were fixed into holes (a 1 by 36 array; 25 cm between adjacent holes) on the mid-shore at Port St Mary. During August 1993 grazing intensity was recorded *in situ* over a 17-d period using a hand lens to help estimate the percentage of each disc which had become scraped. Discs were then removed, and the total area of each disc scraped was estimated in the laboratory using a binocular microscope to aid viewing. Scratch marks were compared with those of limpets and other grazing molluscs (Hickman and Morris, 1985; Hawkins *et al.*, 1989).

4.3.4 Spatial and Temporal Variations in Grazing Intensity

To demonstrate the use of the wax-disc technique to investigate spatial and temporal variation in grazer intensity, discs were set out in three regular 4 by 4 arrays (25 cm spacing) at mid-tide level, Port St Mary. The arrays were positioned in open areas away from rock pools, clumps of seaweed or dense patches of barnacles that might have constrained grazer movement. The density and average size of limpets within these arrays was typical of the

surrounding area (approximately 30 individuals m^{-2}; average shell length, 37 mm, range 10mm - 55mm). In order to reduce possible variations in grazer activity related to the spring-neap tidal cycle (Little *et al.*, 1990; Della Santina *et al.*, 1994) discs were left *in situ* for two weeks (a full spring-neap cycle) before removal. Using a new set of discs on each occasion, grazing was recorded for a two week period each month from February to May 1993. The abundance of limpets within a 25cm radius of each disc site was compared with the percentage area of disc scraped at that site using Spearmans Rank Correlation.

4.4 RESULTS

4.4.1 Recording the Grazing Marks of Various Molluscs

Distinct grazing marks were apparent for all species except *Littorina neglecta,* the smallest gastropod. *Patella vulgata*, the largest grazer, made a series of deep parallel gouge marks approximately 0.5 to 0.8 mm in length (Figure 2). *Gibbula* spp. and *Calliostoma zizyphinum* both gave sets of parallel arc shaped scratches up to 0.3 mm long. *Littorina obtusata* produced slightly longer marks than *Gibbula* spp. or *C. zizyphinum* which were characterised by two parallel grooves with a series of oblique scratches (Figure 3). Faint scratches could just be discerned on discs from the tank with *Littorina neglecta*, the smallest grazer, but these could not be clearly resolved and are not shown.

4.4.2 Period of Exposure and Scoring of Discs

At Derbyhaven, all discs left out for the 7-d period were grazed at the lower shore site, while only 42% were grazed at the upper shore site. These patterns were consistent with the results from the discs replaced daily during this period: at the lower shore site 16 to 50% (mean $\pm$ SE = 29.4 $\pm$ 4.4) of the discs were grazed on each day of the one week period and each of the 'one-day' disc sites was scraped at least once during this period (mean $\pm$ SE =

2.0 ± 0.3). At the higher shore site 0 to 17% (mean ± SE = 6.0 ± 2.4) of the discs were grazed each day and only 33% of the 'one-day' disc sites were scraped during the week period. Thus the cumulative results of the short-term measurements (i.e. daily) were consistent with the long-term measurement with respect to the number of discs scraped.

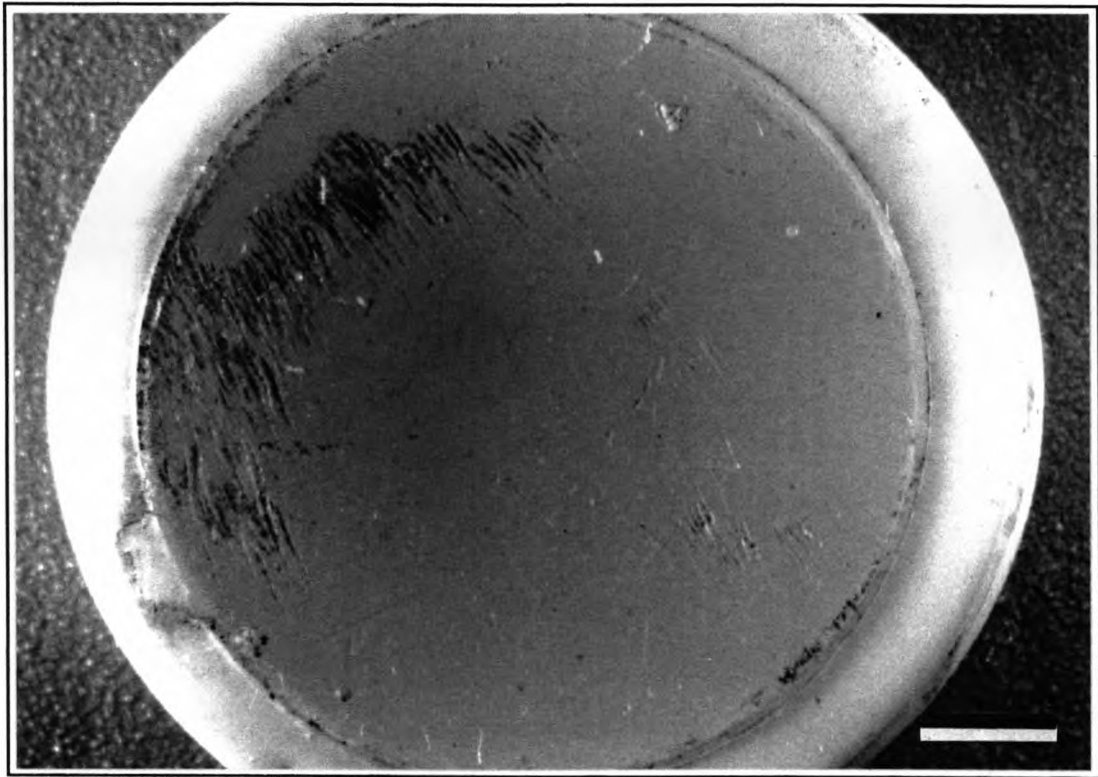


Figure 2) Photograph of wax disc showing grazing marks left by the radulae of *Patella vulgata* during a 14 day exposure on the mid-shore at Port St Mary, Isle of Man. bar = 2mm.

In the study at Port St Mary seven of the 36 discs had been scraped within the first tidal cycle (a night-time high tide), but during the following few days, only 3 more discs were scraped (Figure 4). By the end of the 17-day period, 85% of the disc sites had been grazed. Where clear radulae marks could be discerned they corresponded to those of *Patella vulgata*. The area of the discs scraped followed a similar trend to the number of discs scraped although by the end of the experiment only 23% of the total available surface had been grazed. There was considerable 'between disc' variation in the area scraped; some discs had only a single set of marks whereas others

were extensively scraped or gouged (e.g. 95% of the area). Scratch marks sometimes crossed the surface in several different directions, suggesting that the discs had been grazed on several occasions.

Estimating the area of discs scratched was difficult in the field, especially when the surface of the disc was wet. Scoring dry discs under a binocular microscope with side illumination was much easier.

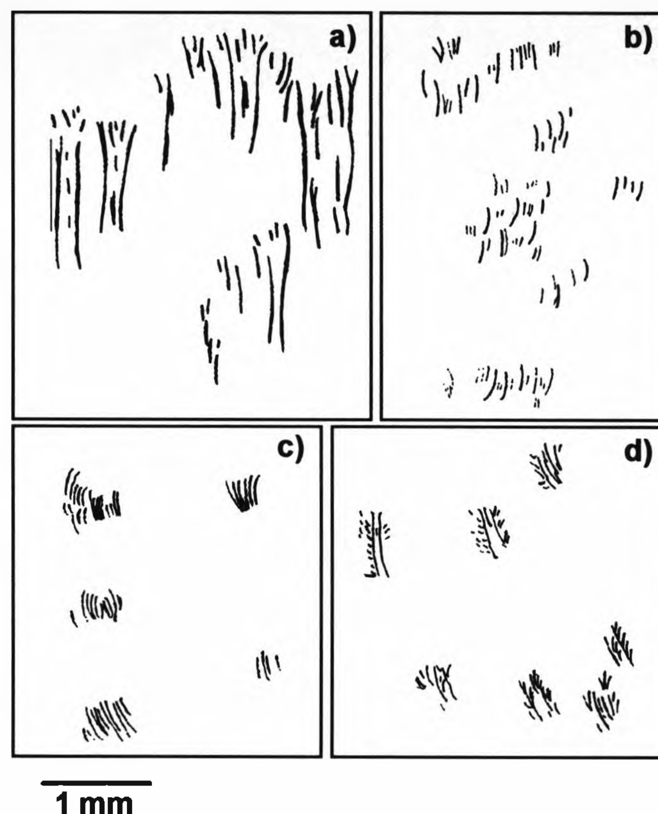


Figure 3) Scratch marks made on wax discs by a) *Patella vulgata* (Linnaeus), b) *Calliostoma zizyphinum* (Linnaeus), c) *Gibbula* Spp. and d) *Littorina obtusata* (L.).

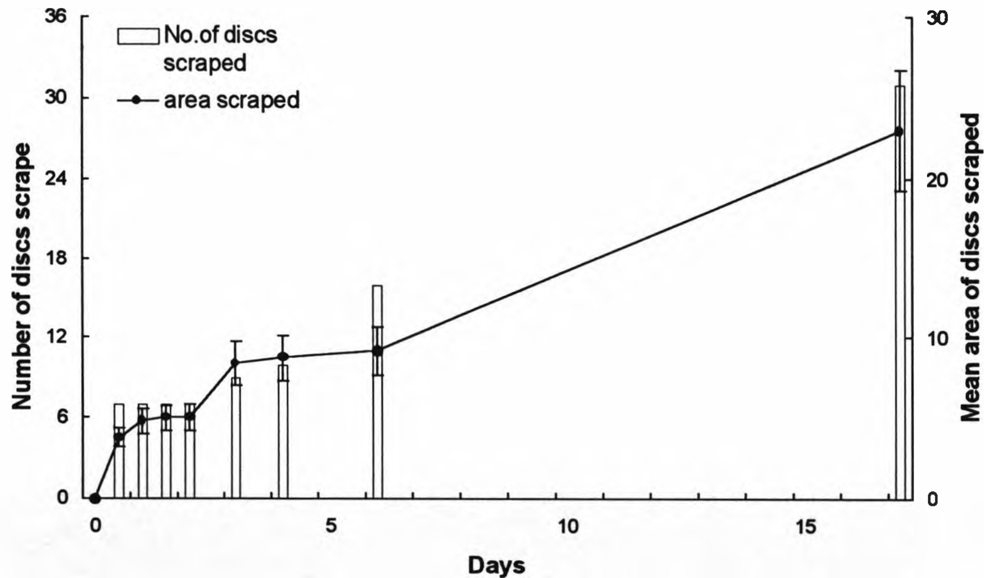


Figure 4) Cumulative number and area (mean ± SE, n = 36) of wax discs (n = 36) scraped by limpets during a 17 day period at mid-tide level, Port St Mary, Isle of Man.

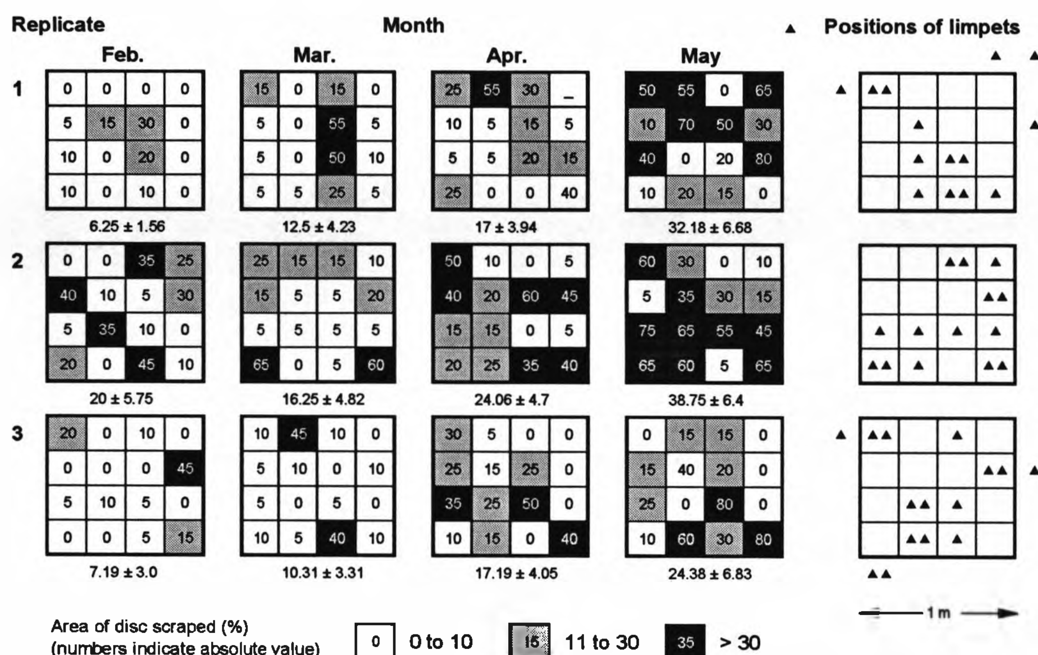
4.4.3 Spatial and Temporal Variations in Grazing Intensity

Within each array grazing was highly spatially and temporally variable between disc sites (Figure 5a). Each month some discs were heavily grazed whilst others remained untouched. At any particular disc site the grazing also varied from month to month. The abundance and orientation of limpets within the arrays remained virtually constant throughout and there was no correlation between abundance of limpets within a 25cm radius around each disc site and the percentage area of disc scraped at the site ($r_{s\ 0.05\ (1)\ ,\ 47}$ = 0.243; r_s Feb. = 0.083, r_s Mar. = 0.011, r_s Apr. = -0.146, r_s May = -0.029).

Overall, grazing intensity increased steadily in each of the arrays between February and May. This trend is shown most clearly by averaging the percentage area scraped across each of the three replicate arrays and comparing between months (Figure 5b). However, averaging the total number of discs scraped in each of the three grids did not show any seasonal trends in intensity since most of the discs were scraped during the two-week exposure period (Figure 5b). Since the same arrays of holes were

used each month, the data were not independent with time, and thus statistical comparisons between months were not made (This statistical problem could be overcome by having many more holes than discs and randomly using a subset on each sampling occasion).

a) Map of grazer activity in each of three replicate plots



b) Plot of combined data from replicates 1,2 and 3

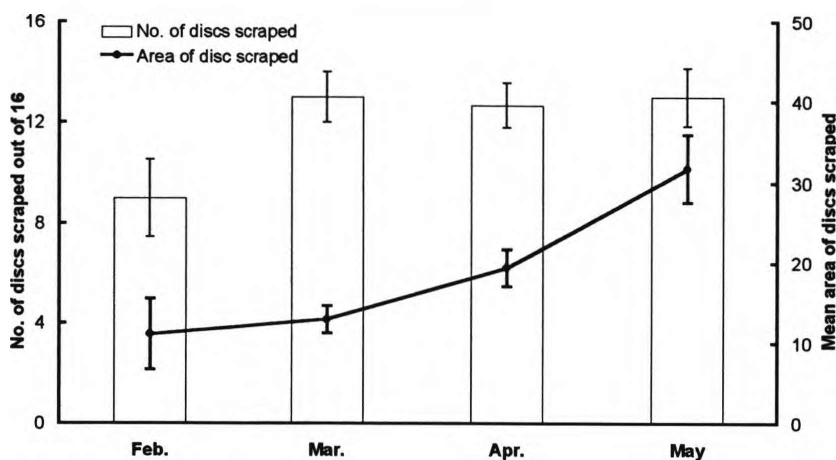


Figure 5a) Spatial pattern of grazing intensity: percentage areas of wax discs scraped (one disc per. 25 cm² cell) by limpets, at Port St Mary, Isle of Man. Discs in each of 3 replicate arrays were positioned at mid tide level for fortnightly periods each month during spring 1995. Mean ± SE (n = 16) is shown below each array. b) Temporal trends of combined data from all three replicate plots (mean ± SE).

4.5 DISCUSSION

4.5.1 Method Development

The wax discs provided an inexpensive, but versatile, new method of recording the grazing intensity of populations of limpets in the field. Preliminary work preparing the disc sites and making the discs was relatively simple compared to that required with methods such as sonography or actography. Placement, recovery and scoring of the wax discs were also simple and rapid. Recessing discs such that their surface was level with the surrounding substratum provided a natural presentation of the wax to the grazers and avoided problems associated with topographical irregularities (Johnson, 1989). Indeed, on one occasion, a limpet was observed to be resting, during low tide, directly over a wax disc suggesting that the presence of the discs did not deter it from establishing a temporary home. The recesses also provided a secure method of fixing discs to the shore, and overall, less than 5% of the discs were dislodged.

Laboratory observations clearly demonstrated that common intertidal grazers leave distinctive marks on these wax surfaces. Of the species examined, limpets left the most easily distinguishable gouges. This is not surprising as they are the largest molluscan grazer on the shore and have exceptionally hard docoglossan radulae. Moreover, the discs were also able to record the feeding activity of top shells (rhipidoglossan grazers) and littorinids (taenioglossan grazers) which have much finer radulae (Hawkins *et al.*, 1989; Fretter and Graham, 1994). The discs could therefore be used to provide estimates of total grazing on shores where several species of grazer are abundant. Alternatively, the relative contribution of different grazing species might also be estimated if discs were replaced frequently enough to avoid initial grazing marks from being obscured by subsequent grazing activity.

The best method for scoring discs depended on the period of exposure. Scoring discs for the absence or presence of grazing marks was the simplest

method and was less prone to variation in measurement, especially between investigators. However, it does not take into account any variation in the intensity of grazing at a disc sites. Thus it cannot integrate the effect of multiple encounters with grazers within the exposure period and therefore may underestimate the grazing activity. It also does not account for any variation in grazing during any given encounter with a grazer, i.e. a disk with 100% of its wax surface grazed is scored the same as one with a single grazing mark. At the same time, this method makes no assumptions regarding the response of grazers after the first “bite” (see below).

A final limitation is that the number of discs "missed" by grazers declines with increasing deployment time, and at some point this method loses its ability to distinguish sites or times with different levels of grazing activities (Figure 5). For these reasons, this scoring method must be used for relatively shorter periods of exposure or in areas of either low grazer numbers or short foraging periods. For example, a 7-d period was appropriate for the high shore site at Derbyhaven but not for the lower site where limpets were more abundant and had longer periods to feed.

For longer periods of exposure, estimating the area scraped on each disc provided a better measure of grazing intensity because radula scrapes could accumulate on the wax surface. However, this method may underestimate grazing intensity if portions of the surface are grazed more than once. For the limpet populations studied here, a 14 day exposure period and scoring the percentage area of the discs scraped, is advocated. Standardising the exposure period in this way avoids variations in foraging activity associated with the spring-neap tidal cycle (Little *et al.*, 1990; Della Santina *et al.*, 1994) and any problems of non-additivity between exposure periods.

The presence of only one or several scrapes on some discs suggests that limpets sometimes move quite rapidly across the substratum perhaps as they search for dense patches of microalgae. In contrast, other discs were

heavily scraped and at times deeply excavated even after only 1-d exposures. Direct observations of groups of limpets have also shown that grazing intensity varies during foraging excursions (Hartnoll and Wright, 1977; Chelazzi *et al.*, 1994a). These excursions were usually characterised by relatively rapid movement to and from the home scar, separated by periods of more intensive and localised foraging. However, comparisons between months did not reveal consistent spatial patterns in scraping activity and possibly indicated a more uniform pattern of grazing per unit area when assessed over a longer time frame.

These observations certainly demonstrate that the wax was not unpalatable to the limpets, but field observations during foraging periods will be needed to determine if the limpets alter their behaviour when they encounter disc sites. Finally, it is not clear whether discs were scraped in the same proportion as the natural rock surface, and so both the number of discs scraped and the area scraped can only be used as an index of grazing intensity, rather than an absolute value. However, neither systematic selection nor rejection of the discs was evident from daily or annual patterns of grazing activity (R. C. T. unpublished data).

4.5.2 Spatial and Temporal Patterns of Grazing Intensity

Regular arrays of discs allowed mapping of grazing patterns. Larger arrays could provide a broader view of spatial variations in foraging intensity. For example, spatial correlations between grazing intensity and macroalgal escapes (growth of the algal germling beyond the size normally eaten by the grazers) could be examined in a more natural manner than with experiments where grazers are just excluded or removed. Temporal variations in grazer intensity could also be recorded from scales ranging from days to years. The technique has also be recently been used with larger numbers of discs (108 at each of 3 sites) to determine diel variations in grazing intensity between calm and rough sea conditions (R. C. T. and R. Whitehead, unpublished data). In a similar manner other short term variations in grazer activity could be determined.

SECTION III

Monitoring and Experimental Studies

CHAPTER FIVE

Spatial and Temporal Variability in Epilithic Biofilms

5.1 ABSTRACT

Spatial and temporal variations in the abundance of intertidal epilithic microalgae were recorded on both moderately wave exposed and sheltered shores, on the Isle of Man. Microalgae were quantified using a combination of direct counts made with a scanning electron microscope, and estimates of total standing stock obtained by chlorophyll extractions. Electron microscope analyses were time consuming and gave highly variable results. Chlorophyll extractions provided a rapid alternative, and gave reliable estimates of abundance which were positively correlated with direct counts of diatoms, but not cyanobacteria.

Microalgal biomass (determined by chlorophyll extractions) varied seasonally with reduced abundance during the summer. During the mid-winter, microalgae were less abundant on sheltered shores than on wave exposed shores. However, it was not possible to generalise about variations in standing stock between shores of differing exposure exposure, tidal level, or about the effects of cover by macroalgal canopy.

Detailed monitoring at one moderately wave exposed site, Port St Mary, showed that microalgal biomass (in terms of chlorophyll) and the abundance of diatoms and cyanobacteria varied with tidal height and sampling date. Microalgal standing stock was least abundant on the mid shore on all sampling occasions and was most abundant on the upper shore during the early spring. Abundance declined markedly during the summer, especially on the upper shore. At this time diatoms were virtually absent from the shore, but cyanobacteria persisted in reduced abundance. Limpet grazing activity increased during the summer and was correlated with changes in both air and seawater temperatures but not with microalgal biomass. At Port St Mary microalgal abundance appeared to be regulated by seasonal changes in emersion stresses experienced during low tide exposure to air whilst vertical zonation in microalgal abundance was regulated by limpet density.

5.2 INTRODUCTION

Most of the studies examining spatial and temporal variability of intertidal communities have focused on macroalgae and invertebrates (e.g. Lewis, 1964; Southward, 1964; Hawkins *et al.*, 1992). Microflora, which influence settlement (see reviews by Crisp, 1984; Rodriguez *et al.*, 1993) of these larger shore dwellers, provide a food resource for grazers (Southward, 1964; Branch, 1981; Hawkins *et al.*, 1989) and are a source of primary production (Bustamante *et al.*, 1995), have been relatively neglected (but see Underwood, 1984c; MacLulich, 1987; Dye and White, 1991; Hill and Hawkins, 1991). This deficiency is partly the result of technical difficulties in studying microbial films (Underwood, 1984c; MacLulich, 1986; Dye and White, 1991; and see comments by Hall, 1992).

Chlorophyll extraction and direct counts of abundance have been used to determine microalgal standing stock, but neither method is ideal. Chlorophyll a extractions from microalgae provide a reasonable index of total abundance (Underwood, 1984c; Hill and Hawkins, 1990), but give no information on the relative composition of the film. In addition, the amount of pigment within each algal cell may vary between sampling occasions depending on the physiological state of the cells (Ryther, 1956; Humphrey, 1961; Oquist, 1974; Foy and Gibson, 1982). Alternatively, scanning electron microscopy (SEM) can be used to make direct counts of microalgal cells. However, this technique views only the surface of the film and gives no indication of the abundance of organisms in lower layers (Hill and Hawkins, 1990). Despite the difficulties, these techniques have successfully been used to record temporal and spatial variations in microalgal abundance (e.g. Aleem, 1950; Castenholz, 1963; Underwood, 1984c; 1991; Hill and Hawkins, 1991).

Seasonal variations in microalgal biomass are usually characterised by reduced abundance during the late spring and summer (Aleem, 1950; Castenholz, 1963; Nicotri, 1977; Underwood, 1984c; MacLulich, 1987; Dye

and White, 1991). Vertical zonation of microalgae has also been recorded with maximal abundance on the lower shore (Aleem, 1950; Castenholz, 1963; Underwood, 1984c; MacLulich, 1987).

In the summer conditions in the intertidal zone are especially harsh during daytime low tides and the principal causes of reduced microalgal standing crop have frequently been attributed to factors associated with emersion, such as increased desiccation (Underwood and Jernakoff, 1984), insolation and thermal stress (Aleem, 1950; Castenholz, 1963; Dye and White, 1991). These factors are likely to have the greatest impact on the upper shore where algae are exposed to the air for the longest periods of time. Other explanations for the patterns in temporal and spatial variability have included variations in grazing activity (Castenholz, 1963), nutrient availability and / or the supply of microalgae from the plankton (MacLulich, 1987). The relative importance of each factor is not known.

Insolation and desiccation stresses should be considerably reduced on areas of shore beneath a macroalgal canopy and comparison between these areas and those outside the canopy may provide a considerable insight into the mechanisms regulating microalgal production. Most of the studies on microalgal distribution have been conducted on moderately wave exposed shores (Castenholz, 1963; Hill, 1990; Dye and White, 1991) where macroalgal abundance is patchy. A previous study at Port St Mary did not reveal any differences in microalgal abundance between areas beneath the macroalgal canopy and adjacent areas of open rock (Hill and Hawkins, 1991). However, sheltered shores, which are dominated by a dense macroalgal canopy, have not been examined.

In addition to differences in canopy cover, exposed shores, by definition, receive greater wave action than sheltered shores. Strong water currents, experienced in river systems during storm flows, have been shown to damage diatoms, but not cyanobacteria (see Lock, 1993). In a similar

manner sheltered shores may offer a refuge from wave action as well as from emersion stresses.

In this study a combination of direct counts and chlorophyll analyses were used to explore the temporal and spatial variability of microalgal abundance on sheltered shores (which had dense macroalgal cover) and moderately wave exposed shores (where macroalgal abundance was more patchy). Temporal and spatial variation in microalgal abundance were also compared between tidal levels and between areas covered by macroalgal canopy and those exposed to the air during low tide.

Differences in microalgal standing stock between tidal heights and between sampling dates were considered in more detail at one moderately wave exposed location. Meteorological data (air temperature, sea water temperature and sunshine hours), dissolved nutrient concentrations, the abundance of phytoplankton and estimates of limpet grazing intensity were recorded in parallel. The relative strength of correlations between these factors and microalgal abundance were used to provide an insight into possible explanations of seasonal variations observed for the microalgae.

5.3 METHODS

5.3.1 Study sites

This study was made between November 1992 and April 1996 on rocky shores on the Isle of Man, U.K. (4°W, 54°N). Two moderately wave exposed shores (Port St Mary and Derbyhaven) and two sheltered shores (Castletown and Langness) were selected in the south of the Island (Figure 1).

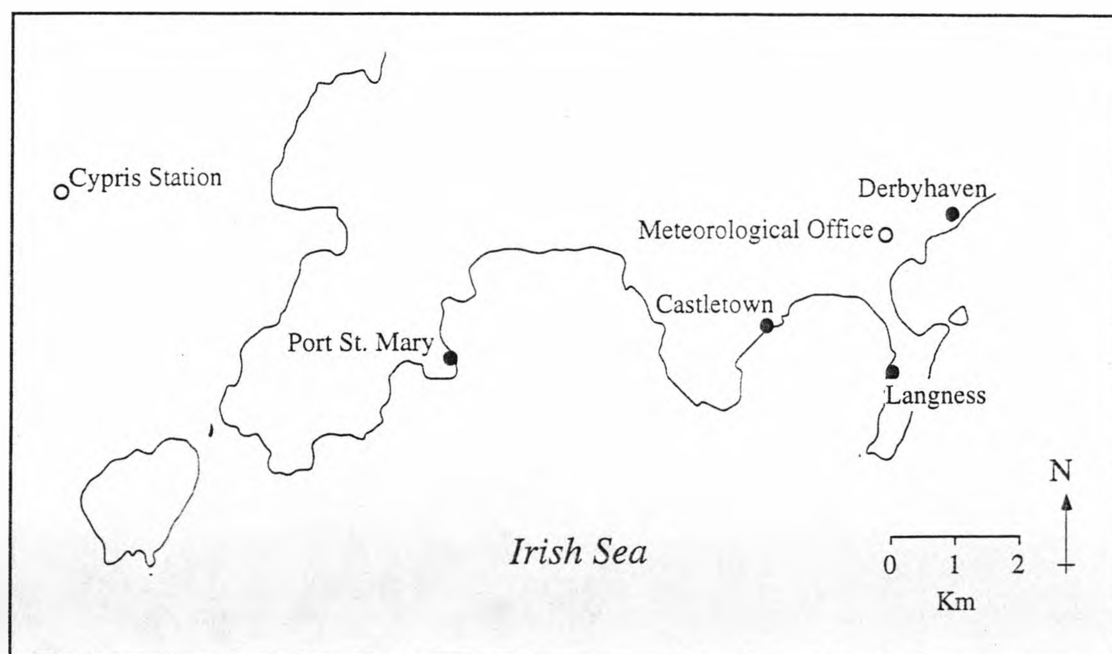


Figure 1) Map showing four study sites in of the south of the Isle of Man and the location of the 'Cypris' monitoring site and the Meteorological Office at Ronaldsway.

At all sites the substrata were gently sloping planes of carboniferous limestone (Ford, 1993). Moderately wave exposed shores at Port St. Mary and Derbyhaven were dominated by a mosaic of fucoid algae and barnacles interspersed with areas of open rock (Figures 2 and 3, Chapter 2). The macrobiota on these shores has been studied extensively (for site description see: Southward, 1951; Southward, 1953; Hawkins, 1979; Hill, 1990). Sheltered shores at Castletown and Langness were dominated by a dense canopy of *Ascophyllum nodosum* (L.), beneath which were patches of turf forming algae, coralline algae and open rock (Figures 4 and 5, Chapter 2; for site description see: Southward, 1951; Southward, 1953; Hawkins, 1979). Limpets, mainly *Patella vulgata* (L.), with some *P. aspera* (Lamark) lower on the shore, were the principal grazing molluscs at all locations.

Comparisons of microalgal abundance were made at several tidal levels between M.L.W.N. and M.H.W.N. The vertical distance between these levels was measured using a Cowley Automatic Level and values were converted to heights above lowest astronomical tide (LAT, for Port St Mary), from the level of low water on calm days (Southward, 1953).

5.3.2 Abundance of Macrobiota

The dominant macrobiota on each shore was recorded from a series of 18 50cm x 50cm quadrats which were positioned randomly at each tidal level. These data were used to calculate the density of limpets per unit area of rock available for grazing. Rock available for grazing was taken as the area not occupied by macroalgal holdfasts, and assumes that foraging on algal turf (on sheltered shores) was minimal. The abundance of other grazers, such as littorinids and top shells, was recorded where these were found on the open rock but not when they were amongst macroalgal fronds.

5.3.3 Estimation of Microalgal Standing Stock

Microalgal standing stock was determined spectrophotometrically using extracted chlorophyll as an index (H.M.S.O., 1983; Hill and Hawkins, 1990), and directly by counts made with SEM (Hill and Hawkins, 1990). Both types of analyses were made using rock chips which were chiselled from the shore complete with epilithic algae. Samples were approximately 2cm x 2cm for chlorophyll analyses and 1cm x 1cm for SEM examination, and were randomly collected from areas of 'bare rock' (not colonised by macrobiota such as barnacles or encrusting algae).

The type of habitat sampled was standardised wherever possible. Sheltered shores were covered by a dense macroalgal canopy and samples were collected from areas of uncolonised rock beneath this. Moderately wave exposed shores had a more patchy distribution of macroalgae, here, samples were collected from areas beneath the canopy (to provide a comparison with the sheltered shores), and from areas which were neither covered nor swept by macroalgae (to examine the effects of the canopy on microalgal abundance).

For chlorophyll analyses, 18 rock chips were collected from each shore level on each sampling occasion. These gave a total area of rock surface greater than $30cm^2$ which was adequate to overcome natural spatial variation (R. C.

T. unpublished data). Chlorophyll was extracted from each sample in hot 100% methanol (Chapter 3; H.M.S.O., 1983; Hill and Hawkins, 1990). From January 1994 this technique was modified to include a hydration step which greatly enhanced extraction (Chapter 3). Estimates obtained prior to this date were adjusted using a formula calculated from regression of hydrated extraction against dry extraction (Chapter 3).

Direct counts of microbiota were made using a Philips XL 30 SEM (Hill and Hawkins, 1990). Six rock chip samples were collected from each shore level on each occasion and fixed in 2.5% gluteraldehyde in filtered sea water. These were viewed at 480x magnification and six randomly located black and white photographs were taken on the surface of each. The photographs were processed into negatives and examined at six times magnification with a binocular microscope. This method provided reasonable resolution for identification with a large field of view (modified after Patterson *et al.*, 1986). Cyanobacteria were quantified in terms of percentage cover whilst diatoms and other microbiota were recorded as numbers of cells present. Data from the six photographs on each sample were then averaged to provide estimates of abundance.

5.3.4 Comparisons Between Shores

Microalgal abundance was estimated at all four sites at approximately three-monthly intervals between August 1993 and July 1994. On each occasion standing crop was assessed at three shore levels, lower, mid and upper. These zones were biologically defined as follows: lower shore was at the upper limits of the *Fucus serratus* (L.) zone, mid shore was in the middle of the *F. vesiculosus* (L.) - *Semibalanus* zone, and upper shore was just below the *F. spiralis* (L.) zone. An index of microalgal standing crop was obtained for each of these areas using chlorophyll analyses. Comparisons with direct counts were made on one occasion (July,1993).

5.3.5 Comparisons Along a Vertical Transect

Vertical zonation of microalgae was examined in detail at approximately three monthly intervals between February 1993 and April 1994 at Port St Mary. Rock chips, for both chlorophyll analyses and direct observation, by SEM, were collected from areas of rock outside the *Fucus* canopy at each of eight tidal levels between MLWN and MHWN. At the lowest sampling level, the shore was covered by a continuous canopy of *F. serratus* and samples were collected from bare rock beneath this.

5.3.6 Detailed Seasonal Monitoring

Microalgal abundance was monitored regularly at Port St Mary between November 1992 and June 1996 using a combination of chlorophyll analyses and direct counts with a SEM. Samples were collected at approximately monthly intervals, at the same three shore levels used for the between shores comparisons.

Estimates of microalgal standing stock and counts of cyanobacteria and diatoms were compared with possible regulating factors, including limpet grazing activity and various physico-chemical parameters, using correlation analyses (see below). Variations in the microalgal biomass and cell counts were 'smoothed' using a five point running mean, to give an annual cycle of average monthly values. Here, data from all of the sampling occasions were initially rearranged in order of the day of the year on which they were collected (0 - 365), irrespective of the sampling year. For example, January 3rd 1993 equals day 3, January 21st 1995 equals day 21, etc.

5.3.7 Limpet Grazing Activity

During 1993 a technique for quantifying limpet foraging activity was developed using discs made of dental wax which were recessed into small holes drilled in the shore (Chapter 4). These discs became scratched by limpet radulae during foraging excursions, and an index of grazing intensity was obtained by averaging the percentage area of these discs scraped

during a two week period in three replicate arrays of 16 discs each (Chapter 4). This method was used to estimate grazing intensity on the mid shore at monthly intervals from January 1994 to April 1996. The abundance of limpets in the area around the discs was recorded in parallel.

5.3.8 Climatological Data and Nutrient Availability

Weather data for the period (1992 - 1996) were obtained from the Meteorological Office at Ronaldsway Airport (Figure 1). Seawater temperature and the concentrations of dissolved nutrients were also available for the survey period from data collected at the 'Cypris' Monitoring Station, which is located 5km West of Port Erin (Figure 1). To confirm that 'Cypris' Station data provided reliable estimates of dissolved nutrients for the intertidal zone at Port St Mary, water samples were collected from the shore there at approximately six-monthly intervals and compared with samples collected at the 'Cypris Station'. The nutrient composition of seawater was determined using an Alpkem® RFA/2 rapid flow analyser.

5.3.9 Statistical Analyses

In general, temporal and spatial variability were analysed using Generalised Linear Models, with the 'PROC GLM' command in SAS® v.6.03 (SAS, 1988) on an IBM personal computer. Microalgal abundance data were analysed using mixed model ANOVA. 'Site' was considered as a random factor nested within 'exposure' whilst 'sampling date', 'exposure' (sheltered or moderately wave exposed) and 'tidal height' were considered as fixed factors. Data from areas beneath and outside the canopy, on moderately wave exposed shores were analysed in a similar way. Here 'site' was considered as a random factor (but was not nested), whilst 'sampling date', 'canopy', and 'shore level' were treated as fixed factors in the ANOVA.

Comparisons between levels within treatment factors were selected *a priori* and determined using Bonferroni Comparisons. Any additional *a posterioi.*

comparisons were made using Tukey's least significant difference tests (Maxwell and Delaney, 1990). Correlations between microalgal abundance and various physico-chemical and biological regulating factors were calculated with Bonferroni adjusted Pearson's Correlation Coefficient using Systat v.5.2 on a Macintosh computer (Systat, 1992). This rather conservative approach minimised type 1 error and so focused on the factors which were most strongly correlated with microalgal abundance. The direction of non-significant correlations were considered in order to help describe possible temporal and spatial trends in the data.

For parametric analyses, data were transformed where appropriate. Cochran's test was performed to check for homogeneity of variance prior to analyses (Winer *et al.*, 1971; Underwood, 1981b). Plots of residuals were examined after ANOVA to check that error terms were normally distributed and to confirm homogeneity of variance where the number of variances being examined exceeded tabulated values for Cochran's test (M. Mortimer pers. comm.). Where data were not normally distributed one-way non-parametric analyses were made with Kruskal-Wallis tests, using Minitab® v. 10.1 for Windows® on an IBM personal computer.

5.4 RESULTS

5.4.1 Comparison Between Shores and Between Areas with Differing Macroalgal Cover

Microalgal standing stock, in terms of chlorophyll, varied between sites, but these differences were not related to shore exposure (Figure 2, Table 1 and Table 2). Port St Mary (wave exposed shore) had the greatest microalgal biomass whilst Castletown (sheltered shore) had the least, Derbyhaven (wave exposed shore) and Langness (sheltered shore) had intermediate microalgal abundance. The abundance of diatoms and cyanobacteria was similar at all sites during July 1993 (Table 3). Differences in microalgal abundance between tidal levels were small and non-significant.

Table 1) Analyses of variance for factors affecting microalgal abundance (determined by chlorophyll extraction) at four sites in the south of the Isle of Man during 1993 and 1994. 'Site' was treated as a random factor nested within 'exposure', while 'exposure' 'sampling date' and 'tidal height' were treated as fixed factors. n.s. = not significant, * = P<0.05, ** = P<0.01, *** = P<0.001.

source of variation	df denominator	Type III MS	df numerator	MS numerator	F	P
Sampling date (Sd)	3	0.749	773	0.014	54.35	***
Exposure (Exp)	2	1.340	2	1.094	1.22	n.s.
Site (Exp) (Si(Exp))	2	1.075	773	0.014	77.93	***
Tidal height (Th)	2	0.041	773	0.014	2.98	n.s.
Sd x Exp	3	0.412	773	0.014	29.86	***
Sd x Si(Exp)	6	0.047	773	0.014	3039	**
Sd x Th	6	0.006	773	0.014	0.45	n.s.
Exp x Th	2	0.041	773	0.014	2.94	n.s.
Th x Si(Exp)	4	0.171	773	0.014	12.40	***
Exp x Sd x Th	6	0.052	773	0.014	3.79	**
Sd x Th x Si(Exp)	10	0.055	773	0.014	3.98	***
Error	773	0.014				

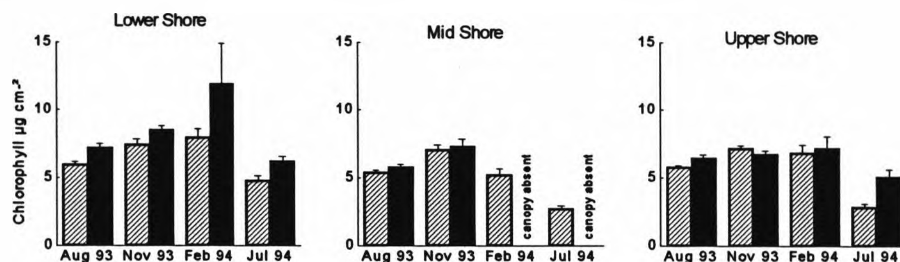
Bonferroni analysis of differences between levels within 'sites' and 'sampling dates'

Port St Mary (P)	-				Aug. 93 (A)	-			
Derbyhaven (D)	*	-			Nov. 93 (N)	n.s.	-		
Castletown (C)	*	*	-		Feb. 94 (F)	n.s.	*	-	
Langness (L)	*	n.s.	*	-	Jul. 94 (J)	*	*	*	-
	P	D	C	L		A	N	F	J

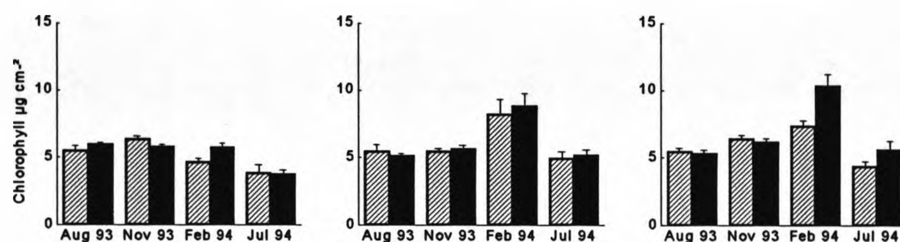
The abundance of limpets and the number of limpets relative to the area of rock available for grazing varied between sites but these variations were not correlated with microalgal biomass (in terms of chlorophyll), or the abundance of either diatoms or cyanobacteria (Table 2). However, differences in microalgal standing stock were negatively correlated (P < 0.05) with the abundance of other grazing molluscs (Table 2).

Moderately wave exposed shores

Port St Mary

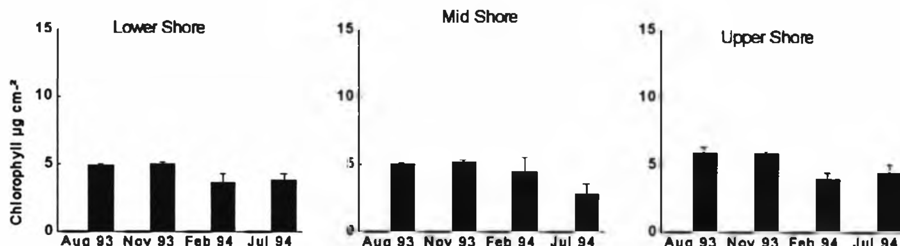


Derbyhaven

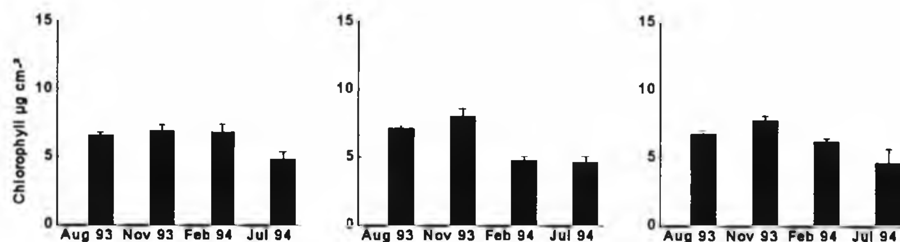


Castletown

Sheltered shores



Langness



Open rock Rock beneath macroalgal canopy

Figure 2) Epilithic microalgal standing crop (measured using extracted chlorophyll as an index), at three tidal heights, on two moderately wave exposed and two sheltered shores in the south of the Isle of Man, between August 1993 and June 1994 (bars = 1SE). Solid columns represent samples collected from beneath macroalgal canopy, hatched columns represent samples from open rock outside the canopy (this habitat was only present on moderately wave exposed shores).

Table 2) Abundance of limpets, other grazing molluscs, area of rock available for grazing, limpet density per area of rock available for grazing, microalgal standing stock (expressed as extracted chlorophyll), and the abundance of diatoms and cyanobacteria at various tidal levels at four shores in the South of the Isle of Man. Abundance of other grazers does not include individuals amongst macroalgal fronds. Pearson's Correlation Coefficients between the abundance of microalgae and grazers are shown. The number of sampling units (quadrats or rock chips) is given.

Location	Shore level	Height above Lowest astronomical tide (m)	Limpet abundance (average per 50 x 50 cm quadrat) *n* = 18	Abundance of other grazers (average per 50 x 50 cm quadrat) *n* = 18	Area of rock available for grazing (% per 50 x 50 cm quadrat)	Limpet density per m-² of area of rock available for grazing	Abundance of diatoms K. cm-² during July 1993 (rock chips) *n* = 6 under canopy	*n* = 6 outside canopy	Abundance of cyanobacteria % during July 1993 (rock chips) *n* = 6 under canopy	*n* = 6 outside canopy	Chlorophyll per cm-² overall (Aug. 93 to Jul. 94) (rock chips) *n* = 18
Port St Mary	Lower	2.1	5.39 ± 0.56	0	79	27	1.1 ± 1.2	0	2 ± 3	28 ± 14	8.5 ± 0.07
	Mid	3.2	6.33 ± 1.12	0	87	29	1.3 ± 0.9	0.3 ± 0.2	4 ± 2	1 ± 1	6.75 ± 0.12
	Upper	4.4	3.11 ± 0.70	0	87	14	0.3 ± 0.2	0	0	24 ± 10	6.37 ± 0.07
Derbyhaven	Lower	2.5	8.37 ± 1.97	0	71	47	4.9 ± 2.1	0.1 ± 0.1	0	14 ± 7	5.27 ± 0.07
	Mid	3.6	6.39 ± 0.72	0.01 ± 0.001	92	27	22 ± 20	0.6 ± 0.6	0	11 ± 7	6.22 ± 0.07
	Upper	4.5	1.72 ±0.46	0.03 ± 0.05	96	7	0	3.8 ± 3.8	4 ± 2	4 ± 3	6.80 ± 0.07
Castletown	Lower	2.2	1.61 ± 0.42	9.17 ± 1.02	37	17	0		0		4.29 ± 0.07
	Mid	3.5	2.56 ± 0.70	5.94 ± 0.78	20	51	0.3 ± 0.3		0		3.85 ± 0.07
	Upper	4.2	0.89 ± 0.31	5.38 ± 1.89	62	6	1.9 ± 1.4		0		5.10 ± 0.07
Langness	Lower	2.5	2.5 ± 0.63	3.85 ± 0.91	7	143	0		1 ± 1		6.30 ± 0.07
	Mid	3.6	1.22 ± 0.47	6.11 ± 0.99	4	122	1.7 ± 1.7		0		6.25 ± 0.07
	Upper	4.2	0.50 ± 0.21	4.23 ± 1.01	25	8	2.1 ± 2.1		0		6.31 ± 0.07
Correlation with chlorophyll concentration	*r*	not examined	0.23 n.s.	-0.66*	not examined	0.01 n.s.					
Correlation with abundance of diatoms	*r*	not examined	0.47 n.s	0.32 n.s.	not examined	-0.12 n.s.					
Correlation with abundance of cyanobacteria	*r*	not examined	0.2 n.s.	-0.51n.s.	not examined	-0.19 n.s.					

Table 3) Abundance of diatoms and cyanobacteria during July 1993 at four sites in the south of the Isle of Man (averaged across three shore levels), mean ± 1SE. The number of rock chips examined (*n*) and the significance of any differences in abundance between sites is given.

Site	*n*	Diatoms cm^{-2}	Cyanobacteria % cover
Port St Mary (wave exposed)	11	11.69 ± 8.61	1.48 ± 0.21
Derbyhaven (wave exposed)	8	100.46 ± 11.86	1.46 ± 0.28
Castletown (Sheltered)	11	6.27 ± 8.61	0 ± 0
Langness (Sheltered)	9	12.63 ± 10.52	8.37 ± 0.25
Kruskal-Wallis test		$H_{0.05, 11,8,11,9,}$ = 4.92, n.s.	$H_{0.05, 11, 8, 11, 9,}$ = 4.56, n.s.

There were variations in microalgal standing stock between areas beneath and outside *Fucus* on some sampling occasions (e.g. Port St Mary, lower shore, and Derbyhaven, upper shore during February 1994; Figure 2). However, abundance between areas beneath and outside canopy were similar when averaged across all data (Table 4). At Port St Mary diatoms were less abundant on rock outside the macroalgal canopy than on rock beneath the canopy. This difference was not evident at Derbyhaven (Table 5), but cyanobacteria were more abundanton rock outside the canopy there (Table 5). Overall, these data were inconclusive, and clear trends were not evident between areas beneath and outside macroalgal canopy.

Seasonal differences in microalgal standing stock accounted for a considerable amount of the variation in comparisons between shores and between canopy cover. Standing stock was lowest during July 1994 in samples from both data sets (Tables 1 and 4). Seasonal trends were evident in samples collected for the comparisons between areas of differing canopy cover, with greater abundance during the winter months (November 1993 and February 1994) than during the summer (August 1993 and July 1994). However, similar trends were not apparent in samples collected for comparisons between shores of differing exposure. Here, August 1993 had similar standing stock to that in November 1993 and February 1994.

Table 4) Analyses of variance for factors affecting microalgal abundance (determined by chlorophyll extraction) at four sites in the south of the Isle of Man during 1993 and 1994. 'Site' was treated as a random factor while 'canopy', 'sampling date' and 'tidal height' were treated as fixed factors. n.s. = not significant, * = $P<0.05$.

source of variation	df denom-inator	Type III MS	df numerator	MS numerator	*F*	*P*
Canopy (Can)	1	0.257	0.92	0.026	9.67	n.s.
Site (Si)	1	0.110	1.52	0.432	0.25	n.s.
Sampling dates (Sd)	3	1.236	2.91	0.086	14.46	*
Tidal height (Th)	2	0.032	1.99	0.511	0.06	n.s.
Can x Si	1	0.025	2.49	0.034	0.72	n.s.
Can x Sd	3	0.050	2.96	0.04	1.25	n.s.
Can x Th	2	0.004	1.45	0.006	0.61	n.s.
Si x Sd	3	0.082	7.65	0.155	0.53	n.s.
Si x Th	2	0.480	5.67	0.119	4.05	n.s.
Sd x Th	6	0.025	5.93	0.128	0.20	n.s.
Can x Si x Sd	3	0.037	3.99	0.009	4.34	n.s.
Can x Sea x Th	6	0.016	4.0	0.009	1.87	n.s.
Can x Si x Th	2	0.007	4.06	0.009	0.79	n.s.
Si x Sd x Th	6	0.118	3.99	0.009	13.97	*
Si x Can x Sd x Th	4	0.009	772	0.011	0.77	n.s.
Error	772	0.01				

Bonferroni analysis of differences between 'sampling dates'

Aug. 93 (A)	-			
Nov. 93 (N)	*	-		
Feb. 94 (F)	*	n.s.	-	
Jul. 94 (J)	*	*	*	-
	A	N	F	J

Table 5) Abundance of diatoms and cyanobacteria between areas of open exposed rock and areas beneath macroalgal canopy, at two sites in the south of the Isle of Man (averaged across three shore levels and four sampling dates), mean ± 1SE. The significance of any differences in abundance between canopy cover is given. n.s. = not significant, * = $P<0.05$.

	n	Diatoms cm^{-2}	Cyanobacteria % cover
Port St Mary (open rock)	9	8.06 ± 1.33	13.29 ± 3.77
Port St Mary (canopy cover)	9	13.50 ± 1.33	1.82 ± 3.77
Kruskal-Wallis test		$H_{0.05,9,9,}$ = 5.43, *	$H_{0.05,9,9}$ = 3.57, n.s.
Derbyhaven (open rock)	9	18.98 ± 21.19	9.81 ± 0.28
Derbyhaven (canopy cover)	8	100.46 ± 18.46	1.15 ± 0.96
Kruskal-Wallis test		$H_{0.05,9,9,}$ = 1.84, n.s.	$H_{0.05,9,9,}$ = 6.56, *

There were significant interactions between the factors considered in both comparisons between shores, and comparisons between canopy cover. Consequently, differences between 'sampling dates' and 'sites' (described above) should be regarded with caution. For comparisons between shores, there were significant interactions between 'sampling date' and 'exposure', between 'sampling date' and 'site', and between 'tidal height' and 'site' (Table 1). These differences were examined using *a posterioi* multiple comparison tests.

Interactions between 'sampling date' and 'site', and 'sampling date' and 'exposure' were predominantly caused by differences in microalgal abundance between sheltered and wave exposed shores during the winter. On sheltered shores standing stock declined markedly between November 1993 and February 1994 whilst on wave exposed shores standing stock increased slightly during the same period. Consequently, microalgal abundance differed substantially during February 1994, both between sites considered individually, and when combined as levels of 'exposure' (for both comparisons $q_{0.001,\infty,48} = 7.30$; $P<0.001$, Tukey test).

Interactions between 'sites' and 'shore levels' were mostly caused by differences in standing stock on the lower shore at the two wave exposed sites. Port St Mary had considerably greater standing stock on the lower shore than Derbyhaven ($q_{0.001,\infty,48} = 7.30$; $P<0.001$, Tukey test). At this tidal level limpets were significantly less abundant at Port St Mary than at Derbyhaven ($F_{1,34} = 7.68$, $P<0.01$, one-way ANOVA) and so variations in grazing intensity may have caused the difference in microalgal standing stock between the sites. Two multiple interactions were also present (Table 1). These were most probably caused by combinations of the two factor interactions described above, and were not considered further.

5.4.2 Comparisons Along a Vertical Transect at Port St Mary

There were clear differences in microalgal abundance between shore levels and between sampling dates (Figure 3). Abundance was greater on the upper shore (4.7m above L.A.T.) than at all other levels, whilst the mid shore (2.4 to 4.0m above L.A.T.) had significantly lower standing stock than either the upper or the lower shore (Table 6). Limpet abundance varied between shore levels and was negatively correlated with microalgal standing stock (Table 7).

Table 6) Analyses of variance for factors affecting microalgal abundance (determined by chlorophyll extraction) along a vertical transect at Port St Mary, Isle of Man. 'Sampling date' and 'Tidal height' were both treated as fixed factors, *** = $P<0.001$. Differences between sampling dates and between levels are shown ($P<0.05$ Bonferroni adjusted *a priori* tests). Differences between underlined values were non-significant.

source of variation	df	MS	*F*	*P*
Sampling dates	4	1.78	125.36	***
Tidal height	7	0.49	51.65	***
Sampling dates x Tidal height	28	0.11	11.38	***
Error	656	0.01		

Bonferroni analysis of differences between levels within 'sampling dates' and 'tidal height'

Differences between sampling dates   Feb. 93 > Jan. 94 = Jun. 93 = Apr. 94 = Sep. 93

Differences between tidal heights (m)   4.7 > 1.7 = 4.4 = 2.1 > 2.4 = 4.0 = 3.2 = 3.6

Table 7) Correlation between limpet abundance and microalgal standing stock, sampled at a range of tidal heights, at Port St Mary, Isle of Man. (* $P<0.05$, one tailed test).

		n	Microalgal standing stock	Diatoms	Cyanobacteria
Correlation with limpet abundance	*r*	8	-0.736*	-0.615*	-0.324

Total microalgal standing stock, in terms of chlorophyll, was significantly lower during the spring and summer (Jun. 93, Sep. 93 and Apr. 94) than in the winter months (Feb. 93 and Jan. 94; Table 6). However, there was a significant interaction between 'tidal height' and 'sampling date' (Table 6), and so differences within sampling dates and shore levels (described above)

should be viewed with caution. Inspection of the graphs (Figure 3) indicated that microalgae were least abundant on the mid shore on most sampling occasions, whilst abundance on the upper and lower shore varied considerably between sampling dates. *A posterioi* multiple comparisons tests confirmed that variations in microalgal abundance between sampling occasions were highly significant on both the upper and lower shore. For example, at the highest site on the shore (4.7m above L.A.T.) microalgal abundance during February 1993 and January 1994 was greater than that from most of other 'tidal height' - 'sampling date' combinations (34 out of 40 comparisons were significant, $p<0.05$, Tukey test $q_{0.05, \infty, 40} = 0.125$).

Diatoms were significantly less abundant on the shore as a whole (averaged across all shore levels), during September 1993 than at all other times. Greatest abundance was during February 1993 and January 1994 (Table ,8 Figure 3). The abundance of diatoms varied between sampling dates (Figure 3) in a similar manner to variations recorded for microalgal standing stock. However, the significance of these seasonal variations could not be determined by one-way analysis as there was a strong seasonal interaction. Comparisons between shore levels were made by visual inspection of graphs (Figure 3). Generally, abundance increased with tidal height, and greatest density was recorded on the upper shore during April 1994 (approximately 60 k. cells per cm^{-2}). The mean abundance of diatoms (averaged across all shore levels) was negatively correlated with the abundance of limpets at each tidal level (Table 7).

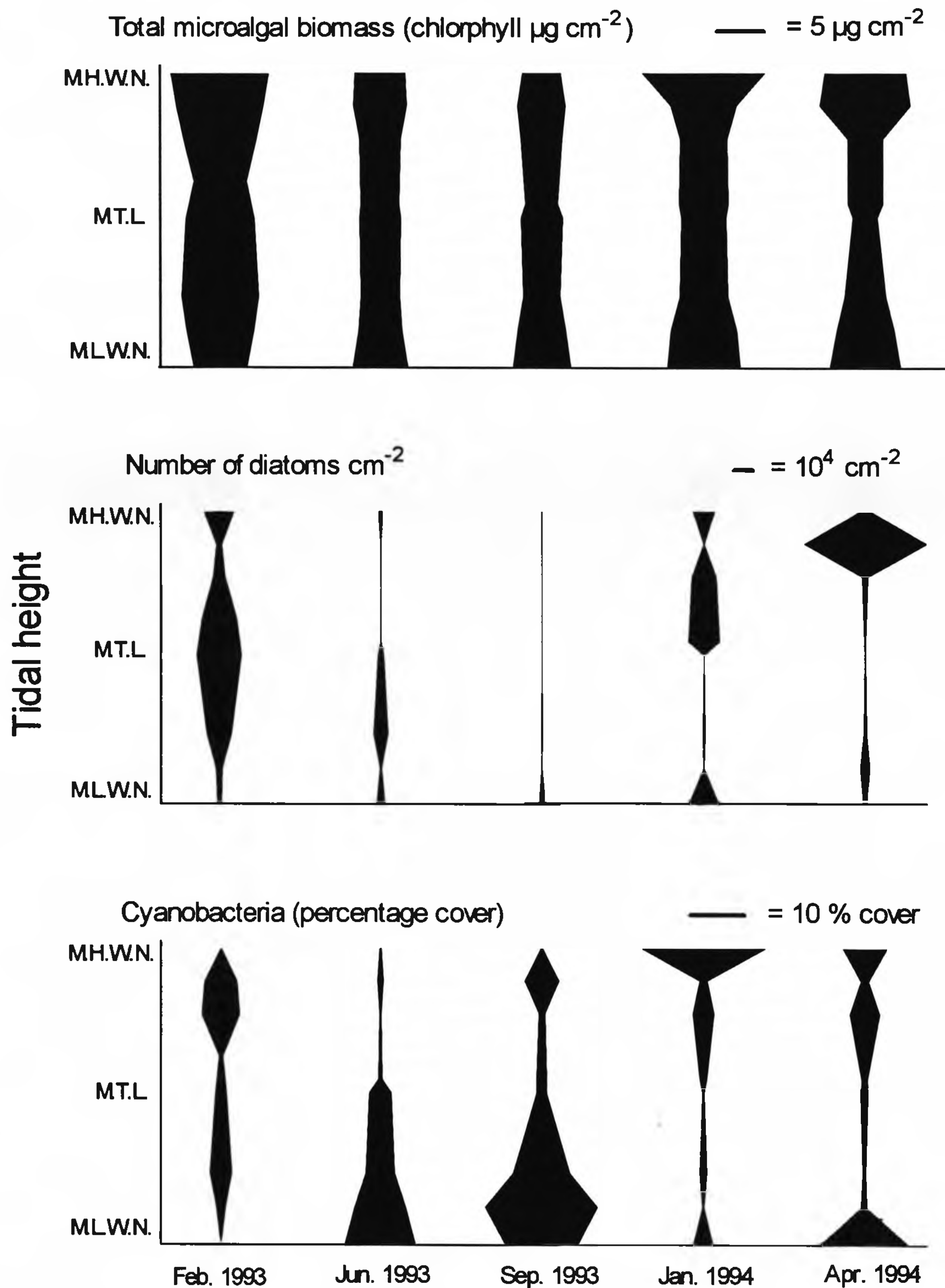


Figure 3) Abundance of benthic diatoms, cyanobacteria and total epilithic microalgal biomass (measured using extracted chlorophyll as an index) along a vertical transect at Port St Mary, Isle of Man. M.H.W.N. = mean high water neaps (~ 5.0m above L.A.T.), M.T.L. = mid tide level (~ 3.5m above lowest astronomical tide or L.A.T.), M.L.W.N. = mean low water neaps (~ 2.0m above L.A.T.).

Table 8) Kruskal-Wallis test for differences in the abundance of intertidal diatoms between sampling dates at Port St Mary, Isle of Man,. Average ranks were compared using a Tukey-type non-parametric multiple comparisons test and the direction of any significant differences are shown. All dates which differed did so to a high level of significance $P < 0.001$.

Date	*n*	Diatoms average rank	Direction of significant differences
Feb. 93	38	154.9	
Jun. 93	42	94.7	
Sep. 93	44	72.6	Feb. 93 > Jan. 94 > Apr. 94 = Jun. 93 > Sep. 93
Jan. 94	48	122.3	
Apr. 94	48	112.2	
Overall	220	110.5	

$H_{0.001,38,42,44,48,48} = 42.02$, $P < 0.001$

The abundance of cyanobacteria for the shore as a whole (averaged across all shore levels), did not differ significantly between sampling dates (Table 9). Inspection of the graphs (Figure 3) revealed interactions between 'tidal level' and 'sampling date'. On the upper shore, abundance was greatest during the winter months (February 1993 and January 1994) whilst on the lower shore abundance was greatest during the spring and summer. Cyanobacteria were less abundant at the mid shore level on four of the five sampling occasions, and their average abundance at each tidal level (averaged across all sampling dates) was negatively, but not significantly correlated with the abundance of limpets at each tidal level (Table 7).

Table 9) Kruskal-Wallis test for differences in the abundance of intertidal cyanobacteria between sampling dates at Port St Mary, Isle of Man.

Date	*n*	Cyanobacteria average rank	Direction of significant differences
Feb. 93	38	96.9	
Jun. 93	42	111.4	
Sep. 93	44	128.6	not tested as no differences between dates overall
Jan. 94	48	102.2	
Apr. 94	48	112.4	
Overall	220	110.5	

$H_{0.05,38,42,44,48,48} = 6.61$, n.s.

5.4.3 Detailed Seasonal Monitoring at Port St Mary

The results from SEM analyses were very variable and were discontinued in May 1995. Chlorophyll analyses of samples from lower and upper shore sites were discontinued in August 1995 as broad scale seasonal patterns appeared to be adequately described by sampling at a single tidal level. Chlorophyll analyses from the mid-shore continued until June 1996.

Seasonal variations in abundance of the major constituents of intertidal biofilms are listed (Table 10). All species, with the exception of cyanobacteria on the lower shore, were least abundant during the summer and most abundant during the winter and early spring. There were clear differences between shore levels, and with the exception of coralline algae, abundance was maximal on the upper shore. For *Fucus* germlings and ephemeral algae, this pattern was opposite to that observed for the adult population (Table 10). The abundance of the two most common constituents of the film, diatoms and cyanobacteria, were considered in detail, together with estimates of total standing stock.

Total microalgal standing stock showed considerable seasonal variation with peaks in abundance during the winter at all three shore levels examined (Figure 4). The abundance of diatoms followed the same seasonal pattern (Figure 5, Table 10) and was correlated positively with measures of standing stock (Table 11).

Seasonal variation in the abundance of cyanobacteria differed between the lower shore and the upper shore (Figure 6) in a similar way to that observed with data from the vertical transect. Abundance was greatest during the winter on the upper shore, and during the summer on the lower shore. The abundance of cyanobacteria was not significantly correlated with microalgal standing stock (Table 11).

Table 10) Seasonal abundance of some of the major constituents of intertidal microbial films at three shore levels at Port St Mary, Isle of Man. The abundance of macroalgae at each shore level is shown for comparison (see text for details).

		Lower Shore			Mid shore			Upper shore		
	Units	Overall abundance mean ± se	Period of greatest abundance	Peak abundance	Overall abundance mean ± se	Period of greatest abundance	Peak abundance	Overall abundance mean ± se	Period of greatest abundance	Peak abundance
Diatoms mostly *Achnanthes* spp.	x 10^3 cm^{-2}	2.0 ± 4.0	Dec. - Apr.	8.0	6.5 ± 2.2	Dec. - Mar.	18.0	16.0 ± 5.4	Dec. - Apr.	110.0
Cyanobacteria mostly	%	6.7 ± 1.5	Apr. - Sept.	25	2.7 ± 0.7	Feb. -Apr. & Sep.	13	10.9 ± 1.3	Dec. - Apr.	23
Protozoans	cm^{-2}	27 ± 14	Jan. - Feb.	130	66 ± 18	all year	300	210 ± 53	Dec. - Apr.	770
Ephemeral algae	cm^{-2}	180 ± 65	Apr. - Jul. & Sep.	830	2.5 ± 2.5	only one occurrence		275 ± 130	Apr. - Jul. & Sep.	2360
Macroalgal germlings probably Fucus spp.	cm^{-2}	17 ± 8	Apr. - May & Sep.	170	11 ± 6	Nov. - Feb.	130	54 ± 32	Apr. - Sep.	700
Crustose corallines mostly *Phymatolithon lenormandii*	%	2.6 ± 0.6	all year	8.6	0.1 ± 0.1	only three occurrences		0.1 ± 0.1	only four occurrences	
Macroalgal canopy	%	36.0	all year	-	0.5	all year	-	1.3	all year	-

Table 11) Pearson's Correlation Coefficients for comparisons between microalgal abundance calculated using extracted chlorophyll and the abundance of diatoms and cyanobacteria determined by direct counts from samples collected at Port St Mary, Isle of Man. Sample size (*n*) is given after list-wise deletion of missing values. Bonferroni corrected probabilities are shown n.s. = not significant, * = $P<0.05$, ** = $P<0.01$.

Chlorophyll concentration	*n*	Abundance of diatoms *r*	Abundance of cyanobacteria *r*
Upper shore	21	0.67**	0.36ns
Mid shore	24	0.44ns	-0.12ns
lower Shore	22	0.53*	-0.45ns

Data for microalgal standing stock, abundance of diatoms and cyanobacteria were averaged using a five point running mean of values arranged in order the day of the year in which they were collected (see methods). This presentation gave average estimates for monthly variations in microalgal abundance (Figure 7). Microalgae were always less abundance on the mid shore than on the upper or lower shore, whilst seasonal variations in standing stock were greatest on the upper shore and least on the lower shore. Diatoms increased in abundance from the lower to the upper shore and were more abundant during the winter and early spring than during the summer. For cyanobacteria seasonal patterns in abundance were not evident on the mid shore. However, the upper and lower shore clearly showed the opposing seasonal trends in abundance described earlier.

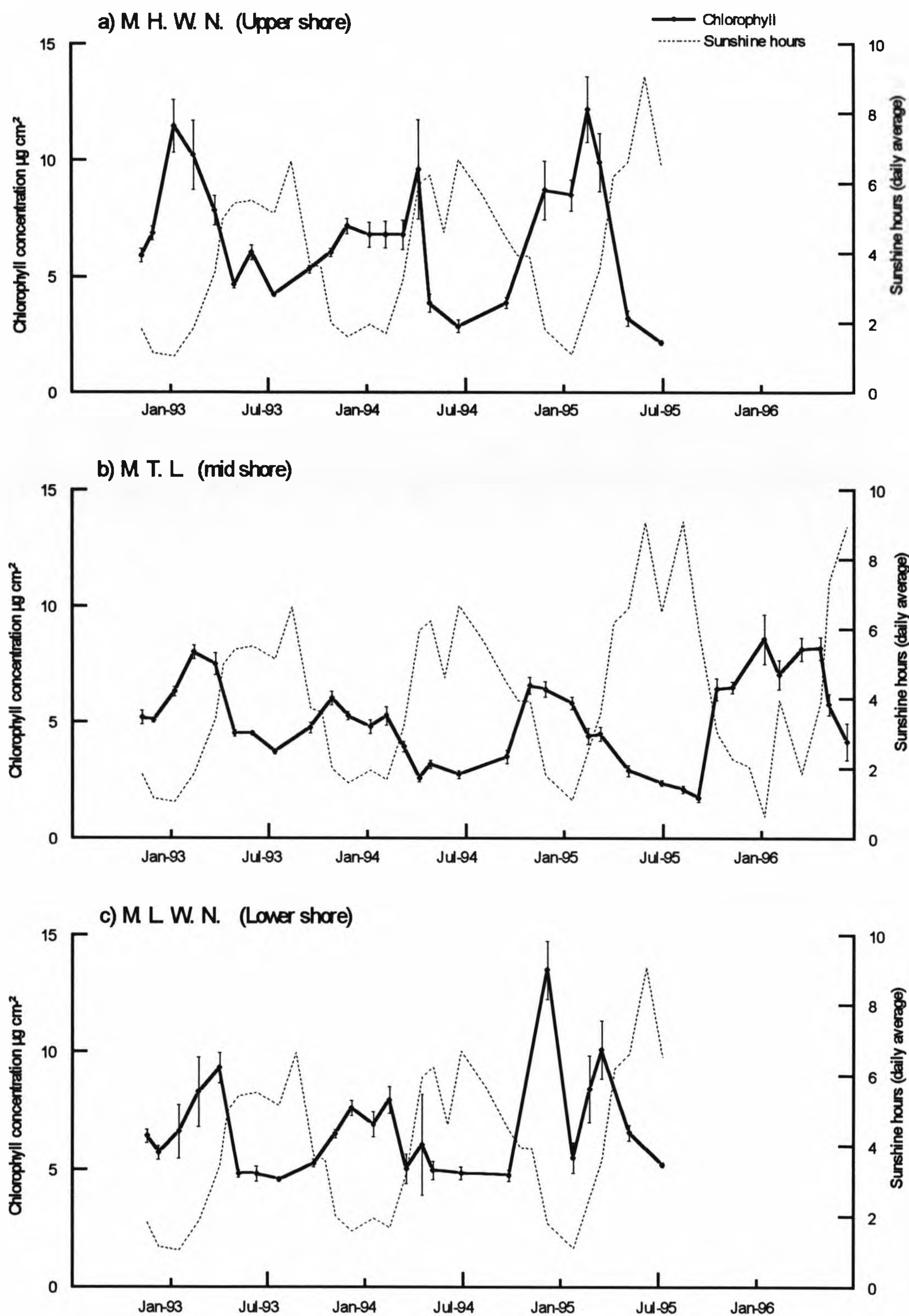


Figure 4) Seasonal changes in total microalgal standing stock (measured using extracted chlorophyll as an index) at three tidal heights on the shore at Port St Mary, Isle of Man (bars = 1SE). Mean daily sunshine hours are shown for comparison (see text for details). M.H.W.N. = mean high water neaps (~ 5.0m above lowest astronomical tide or L.A.T.), M.T.L. = mid tide level (~ 3.5m above L.A.T.), M.L.W.N. = mean low water neaps (~ 2.0m above L.A.T.).

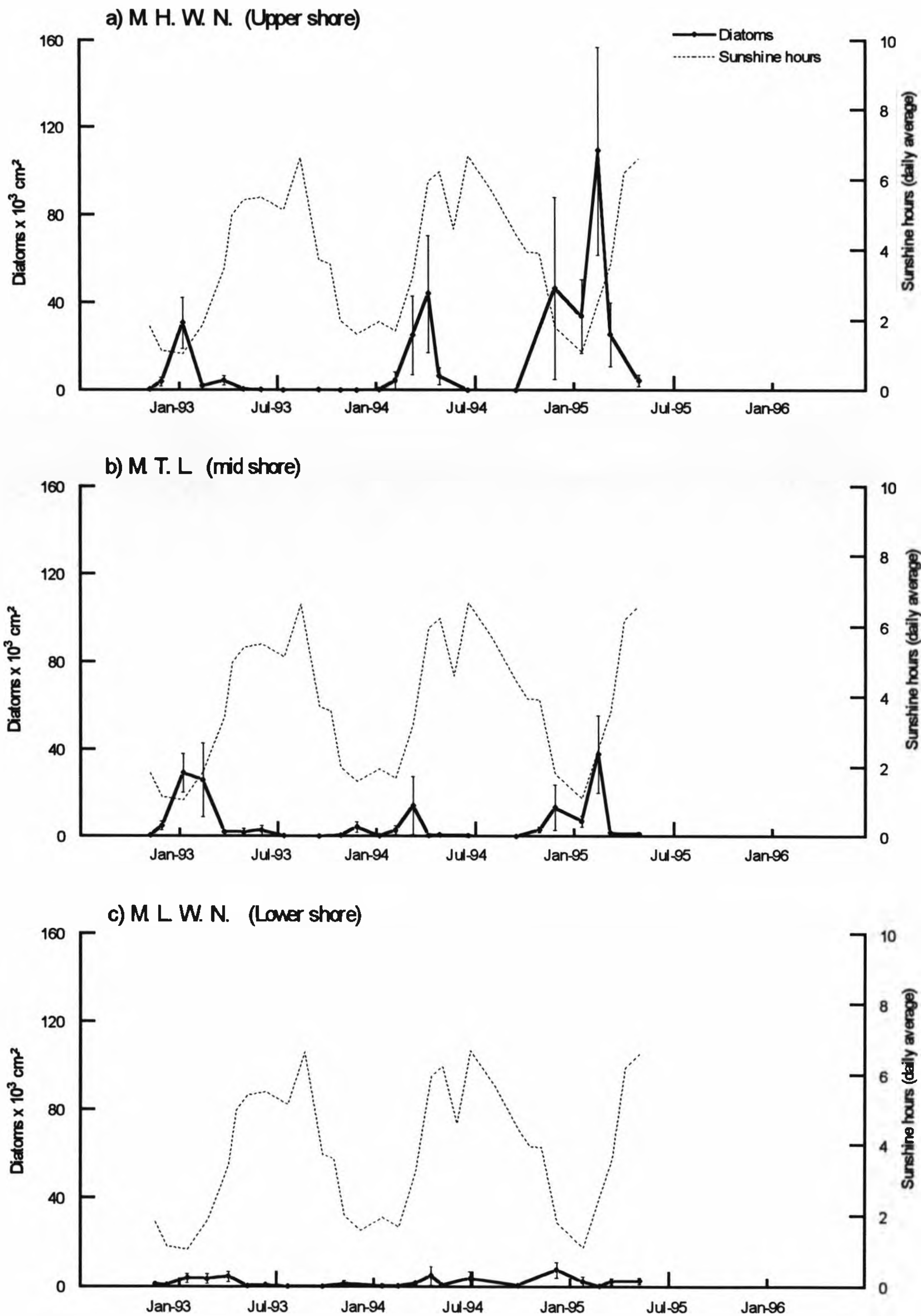


Figure 5) Seasonal changes in abundance of benthic diatoms at three tidal heights on the shore at Port St Mary, Isle of Man (bars = 1SE). Mean daily sunshine hours are shown for comparison (see text for details). M.H.W.N. = mean high water neaps (~ 5.0m above lowest astronomical tide or L.A.T.), M.T.L. = mid tide level (~ 3.5m above L.A.T.), M.L.W.N. = mean low water neaps (~ 2.0m above L.A.T.).

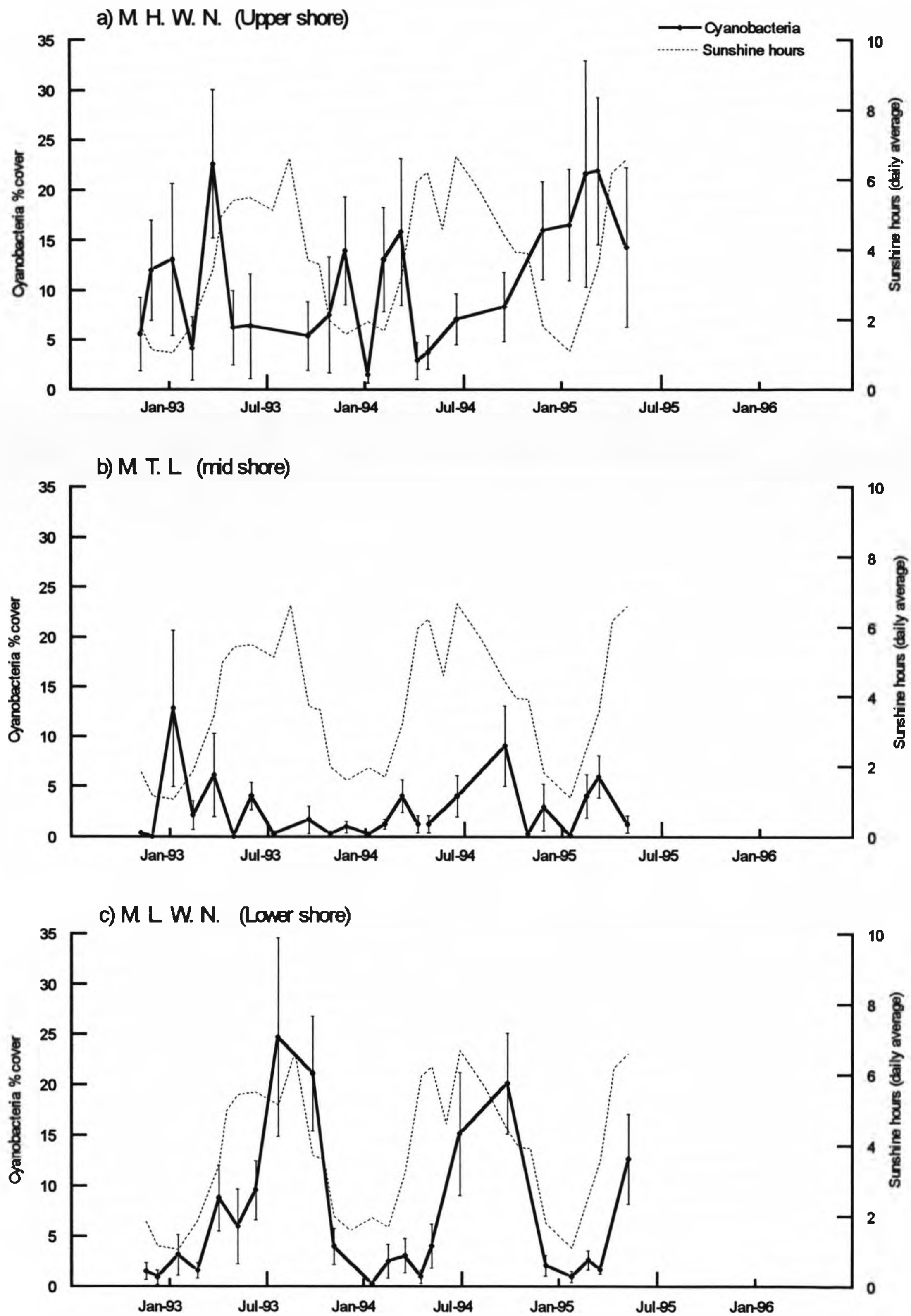


Figure 6) Seasonal changes in abundance of benthic cyanobacteria at three tidal heights on the shore at Port St Mary, Isle of Man (bars = 1SE). Mean daily sunshine hours are shown for comparison (see text for details). M.H.W.N. = mean high water neaps (~ 5.0m above lowest astronomical tide or L.A.T.), M.T.L. = mid tide level (~ 3.5m above L.A.T.), M.L.W.N. = mean low water neaps (~ 2.0m above L.A.T.).

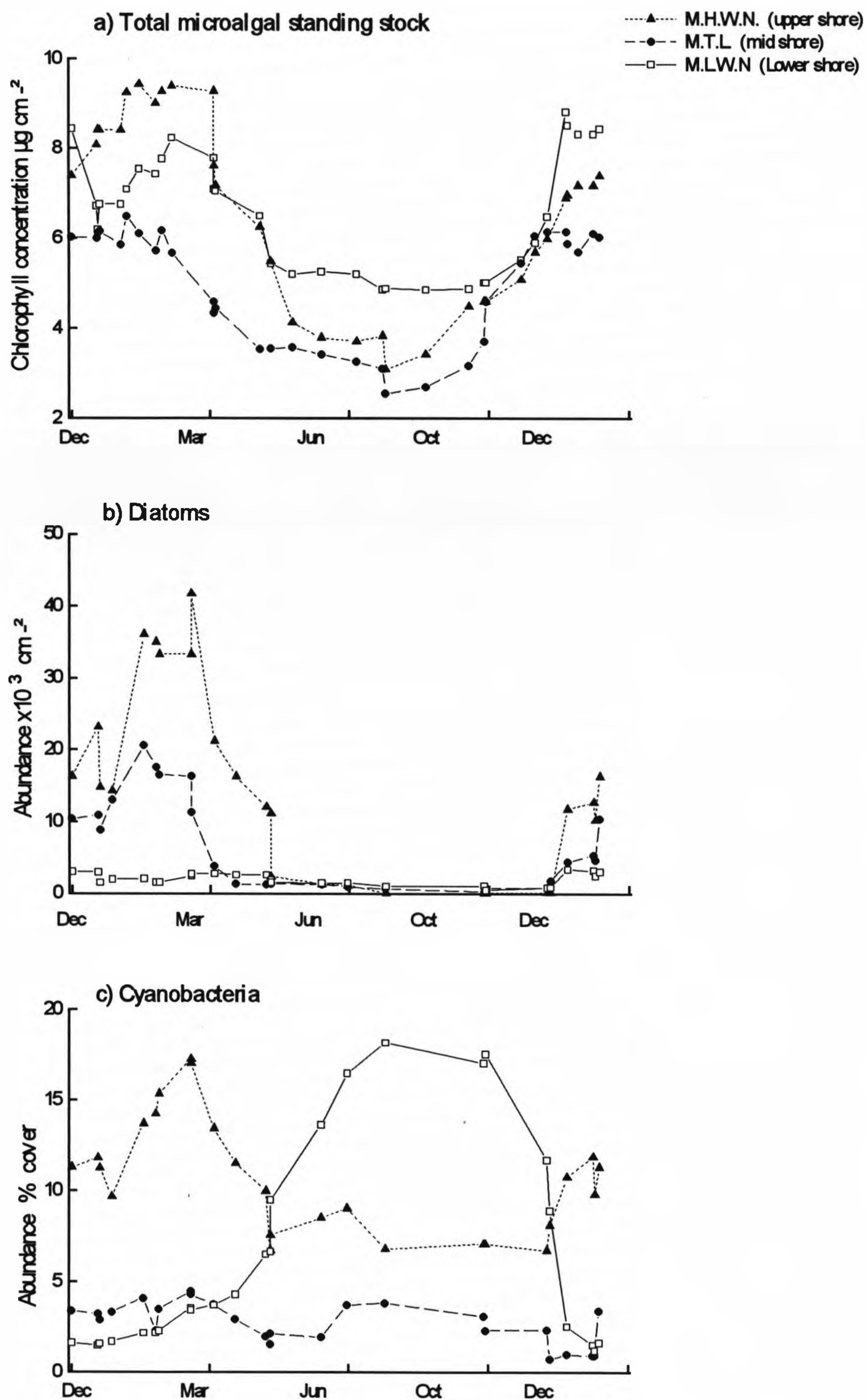


Figure 7) Monthly variations in abundance of (a) total microalgal standing stock (measured using extracted chlorophyll as an index), (b) diatoms and (c) cyanobacteria, at three tidal heights on the shore at Port St Mary Isle of Man. Each value represents a five point running mean of data collected between November 1992 and June 1996 (see text, Page 114). M.H.W.N. = mean high water neaps (~ 5.0m above lowest astronomical tide or L.A.T.), M.T.L. = mid tide level (~ 3.5m above L.A.T.), M.L.W.N. = mean low water neaps (~ 2.0m above L.A.T.).

Water samples collected from the shore at Port St Mary gave similar estimates of dissolved nutrients to those obtained from the Cypris Station (Table 12), and so it was assumed that Cypris data provided a reliable estimate of nutrient supply to the shore. These data and those for air temperature and sunshine hours, from Ronaldsway Meteorological Office, were compared with microalgal abundance data from Port St Mary.

Table 12) Comparison of dissolved nutrients, on various dates, from the Cypris station (5 km off shore from Port Erin) and from inshore at Port St Mary, Isle of Man.

Date	Nitrate Nμg at l^{-1}		Phosphate $PO_4$$\mu g$ at l^{-1}		Silicate $SiO_2$$\mu g$ at l^{-1}	
	Cypris	Port St Mary	Cypris	Port St Mary	Cypris	Port St Mary
Jun. 93	< 0	< 0	0.10	0.57	< 0	2.41
Feb. 94	no data		0.65	0.67	5.03	3.57
Jul. 94	0.58	2.58	0.13	0.53	1.24	2.00
Dec. 94	6.16	5.45	0.56	0.55	3.85	5.47
Jul. 95	0.67	0.43	0.11	0.36	1.09	1.82

Variations in microalgal standing stock, in terms of chlorophyll, were negatively correlated with both air temperature and sunshine hours. These correlations were significant for the mid and upper shore (Table 13). Seasonal variations in sunshine hours and microalgal abundance are presented together for comparison (Figure 4). Standing stock was negatively correlated with the abundance of planktonic algae on the mid and upper shore, whilst standing stock on the lower shore showed the same trend, but was not significantly correlated. On the upper shore microalgal abundance was positively correlated with dissolved silicate. Possibly this reflected the large proportion of diatoms at this level. However, counts of diatoms made with the SEM were positively, but not significantly, correlated with silicate.

The abundance of diatoms was negatively (but not significantly) correlated with air temperature and sunshine hours at all three shore levels (Table 13). Cyanobacteria showed a more confusing relationship and were negatively (but not significantly) correlated with air temperature and sunshine hours on the upper shore but positively and significantly correlated with the same

Table 13) Pearson's Correlation Coefficients for comparisons between seasonal variations in biological and physico-chemical variables and the abundance of epilithic microalgae (calculated per unit chlorophyll), diatoms and cyanobacteria at Port St Mary, Isle of Man. Sample size (*n*) is given after list-wise deletion of missing values. Bonferroni corrected probabilities are shown n.s. = not significant, * = $P<0.05$, *** = $P<0.001$. Correlations between limpet grazing and either diatoms or cyanobacteria (†) were obtained from a subset of the main data (*n*=13) for which simultaneous estimates were available.

Height on shore	*n*	Air temp. (max.)	Sea temp. (mean)	Sunshine (mean hours)	Dissolved phosphate	Dissolved silicate	Dissolved Nitrate	phytoplankton (chlorophyll)	limpet grazing
					r values				
Upper									
Chlorophyll	24	-0.76***	-0.60ns	-0.66*	0.58ns	0.63*	0.42ns	-0.61*	not examined
Diatoms	21	-0.36ns	-0.49ns	-0.17ns	0.23ns	0.47ns	0.58ns	-0.31ns	not examined
Cyanobacteria	21	-0.31ns	-0.23ns	-0.32ns	0.32ns	0.36ns	0.23ns	-0.22ns	not examined
Mid									
Chlorophyll	20	-0.67*	-0.34ns	-0.815***	0.66*	0.60ns	0.25ns	-0.81***	-0.13ns
Diatoms	24	-0.44ns	-0.45ns	-0.49ns	0.40ns	0.41ns	0.27ns	-0.42ns	-0.02ns†
Cyanobacteria	24	0.10ns	-0.16ns	0.07ns	0.11ns	0.22ns	0.18ns	-0.19ns	0.221ns†
Lower									
Chlorophyll	24	-0.50ns	-0.32ns	-0.46ns	0.43ns	0.40ns	0.36ns	-0.47ns	not examined
Diatoms	22	-0.15ns	-0.30ns	-0.10ns	0.10ns	0.24ns	0.39ns	-0.27ns	not examined
Cyanobacteria	22	0.84***	0.66*	0.59ns	-0.43ns	-0.60ns	-0.30ns	0.52ns	not examined

factors on the lower shore (Table 13). This pattern reflected seasonal differences in the abundance of cyanobacteria between tidal levels (Figure 7). Variations in sunshine hours and the abundance of both diatoms and cyanobacteria are presented together for comparison (Figures 5 and 6).

Limpet grazing activity varied seasonally (Figure 8), with reduced grazing during the late winter (Jan. - Feb.; 5-10% of disc area scraped). Grazing intensity was greater during the rest of the year and was characterised by peaks of activity in late spring and late autumn (20-35% of disc area scraped). These differences could not be accounted for by variations in the abundance of limpets (Table 14) and must have been caused by seasonal changes in foraging effort. Variations in foraging intensity were positively correlated with seasonal changes in seawater and air temperatures (Table 14), but were not correlated with changes in microalgal biomass or sea state (estimated in terms of wind strength). Seasonal variations in sea temperature and grazing intensity are presented together for comparison (Figure 8).

Table 14) Pearson's Correlation Coefficients for comparisons between biological or physical variables and seasonal changes in grazing activity recorded on the mid-shore at Port St Mary, Isle of Man. Sample size (*n*) is given after list-wise deletion of missing values. Bonferroni corrected probabilities are shown n.s. = not significant, ** = $P<0.01$, *** = $P<0.001$.

		n	Air temp. (max.)	Sea temp. (mean)	Wind force (monthly mean)	Microalgal abundance (chlorophyll)	Limpet abundance
Correlation with grazing	*r*	21	0.715**	0.753***	-0.12 n.s.	-0.13	-0.16 n.s.

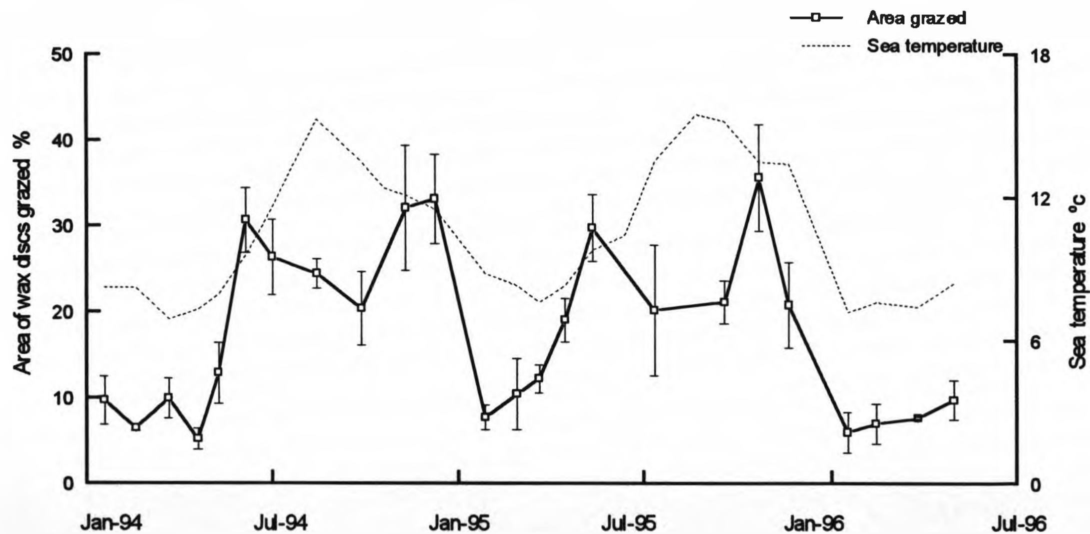


Figure 8) Seasonal changes in limpet grazing activity on the mid shore at Port St Mary, Isle of Man (bars = 1SE). Variations in monthly sea temperature are shown for comparison.

5.5 DISCUSSION

5.5.1 Sampling Methods

Direct counts of microbiota were highly variable and time consuming. Chlorophyll analyses provided a more rapid measure of the total microalgal component of the film. These estimates correlated with direct counts of diatoms but not cyanobacteria, and so provided a reasonably reliable means of determining microalgal abundance. Similar results have been obtained by other workers (Underwood, 1984c; Hill and Hawkins, 1990) and it appears that temporal variations in the chlorophyll content of algal cells (Ryther, 1956; Humphrey, 1961; Oquist, 1974; Foy and Gibson, 1982) were small compared to variations in overall microalgal abundance.

5.5.2 Comparison Between Shores and Between Areas with Differing Macroalgal Cover

Bustamante (1996) found that productivity was greater on wave exposed shores, as a result of locally enriched nutrient supply. In the present study there were differences in microalgal biomass between sites, and between sampling dates, but it was not possible to generalise about the influence of exposure across all shores. Port St Mary (wave exposed site) had greatest standing stock whilst Langness (sheltered site) had the lowest standing stock, the remaining two shores having intermediate microalgal abundance. The cause of this pattern is not clear as differences in exposure to wave action were confounded by variations in limpet density between sites. Similarly, variations in grazing intensity were confounded by differences in wave exposure. Correlations between microalgal abundance and either shore exposure or limpet density were not significant.

Variations in standing stock between sites and between shores were negatively correlated with the abundance of other grazing molluscs (top shells and *Littorina obtusata* (L.)). Top shells are known to feed on microalgae (Hawkins *et al.*, 1989), whilst *Littorina obtusata* usually feeds on the fronds of intertidal seaweeds (Watson and Norton, 1987). However, individuals recorded in the present study were all found on rock surfaces, and it seems likely that they could also feed on microalgae there. These grazers were probably not responsible for variations in microalgal standing stock between sites, but the results do suggest that the abundance of *all* grazers should be considered during future studies. This would be especially important for work on sheltered shores where the area of rock available for grazing is minimal and littorinids and top shells are abundant.

Although it was not possible to generalise about the influence of shore exposure on microalgal standing stock between seasons, significant differences were apparent on some sampling occasions. During the winter, microalgae were less abundant on sheltered shores than on wave exposed

shores. Sheltered shores were covered by a dense macroalgal canopy, consequently during the winter, light may have been more limiting there than on wave exposed shores.

A similar effect of canopy cover was observed on some sampling occasions on moderately wave exposed shores. For example, during July 1993 cyanobacteria were more abundant on areas of open rock outside the *Fucus* canopy. However, diatoms and total microalgal standing stock were more abundant beneath the macroalgal canopy on other shores, and on other occasions. Hill and Hawkins (1991) recorded the abundance of microalgae on the shore at Port St Mary during 1989, but found no difference between areas beneath the macroalgal canopy and adjacent areas of open rock. In conclusion, whilst stresses experienced during low tide emersion (insolation, desiccation and thermal) are important factors regulating microalgal abundance (Aleem, 1950; Castenholz, 1963; Lamontagne *et al.*, 1989) the effects of reduced emersion stresses on microalgae living beneath the macroalgal canopy were not clear from observations on Manx shores.

5.5.3 Vertical Zonation

Microalgal abundance varied between tidal heights at Port St Mary, but it was not possible to generalise about the influence of tidal height across several shores. The study along the vertical transect, at Port St Mary, had greater power to detect differences between tidal heights, than the broader study which compared zonation between shores, since at Port St Mary variation associated with 'sites' and 'exposure' were not considered. However, microalgae were on average less abundant on the mid shore when considered across all sites and all sampling occasions. Variation between shores seems to have been caused by differences in relative abundance on the upper and lower shore, 'between sites'.

Previous research has suggested that microalgal standing stock was affected by both physical stresses experienced during low tide (Castenholz,

1963; Underwood, 1984c), and grazing intensity (Castenholz, 1963; Dye and White, 1991). In the study at Port St Mary, grazers were most abundant on the mid shore and probably caused the reduction in microalgal standing stock, and also reduced abundance of diatoms and cyanobacteria at this shore level.

In contrast to studies by Aleem (1950), Castenholz (1963), Underwood (1984c) and MacLulich (1987), which describe increasing microalgal abundance lower on the shore, during the present study maximum standing stock and abundance of diatoms were recorded on the upper shore. Differences in the vertical distribution of microalgae do not seem to have been caused by variations in grazer density between the various shores studied (e.g. Underwood, 1984c, recorded maximum grazer density on the mid shore, whilst MacLulich, 1987, recorded maximum density on the upper shore), However, relative conditions between the upper and lower shore may vary with latitude and sampling date. For instance, in the present study, a peak in biomass was consistently recorded on the upper shore during late winter, and on the lower shore during the summer. The time of year during which conditions are most favourable for microalgal growth is also likely to vary geographically. Studies of intertidal microalgae have mostly been conducted in temperate regions, and maximal abundance has been recorded during the late winter or early spring, whilst in the Antarctic, for example, subtidal benthic microalgae are most abundant during the summer (Gilbert, 1991). These differences emphasise the importance of season when determining a sampling protocol to record microalgal standing stock.

It seems likely that microalgal abundance varied between tidal levels at the sites examined in this study, but a general pattern of distribution was not evident because of differences between shores and between seasons.

5.5.4 Seasonal Variations in Microalgal Abundance

A considerable proportion of the variation in microalgal standing stock between shores was accounted for by differences between sampling dates. Variations in the abundance of microalgal standing stock, cyanobacteria and diatoms at Port St Mary were characterised by increased standing stock in the early spring and a decline during the summer. Similar results have been obtained in America (Castenholz, 1963), Australia (Underwood, 1984c; MacLulich, 1987), South Africa (Dye and White, 1991), and Northern Europe (Aleem, 1950; Nicotri, 1977; Hill and Hawkins, 1991). The same trends have also been observed for intertidal diatoms and cyanobacteria on soft sediments (Colijn and Dijkema, 1981; Stahl *et al.*, 1985).

In the present study microalgal biomass was strongly correlated with seasonal changes in stresses experienced during low tide exposure to air. The extent of the seasonal variation increased with tidal height and was considerable on the upper shore which is emersed for the longest period of time. For instance, microalgal standing stock on the upper shore decreased from 9.5µg cm^{-2} during the early spring to 3.5µg cm^{-2} during the summer, whilst the abundance of diatoms decreased from over 40,000 cells cm^{-2} in the spring to less than 500 cells cm^{-2} during the summer. Increased seasonal variation in microalgal abundance on the upper shore was also observed by Dye and White (1991), whilst Underwood (1984c) observed the opposite, increased seasonal variation on the lower shore.

The abundance of most microalgal species at Port St Mary was maximal on the upper shore during the late winter and early spring. For the permanent constituents of the film such as diatoms and cyanobacteria, this may reflect a greater recruitment to that level. Macroalgal germlings and ephemeral algae were also more abundant on the upper shore and this contrasted with the distribution of the adult population. The difference could not be entirely explained by spatial variations in grazer density, suggesting that supply of algal propagules may be greater on the upper shore. Similar results have

been recorded for *Ascophyllum nodosum* (L.), with greater numbers of germlings at the upper limits of the *Ascophyllum* zone, but a greater abundance of adult plants on the mid and lower shore (M. Perrins, unpublished observation). Possibly such a distribution could be caused by tidal influences depositing a 'strandline' of germlings on the upper shore.

The abundance of microalgae will be influenced by the relative tolerance of different species to changing physical conditions, to inter-specific competition and to susceptibility to grazing. Intertidal microalgae are sensitive to insolation, thermal and desiccation stress and can become photoinhibited during low tide exposure (Stahl *et al.*, 1985; Lamontagne *et al.*, 1986). Conditions in the intertidal zone on rocky shores can be extremely harsh during daylight low tides with high temperature, insolation and desiccation stresses. However, unlike their counterparts on sedimentary shores, which can migrate downwards into sediments to avoid damage (Patterson, 1993; Blanchard and Gall, 1994; Garcia Pichel *et al.*, 1994; Pinckney *et al.*, 1994b), epilithic algae are fully exposed to emersion stress. This difference between habitats could explain why benthic diatoms in soft sediment have been observed to tolerate high temperatures (Admiraal and Peletier, 1980) and insolation stresses (Taylor, 1964; Colijn and van Buurt, 1975).

Cyanobacteria seemed to be considerably more tolerant to summer conditions than were diatoms, and whilst diatoms were virtually absent from the shore during the summer a reduced cover of cyanobacteria persisted. Cyanobacteria microalgae are renowned for their tolerance to extreme desiccation stress and are able to survive in a senescent form in arid deserts, growing rapidly only after rainfall (Fogg *et al.*, 1973). They are able to dissolve certain rock surfaces and could survive endolithically during adverse conditions (Le Campion Alsumard, 1979). Cyanobacteria could have persisted endolithically in intertidal bedrock at the site studied as they are able to dissolve limestone of which the shore was composed (Le

Campion Alsumard, 1979). If so, their contribution to the total microflora would not have been detected by SEM counts, but would have been incorporated in estimates of total standing stock made using chlorophyll extraction. This could explain why, during the summer, cell counts diminished by a greater proportion than microalgal standing stock (e.g. compare total microalgal biomass with the abundance of diatoms and cyanobacteria during June and September, 1993; Figure 3).

The differences in seasonal abundance of cyanobacteria between shore levels probably reflected a change in the tidal height at which optimum conditions were available for growth on the rock surface. With the onset of summer, favourable conditions were found progressively lower on the shore. Temporal variations in zonation have been observed for ephemeral seaweeds which progressively colonise lower shore levels as emersion stress increases during the summer (Knight and Parke, 1931; Rees, 1935).

Microalgal standing stock was negatively correlated with the abundance of planktonic algae. This was somewhat surprising as both planktonic and benthic microalgae utilise the same resources. Benthic microalgae increased in abundance earlier in the spring (March to April) than did planktonic algae (May to June). Possibly this was caused by beneficial effects of greater light availability and increased temperatures experienced by benthic algae during low tide at that time of year. A similar effect has been observed on mud flats where benthic algae increase in abundance on the upper shore earlier in the spring than on the lower shore, the difference here being attributed to a greater exposure to sunlight during low tide (Admiraal and Peletier, 1980). Hence epilithic algae possibly benefited from aerial exposure during the early spring but were at a disadvantage compared to their planktonic counterparts during the summer when aerial exposure whilst the tide was out became stressful rather than beneficial.

5.5.5 Variations in Limpet Grazing Activity

Seasonal differences in grazing intensity have been considered as possible explanations for seasonal variations in microalgal abundance (Castenholz, 1963; Cubit, 1984; Hill and Hawkins, 1991), but have not previously been quantified in the field. In the present study seasonal patterns were evident from wax disc data with grazing in the winter reduced to 25% of that in the summer. However, these variations alone did not account for changes in microalgal standing stock.

Grazing intensity on the mid shore appeared to be most strongly influenced by seasonal changes in temperature. Similar results were obtained by Boyden and Zeldis (1979) who observed a direct relationship between water temperature and the feeding activity of an individual limpet. Metabolic rate, and feeding activity of marine invertebrates increases with temperature (see Newell and Branch, 1980, for review). Variations in feeding intensity observed in the present study may also have been caused by increased metabolic activity during the warmer months of the year. There was a slight decline in activity during the mid summer. Possibly, this was caused by an increased requirement for a rehydration period after low tide emersion during the summer (Davies, 1969) which would reduced the time available for foraging. Alternatively, since foraging excursions are energetically costly (Davies *et al.*, 1990) the reduced activity may have been a behavioural response to the shortage of food at that time.

5.6 CONCLUSIONS

Microalgal biomass appears to be regulated by a combination of grazing intensity and physical stress experienced during emersion. The relative impact of these stresses varied seasonally between shores and between shore levels. Seasonal variations in standing stock were relatively consistent between shores and between years, and were characterised by reduced abundance during the summer. However, it was not possible to generalise

about the influence of shore exposure, tidal height or canopy cover. Spatial patterns in microalgal abundance were confounded by differences in grazer density both between sites and between shore levels. These difficulties could be overcome by standardising grazer density, for example by caging, or by measuring productivity rather than standing stock. Sampling protocols must also be standardised between seasons and between tidal levels.

CHAPTER SIX

Factors Regulating the Standing Stock of Lower Shore Epilithic Microalgae During the Summer

6.1 ABSTRACT

The abundance of lower shore microalgae varies seasonally, with reduced standing stock during the summer and maximal levels in the winter. However, the causes of these patterns are not known. The influence of various biotic and abiotic factors, on microalgal abundance, was assessed in a multifactorial experiment in which conditions on the shore during the summer were adjusted to resemble those normally experienced during late winter.

Microalgal abundance in terms of chlorophyll increased by around 50% in treatments where grazing was reduced by limpet removal, and by a similar proportion in treatments where insolation was reduced by shading. The abundance of macroalgal germlings and ephemeral algae was also greater in areas where limpet densities were reduced. Cyanobacteria and algal germlings were both significantly more abundant in experimentally shaded areas. Reducing desiccation, by watering with seawater during low tide, had no effect. However, watering with nutrient-enriched seawater increased algal growth under the shade canopies, but not in unshaded areas. Limpets grazed preferentially under shade canopies where microalgal abundance had increased.

The results suggest that microalgal abundance and growth of macroalgal germlings on the lower shore, were regulated by a combination of physical stresses experienced whilst the tide was out and grazing when submerged.

6.2 INTRODUCTION

Physical conditions such as insolation, desiccation, temperature and nutrient availability have a strong influence on both the distribution and abundance of intertidal organisms (Lewis, 1964; Lubchenco and Menge, 1978; Underwood, 1980; Underwood, 1981a; see Underwood and Denley, 1984; Chapman, 1995 for reviews). Excessively elevated or reduced levels of these factors cause stress and damage to shore dwellers (Davies, 1969; Davies, 1970; Wolcott, 1973; Schonbeck and Norton, 1978; Hawkins and Hartnoll, 1985; Davison *et al.*, 1989). An interaction of abiotic and biotic factors, such as nutrient availability and grazing intensity may regulate community structure in the intertidal (Cubit, 1984; Underwood and Denley, 1984 ; Menge, 1992).

Microalgal films, consisting mainly of diatoms, and cyanobacteria, play an important, but little investigated, role in rocky shore ecology (Underwood, 1979; Hawkins and Hartnoll, 1983; Dye and White, 1991). They are an important source of primary production (Bustamante *et al.*, 1995), provide an energy source for grazers (Steneck and Watling, 1982; Underwood, 1984b; Hawkins *et al.*, 1989), and are the site of attachment for invertebrate larvae and algal propagules (see Wahl, 1989; Santelices, 1990; Rodriguez *et al.*, 1993 for reviews). Grazing of these films is important in regulating recruitment and species successions. In particular, macroalgal biomass is largely controlled by grazers which feed on the juvenile stages of these algae (Southward, 1964; Underwood, 1979; Hawkins, 1981; Jernakoff, 1985).

In temperate regions the abundance of intertidal microalgae exhibits clear temporal variation, typically with a winter maximum and a summer minimum (Aleem, 1950; Castenholz, 1963; Nicotri, 1977; Underwood and Jernakoff, 1984; MacLulich, 1987; Dye and White, 1991; Hill and Hawkins, 1991). In the summer conditions in the intertidal are especially harsh during daytime

low tides. However, unlike their counterparts living within soft substrata, microalgae on rocky shores cannot seek refuge amongst sediment grains (Blanchard and Gall, 1994; Garcia Pichel *et al.*, 1994; Pinckney *et al.*, 1994b) and must tolerate the full effects of emersion stresses. Perhaps not surprisingly then the principal causes of reduced microalgal standing crop have been attributed to factors associated with emersion, such as increased desiccation, insolation and thermal stress (Aleem, 1950; Castenholz, 1963; Underwood, 1984c). Other explanations have included increased grazing activity (Castenholz, 1963; Cubit, 1984) or nutrient depletion (MacLulich, 1987). However, the relative importance of these factors have not been established.

For macroalgae, experimental studies have shown that increased desiccation (Johnson *et al.*, 1974; Quadir *et al.*, 1979), insolation and temperature (Quadir *et al.*, 1979) can cause photoinhibition (temporary damage leading to reduced productivity) and bleaching (permanent damage) of photosynthetic organelles and thus reduce survival and growth (Schonbeck and Norton, 1978; Dring and Brown, 1982; Hawkins and Hartnoll, 1985; Norton, 1985; Chapman, 1995). Tolerance to these stresses varies between algae with some species, typically those found on the upper shore, being able to survive prolonged periods of emersion (Schonbeck and Norton, 1978; Norton, 1985; Rugg and Norton, 1987).

Macroalgal abundance is also regulated by grazers which feed on the juvenile stages of these algae within the film. Spatial variations in grazer density provide an opportunity for macroalgal 'escape' (reaching a size which is greater than that normally consumed by the grazers) in areas of lower grazing density (Lubchenco, 1983). In contrast to the substantial amount of work on macroalgae the effect of low tide stresses on microalgae have received little attention

Microalgae and algal germlings have a high surface area to volume ratio and are therefore especially vulnerable to desiccation stress (Brawley and Johnson, 1993). For this reason solitary species such as diatoms or macroalgal propagules may be at a slight disadvantage compared to cyanobacteria which are colonial (Pechar and Masojidek, 1995). Greater microalgal abundance occurs in areas which are naturally shaded (Castenholz, 1963 and R. C. T. pers. obs.) and can be enhanced by experimental shading (Williams, 1994). An excellent study by Lamontagne *et al.* (1989) showed that lower shore microalgae were photo-adapted to low light levels (around 100 - 150 μmol $s^{-1}m^{-2}$), were photosynthetically most active during immersion, became photoinhibited during emersion, and then recovered again several hours after they were resubmerged. Recovery time decreased after subsequent emersions, with the algae apparently acclimatising to the 'sunburn' (Lamontagne *et al.*, 1989).

Experimental studies have shown that increasing nutrient availability enhances the growth of intertidal algae (Bosman *et al.*, 1986). Increased nutrient concentrations enhance the growth of some macroalgae during the summer but not during the winter (Topinka and Robins, 1976). Recently microalgal productivity has also been shown to correlate positively with geographical variations in nutrient availability (Bustamante *et al.*, 1995). In temperate waters the concentration of dissolved nutrients in sea water is generally lower during the summertime than in winter (e.g. Slinn, 1974; Slinn, 1984; see Allen *et al.*, 1994, for summary of over 40 years data at Port Erin) and so reduced nutrient availability could explain the decline in microalgal abundance on Manx shores during the summer.

Molluscs are the principal grazers in the eulittoral zone and their removal or exclusion cause considerable increases in microalgal standing crop (e.g. Southward, 1964; Underwood, 1979; Hawkins, 1981). Grazing and emersion stresses are important factors regulating the vertical zonation of macroalgae (Underwood and Jernakoff, 1984). However, seasonal variations in

microalgal biomass appear not to be related to changes in the abundance of molluscan grazers (Underwood, 1984c). In northern Europe limpets are the principal intertidal grazers (Lewis, 1964). Recent work in the Isle of Man has demonstrated seasonal patterns in grazing by limpets, the principal shore herbivore, which could explain patterns in the abundance of microalgae (Chapter 5).

Clearly, a combination of factors appear to regulate microalgal biomass. Preliminary studies have shown that during the summer microalgae were more abundant on some shores beneath macroalgal canopy than on the surrounding rock (Chapter 5). The lower eulittoral was selected for the present study in order to determine whether microalgal abundance at that level was regulated by a combination of both physical and biological factors.

The study took place at Port St Mary on the Isle of Man where mid shore microalgal abundance exhibits clear seasonal variations (Hill and Hawkins, 1991, Chapter 5). The overall objective was to unravel the factors affecting abundance of microalgae on a temperate rocky shore. This was achieved using a 4-way factorial experiment where summer levels of grazing, insolation, desiccation and nutrient availability were manipulated to levels comparable with conditions experienced during the winter (Table 1). The effect of these treatments on microalgal biomass was measured using extracted chlorophyll (to provide an index of overall microalgal standing stock) and direct counts of microbiota and algal germlings.

6.3 METHODS

6.3.1 Study Site

The study was conducted on a moderately wave exposed rocky shore at Kallow Point, Port St Mary, Isle of Man, (4°44'12W, 54°4'0N) during the summer of 1995. The shore here is comprised of numerous ledges of

carboniferous limestone (Ford, 1993), which have relatively consistent topography and exposure to wave action (for site description see: Southward, 1951; Southward, 1953; Hawkins, 1979). Tides in the Irish Sea are semi-diurnal, usually with one daylight low tide each day (Southward, 1953).

Table 1) Seasonal comparison of physical and biological factors which may influence the standing stock of epilithic microalgae on the shores of the Isle of Man.

Factor	Winter (Jan. - Feb.) average	Summer (Jun. - Jul.) average	Reference
Lower shore microalgal standing stock			
chlorophyll concentration (µg cm^{-2})	7.28	4.85	Chapter 5
Physical factors			
surface irradiance (µmol $s^{-1}m^{-2}$)	130	975	Kain *et al.* 1976
max air temperature (°C)	8.6	17.4	Ronaldsway Meteorological Office
sea temperature (°C)	7.5	12.0	Slinn, 1974; Slinn and Eastham,1984
relative humidity (%)	82.5	72	Ronaldsway Meteorological Office
total organic nitrate (µg at l^{-2} N)	8.4	1.8	Slinn, 1974; Slinn and Eastham,1984
phosphate (µg at l^{-2} P)	0.9	0.3	Slinn, 1974; Slinn and Eastham,1984
silicate (µg at l^{-2} SiO_2)	5.3	1.5	Slinn, 1974; Slinn and Eastham,1984
Biological factors			
limpet grazing (% area scraped $14d^{-1}$)	9.0	25.9	Chapter 5

6.3.2 Experimental Design

Eight areas on the lower shore (~ 2m above L.A.T.), each approximately $100m^2$, were selected on the basis of smooth flat rock surface and similar abundance of the principal space occupying species: limpets (mostly *Patella vulgata* with some *Patella aspera*), barnacles (*Semibalanus balanoides*) and

Fucus serratus. The areas were 10 - 20m apart and each was considered as a separate 'block' in the experimental design. Within each block, 14 circular, 1.3m diameter areas were chosen. These were predominantly open, bare rock from which microalgae could easily be sampled. The areas were not surrounded by or swept by fucoids, and limpet movement both in and out of the areas was not restricted by surface topography. Treatments, consisting of various levels of 'grazing', 'shading' and 'desiccation' (Table 2) were randomly assigned to a 40 x 40cm central portion within each of these circular areas. In order to reduce variation, sampling (see later) was conducted within the central 30 x 30cm portion of these treatment areas (Figure 1).

Table 2) Summary of treatments and experimental design for each block (1-8). See text for explanation of controls.

Factor	Experimental treatments	Controls	Level +	Level -
Block	1 of 8	1 of 8	+	-
Grazing	+ (1-6), - (7-12)	+ (13), - (14)	normal	20% of normal
Shading	+ (1-3), - (4-6), + (7-9), - (10-12)		normal	20% shade
Desiccation / nutrients	a b c a b c a b c a b c	a a	a) normal (not watered) b) watered c) watered with nutrient enriched media	
Treatment	1 2 3 4 5 6 7 8 9 10 11 12	13 14		

'Grazing' was examined at two levels: 'normal' and 'reduced'. The reduced level was created by removing all limpets from within the 1.3m circular areas. Limpets which migrated into these areas during the course of the experiment were also removed. Limpet density in 'normal' areas was not manipulated.

'Shading' consisted of, 'normal' (non-shaded) and 'shaded' (canopy covered) treatments. Shade canopies were made of 40 cm x 40cm sheets of 73% Tildnet black netting, (Tildnet Limited, Bristol, UK) which is manufactured for shading agricultural crops. These were fastened 6cm above the rock surface using rectangular hoops of 6mm galvanised bar which were hammered into holes drilled in the rock (Figure 1).

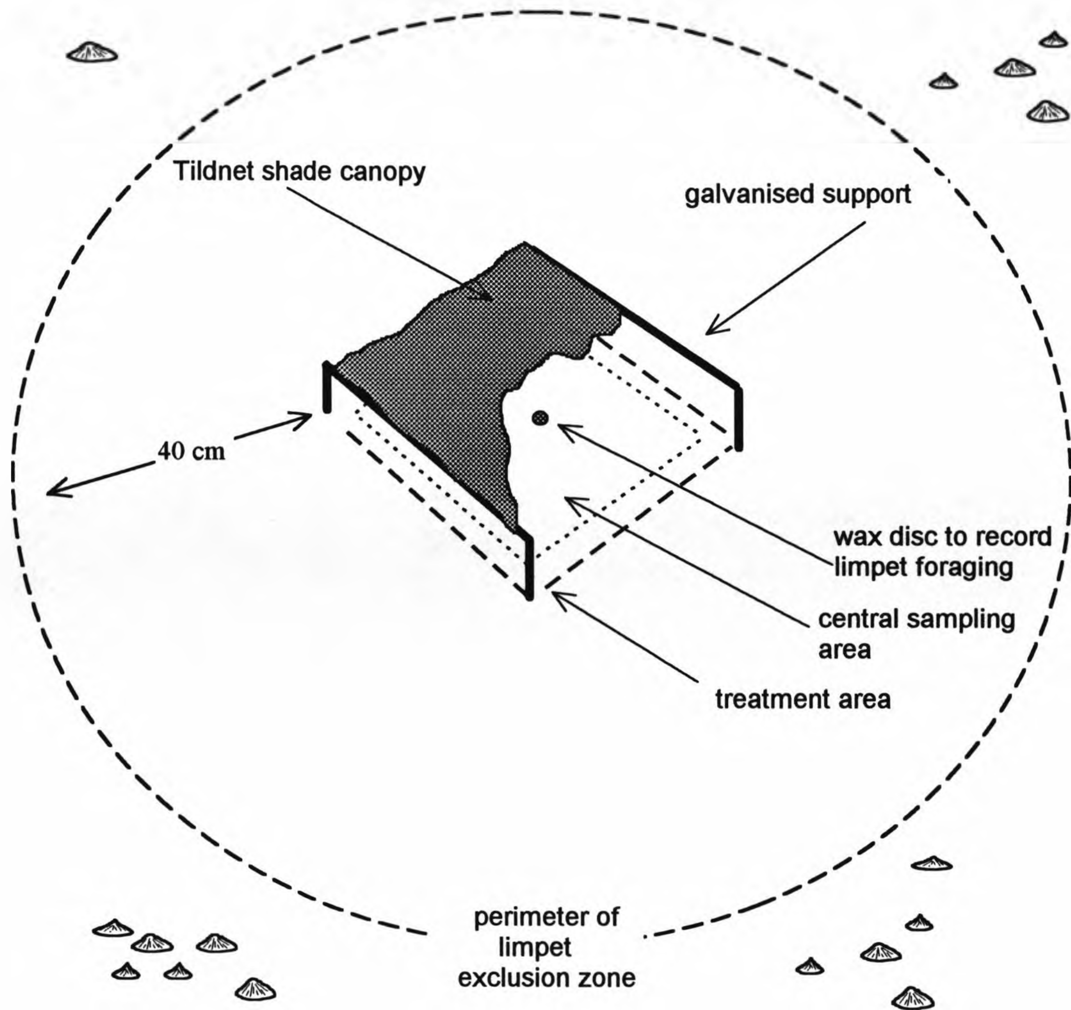


Figure 1) Diagram showing treatment with limpet removal and shading. A cut away section of the shade canopy is shown, revealing a centrally placed wax disc and the boundaries of both the treatment and the sampling areas.

The canopies were inexpensive (approximately £1 sterling each), simple to prepare and were sufficiently robust to survive wave action (maximum during the experiment Beaufort wind force 5 to 6). Similar treatments have been created with opaque and clear perspex (Kain, 1987) or opaque materials with slits cut in them (Williams, 1994). However, opaque panels remove all direct light, whilst panels with slits create highly variable levels of insolation within treatments. These types of shade canopy would not have accurately duplicated winter conditions. Unshaded treatments were marked out with coloured plastic rawlplugs, pushed into holes drilled at the corner of each 40 cm x 40cm area.

The study area was exposed to the air for 2.5 - 3.5 hours (depending on weather conditions and the stage in the spring - neap tidal cycle) during low tide. During daylight low tides 'reduced desiccation' was achieved by sprinkling selected treatment areas with seawater from a watering can until the rock surface was moist. This procedure was repeated twice during tidal exposure, approximately one hour after plots had become uncovered by the tide and then again one hour later. Other treatments were left unwatered (normal desiccation).

Other researchers have reduced desiccation stress by either redirecting streams of seawater drainage or creating small pools (Frank, 1965; Dayton, 1971; Buschmann, 1990). However, such levels of reduced desiccation stress were considered too extreme in relation to natural differences between summer and winter levels which were to be compared.

Ideally nutrient availability should have been considered as a separate factor in the experimental design. However, a convenient method for administering nutrients without also adding water could not devised. Bags of solid nutrients, which would dissolve whilst immersed have been used (Chapman and Craigie, 1977), but this option was rejected since it would not have provided local nutrient enrichment to the treatment areas. Nutrients were added by watering with seawater enriched with Jaworoski's medium (0.01ml l^{-1} stock solution, see Beakes *et al.*, 1988), with added silicate (2.85^{-4} g l^{-1}). Hence the experimental design could not be fully crossed with respect to desiccation and nutrient enrichment. Instead, these treatments were considered as two of three levels within the factor 'desiccation'. (i.e. desiccation reduced, desiccation reduced together with nutrient enrichment and desiccation unaltered, Table 1 a, b & c).

Since limpets were removed rather than excluded or caged, controls were not required for the reduced density treatments. Preliminary studies indicated that support bars did not impede limpet movement around

treatment areas and so a combined control for the shade supports and canopies was used.

Control canopies were problematic since transparent, 'non-shading net' was not available. As an alternative canopies were prepared from sheets of transparent Cryllex (a material similar to Perspex). These were cut to the same size as the shade canopies, perforated with numerous small (6mm) holes to allow air circulation, and fastened on the shore with galvanised supports as before. Controls were prepared in each of the eight experimental blocks at each of the two limpet densities.

The experiment was fully controlled for both grazing and shading which I anticipated would be the major sources of variation in the design. However, because of limitations in the area of shore available and in the amount of time available for processing, it was not possible to duplicate the controls at all three levels of the desiccation factor. Transparent control treatments were not watered as this was the natural condition (treatments 13 and 14, Table 1). This meant that possible interactions between canopy control structures and water or water/nutrients could not be tested. Treatments were applied to all blocks on 25/6/95 and continued for six weeks (until 10/8/95).

During the experiment chlorophyll extracted from rock chippings chiselled from treatment areas was used to provide a reliable index of microalgal biomass (Underwood, 1984b; Hill and Hawkins, 1990) and hence was the dependant variable in the analysis. The amount of chlorophyll per unit area was determined one day before manipulation, then again two weeks and six weeks after manipulation commenced.

Preliminary studies indicated that for chlorophyll analyses at least 20cm^2 of rock surface was required from each sampling area in order to overcome natural variations in microalgal abundance. This was achieved by chiselling nine rock chips from each experimental unit on each occasion. Hence,

sampling was destructive, but the area removed was small compared to the treatment area as a whole (3% on each occasion). Rock chips were rinsed and hydrated in filtered seawater and extracted in 95% hot methanol (see Chapter 3). On the last sampling occasion macroalgal germlings and ephemeral algae were visible in some experimental areas. Areas of rock which were colonised by these algae were not used for chlorophyll analyses.

In addition to samples for chlorophyll analyses five smaller (1 cm^2) samples were collected so that counts of microbiota could be made using a scanning electron microscope (SEM). These samples were air dried and fixed in 2.5% gluteraldehyde in filtered sea water (Hill & Hawkins, 1990). Unfortunately it was only possible to view a small selection from this set of approximately 1700 samples. Rock chips collected at the end of the experiment from areas which were grazed and shaded, and grazed and unshaded (treatments 1 and 4, Table 2) were examined using SEM. This selection was made in order to confirm that, in the absence of any other manipulation, differences between shaded and unshaded areas were caused by an increase in microalgal abundance and not a change in cell pigmentation as a response to the differing light regimes imposed (Oquist, 1974; Foy and Gibson, 1982). Samples were viewed at 480x magnification using a Philips XL 30 scanning electron microscope (SEM). Six randomly located photographs were taken on the surface of each sample, these were later examined at six times magnification to provide reasonable resolution for identification with a large field of view (Patterson *et al.*, 1986). Diatoms were recorded as numbers of cells present whilst percentage cover was used to quantify the abundance of cyanobacteria. Data from the six photographs on each sample were then averaged to provide estimates of abundance.

6.3.3 Assessing the Experimental Treatments

Limpet grazing was quantified using a wax disc positioned in the centre of each treatment area (Figure 1) to record marks of radula scraping (see Chapter 4). The discs were replaced every two weeks, giving three separate

measures of activity during the experiment. Foraging activity was recorded by scoring the percentage area of each disc scraped. Irradiance, temperature and relative evaporation rates were recorded underneath shade canopies, under transparent controls and on the open rock. Irradiance was measured using a Li-cor, LI-185B photometer. Temperature was measured using a mercury thermometer held just above the rock surface. Evaporation was estimated by placing petri dishes filled with moist tissue on the rock surface and determining their weight loss after three hours (a similar method to that of Williams, 1994). The nutrient composition of seawater and of nutrient-enriched seawater was determined using an Alpkem® RFA/2 rapid flow analyser.

At the end of the experiment the abundance of algal germlings and ephemeral algae within the treatment areas was recorded by direct observation and all structures were removed from the shore. The number of germlings present was recorded by averaging counts from ten randomly placed 5 x 5 cm quadrats. Ephemeral algae were most apparent on areas of newly exposed virgin rock created in previous weeks by chiselling during sample removal. Colonisation was recorded by counting the number of these areas which were 'greener' in appearance (visible colonisation) than the surrounding rock. This value is presented as a percentage of the total number of chisel marks present.

6.3.4 Data Analysis

Data were analysed with 4-way ANOVA using Generalised Linear Models, with the 'PROC GLM' command in SAS® v6.03 (SAS, 1988). 'Block', 'grazing', 'shading' and 'desiccation' were all considered as fixed factors (Winer *et al.*, 1971). Comparisons between shade canopies, transparent controls and open rock were tested separately using a 3-way mixed model ANOVA, again 'Block', 'grazing', 'shading' and 'desiccation' were considered as fixed factors. This approach was modified slightly for analyses of germlings, ephemeral algae and counts of microbiota (see results).

Cochran's test for homogeneity of variance was performed to check for homoscedasticity (Winer *et al.*, 1971; Underwood, 1981b) and data were transformed where desirable. Plots of residuals were examined after each ANOVA to check that error terms were normally distributed. Comparisons between levels within treatment factors were selected *a priori* and determined using Bonferroni Comparisons (Maxwell and Delaney, 1990).

6.4 RESULTS

6.4.1 Effectiveness of Experimental Treatments

Removal of limpets reduced grazing to just less than 25% of that in unmanipulated areas (overall means of percentage area of wax disc surface scraped ± 1SE; grazer reduction = 7.3 ± 1.0, normal grazer density 32.2 ± 4.3). Removal was an effective way of manipulating limpet grazing intensity. However, unlike studies where grazers have been caged at various densities (e.g. Castenholz, 1963; Nicotri, 1977), manipulating limpet abundance by removal meant that grazing could not be completely standardised between similar treatments. This problem contributed to variation for all treatments.

Shade canopies provided consistent, diffuse shading and reduced irradiance to around 20% of that in open areas (Table 3). These reductions were similar to levels of insolation and grazing during the winter (compare Table 1 and Table 3). Unfortunately, the reduction in insolation was accompanied by reduced evaporation and lower temperature. Transparent Cryllex canopies caused negligible difference in irradiance, temperature and evaporation compared to open areas and therefore provided reliable shade controls (Table 3).

Table 3) Comparison of physical conditions between open, control and shaded treatment areas, made on warm sunny days during August 1995 (mean ± 1SE). Evaporation of water from small containers was used to provide an indication of desiccation stress (see text). The percentage difference from the open areas is shown (in parentheses)

Treatment	Irradiance (Eμ $s^{-1}m^{-2}$)		Evaporation loss (g)		Temp (°C)	
open - no cover	1500 ± 40		5.05 ± 0.14		29 ± 0.5	
transparent control cover	1400 ± 40	(-6 %)	5.30 ± 0.72	(+5%)	29 ± 0.5	(0%)
net shade cover	290 ± 14	(- 81%)	3.13 ± 0.23	(- 36%)	23 ± 0.5	(- 28%)

A note of caution must be made here, as air temperature was recorded just above the rock surface. A more realistic approach would have been to use a thermocouple to measure the temperature of the rock surface (e.g. Williams, 1994). The technique used was probably reasonably reliable for shaded areas, but may have underestimated the temperature experienced by microalgae on the rock surface in exposed areas.

Watering was a moderately effective method for reducing desiccation stress. Administering fixed quantities (2 waterings each low tide) was satisfactory on overcast days, but was insufficient on hot sunny days, with treatment areas drying and microalgae possibly becoming dehydrated within approximately 25 minutes after watering.

Nutrient-enriched seawater had elevated concentrations of total oxidised nitrogen, phosphate and silicate compared to those in natural seawater. Some concentrations were greater than those experienced during the winter (compare Tables 1 and 4). However, nutrients were only available to the plants whilst the rock surface remained hydrated (~ 25 minutes after watering). Consequently the effective nutrient concentration available to the plants may have been less than that in the winter.

Microalgal abundance (in terms of chlorophyll) on the lower shore as a whole declined naturally by around 35 percent during the course of the experiment (compare treatments 1 and 7 between sampling occasions, Figure 2a, b, c), whilst grazing increased by around 50 percent. Statistical comparisons between sampling dates were not made as sampling was not independent with time.

Table 4) Nutrient content of seawater collected from Port St Mary at Low tide on 30/7/95 and seawater enriched with Jaworski's medium and silicate (mean ± 1SE).

Treatment	Total oxidised nitrogen (μg at l^{-2} N)	Phosphate (μg at l^{-2} P)	Silicate (μg at l^{-2} SiO_2)
Seawater (Port St Mary)	0.32 ± 0.08	0.36 ± 0.01	1.82 ± 0.02
Nutrient enriched seawater	14.20 ± 0.07	4.5 ± 0.08	4.98 ± 0.05

6.4.2 Controls

There were no differences in either chlorophyll concentration or grazing, between open areas and areas beneath transparent control canopies, on any of the three sampling occasions ($F_{1,21}$ $P < 0.31$, 3 way ANOVA). At the end of the experiment, *Fucus* germlings and ephemeral algae were slightly more abundant in open areas than beneath transparent control canopies, but this effect was not significant ($F_{1,7}$ $P < 0.09$, 2 way ANOVA).

6.4.3 Microalgal Biomass

There were significant differences in chlorophyll concentration between experimental blocks, on all three sampling occasions (Table 5). These differences occurred in similar proportions on each occasion but were small compared to the variation between treatment effects and presumably reflected natural spatial variability on the shore.

Microalgal abundance was similar between all treatments at the start of the experiment (Figure 2a). Slight differences between treatments were apparent two weeks after manipulation (Figure 2b), but considerable differences were evident six weeks after manipulation (Figure 2c).

After two weeks of manipulation areas where grazer density had been reduced had around 20 percent more chlorophyll than areas with normal grazer density. This increased to around 50 percent more chlorophyll by the end of the experiment. These differences were highly significant (Figure 2 and Table 5).

Areas with increased levels of shading had significantly higher chlorophyll concentrations than unshaded areas after both two and six weeks of manipulation. The extent of these differences was similar (+20% after two weeks and + 50% after six weeks) to those resulting from reduced grazing (Figure 2 and Table 5). Interactions between grazing and shading were not significant.

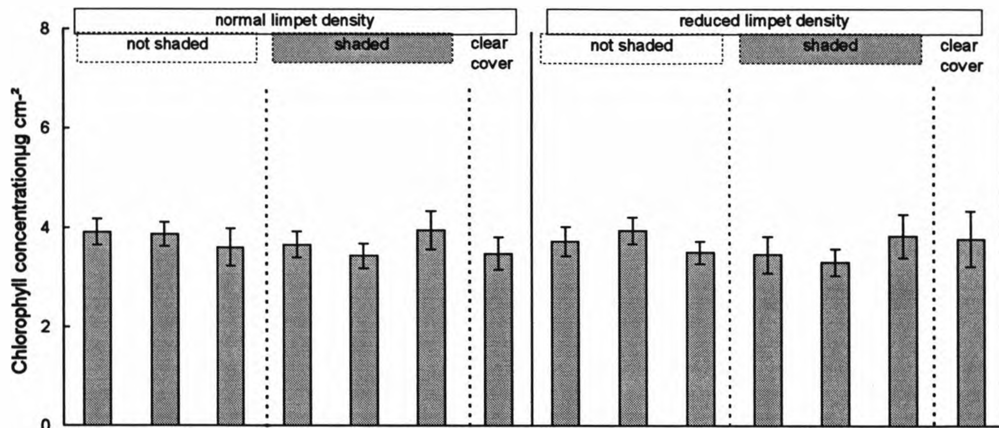
Addition of seawater or nutrient-enriched seawater did not cause any differences in chlorophyll concentrations as a 'main effect' in the ANOVA. There was, however, a significant interaction effect between desiccation and shading treatments at the end of the experiment ($P < 0.01$, Table 5). This occurred as a result of elevated chlorophyll concentrations in treatments receiving both shading and nutrients (Bonferroni Least Significant Difference calculation $F_{(3,77)} = 0.103$, $P < 0.05$). This indicated that nutrients, which are depleted in the Irish Sea during the summer (Table 1), might become limiting if conditions for algal growth were ameliorated, in this case by shading.

Table 5) Analyses of variance for treatment effects on microalgal biomass (measured as extracted chlorophyll) at the time of manipulation and on two occasions thereafter (data were log x+1 transformed). 'Block', 'shade' and 'watering' were treated as fixed factors. n.s. = not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

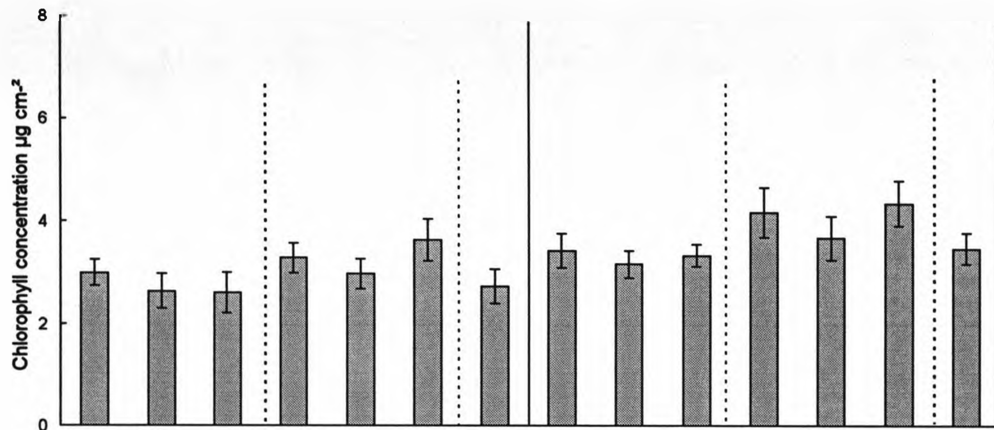
Source of variation	df	Microalgal Biomass (chlorophyll μg cm^{-2}) At manipulation			2 weeks after manipulation			6 weeks after manipulation		
		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Block	7	0.0365	8.82	***	0.0624	12.92	***	0.0875	6.11	***
Grazing	1	0.0028	0.68	n.s.	0.1100	22.77	***	0.5207	36.37	***
Shade	1	0.0067	1.61	n.s.	0.0969	20.05	***	0.4662	32.56	***
Watering	2	0.0002	0.06	n.s.	0.0137	2.83	n.s.	0.0084	0.59	n.s.
Grazing x Shade	1	0.0006	0.14	n.s.	0.0001	0.01	n.s.	0.0158	1.10	n.s.
Grazing x Watering	2	0.0006	0.15	n.s.	0.0005	0.11	n.s.	0.0010	0.07	n.s.
Shade x Watering	2	0.0132	3.19	*	0.0072	1.50	n.s.	0.0768	5.37	**
Grazing x Shade x Watering	2	0.0001	0.02	n.s.	0.0019	0.39	n.s.	0.0095	0.66	n.s.
Error	77	0.0041			0.0048			0.0143		
Cochran's test: C_{crit}, P0.05 = 0.18; C_{crit}, P0.01 = 0.21		*C* = 0.15			*C* = 0.12			*C* = 0.14		

An interaction between plots selected for watering and shading treatments was apparent at a low level of significance before the manipulation (Table 5). This may have simply been caused by natural random variations in grazing intensity between areas prior to manipulation. Comparison of means for these treatments showed that areas which were to receive shading and watering had greater microalgal biomass than those which were to be shaded and left unwatered. However, the Bonferroni Least Significant Difference test was not sufficiently sensitive to confirm this ($F_{(21,77)}$ =0.072, n.s.). Whatever the cause of the 'pre-manipulation interaction effect' it appeared to be unrelated to that of the interaction recorded at the end of the experiment.

a) At manipulation 22/06/95



b) Two weeks after manipulation 09/07/95



c)Six weeks after manipulation 08/08/95

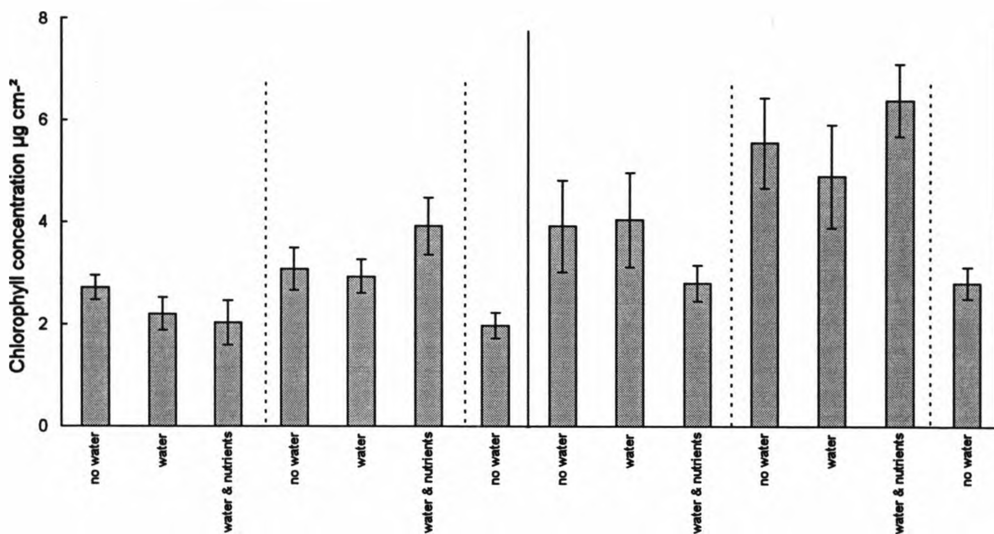


Figure 2) Estimates of chlorophyll concentration from each of 14 treatments (n= 8 for each treatment), obtained at manipulation (a), Two weeks after manipulation (b) and six weeks after manipulations were made (c). Bars show 1SE. Clear covers were control treatments which provided a similar physical structure to the shade canopies but negligible shade.

6.4.4 Abundance of *Fucus* Germlings and Ephemeral Algae

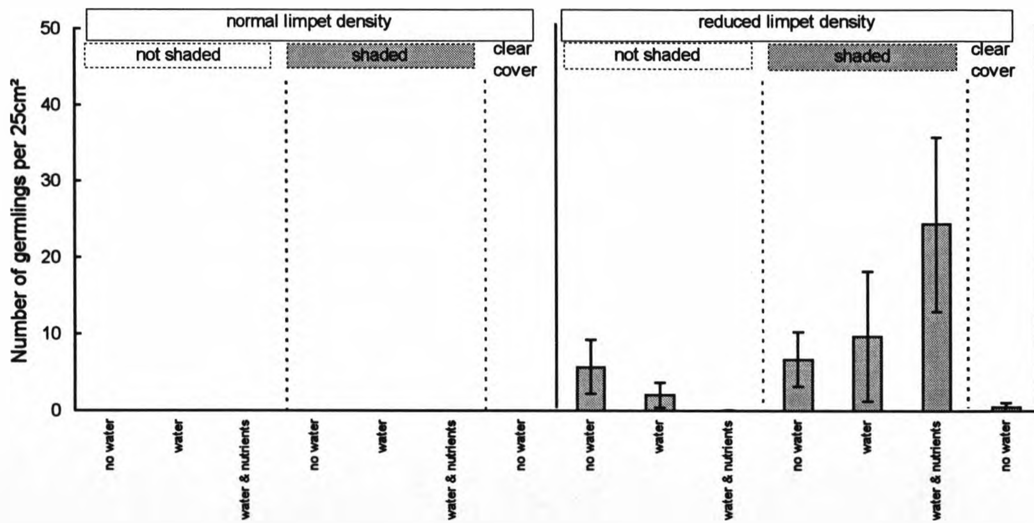
Macroalgal germlings were visible in some treatment areas after six weeks of manipulation. There were clear differences in the abundance of germlings between the two levels of grazing intensity (Figure 3), but statistical comparisons were not made because of heteroscedacity. Differences between treatments at reduced limpet density (i.e. treatments 8 to 13, Table 2) were compared using a 3-way ANOVA ('Block', 'Shade', 'Desiccation'). This analysis showed that, in areas where grazing was reduced, germlings were significantly more abundant beneath shade canopies than in exposed areas (Table 6).

Colonisation by ephemeral algae (mostly *Enteromorpha clathrata* and *E. flexuosa*) was also observed in some treatment areas after 5-6 weeks. Again there were clear differences between the two levels of grazing intensity (Figure 4), but statistical comparisons were not made because of heteroscedacity. Differences between treatments at reduced limpet density were compared, as described for germlings, but were not significant (Table 6).

Table 6) Analyses of variance for treatment effects in areas of reduced grazing (see text) on the abundance of *Fucus* germlings (data were square root transformed) and colonisation by ephemeral algae (data were log x+1 transformed) six weeks after manipulation. 'Block', 'shade' and 'watering' were treated as fixed factors. n.s. = not significant, * = $P<0.05$.

Source of variation	Abundance 6 weeks after manipulation							
	Fucus germlings				Colonisation by ephemeral algae			
	df	MS	*F*	*P*	df	MS	*F*	*P*
Block	7	7.24	1.49	n.s.	7	0.40	0.99	n.s.
Shade	1	30.51	6.26	*	1	0.56	1.37	n.s.
Watering	2	1.73	0.35	n.s.	2	0.12	0.29	n.s.
Shade x Watering	2	11.53	2.36	n.s.	2	0.49	1.20	n.s.
Error	35	4.88			35	0.41		
Cochran's test: C_{crit}, P0.05 = 0.39		0.34				0.26		

a) Fucus germlings



b) Ephemeral algae

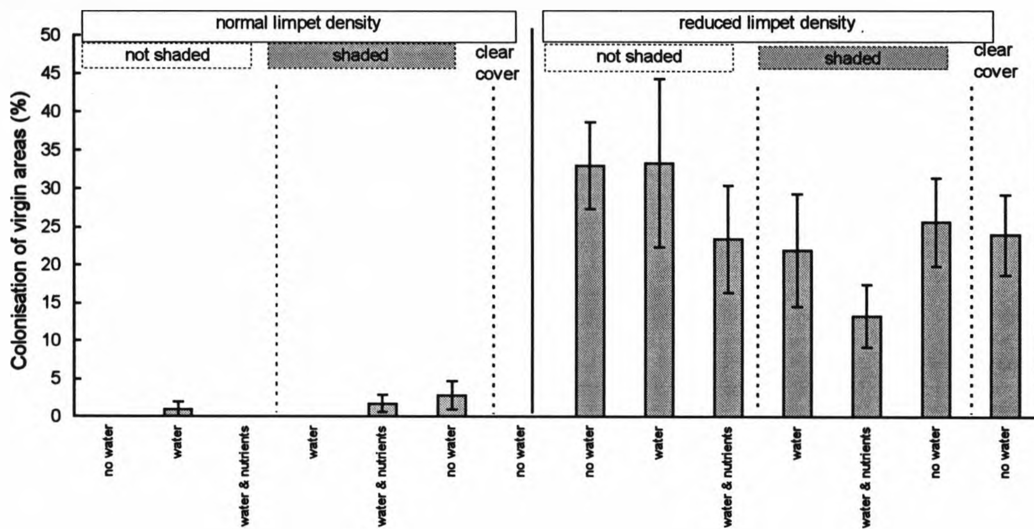


Figure 3a) Abundance of *Fucus* germlings b) Colonisation of virgin rock by ephemeral algae, within each of 14 treatment areas, six weeks after manipulation. Assessment of ephemeral algae was qualitative and based on the percentage of virgin areas which were greener than the surrounding rock. In both figures clear covers were control treatments which provided a similar physical structure to shade canopies but negligible shade. Bars show 1SE. (n= 8 for each treatment).

6.4.5 Abundance of Microbiota

In treatments with normal grazing intensity cyanobacteria and diatoms were more abundant beneath shade canopies than in open areas (means ± 1SE; diatoms unshaded 6.8 ± 3.84, shaded 33.9 ± 24.7; cyanobacteria unshaded 23.4 ± 4.9, shaded 28.9 ± 4.9). This effect was significant for cyanobacteria (Table 7) but data for diatoms were not normally distributed and could not be tested using ANOVA. Average counts of diatom abundance from each from each treatment (n = 8) were not significantly different when compared using nonparametric analyses (Mann-Whitney two-sample rank sum test P = 0.09).

Table 7) Analyses of variance for shaded and unshaded treatments in areas of reduced grazing on abundance of cyanobacteria and diatoms six weeks after manipulation. 'Block' and 'shade' were treated as fixed factors. n.s.: not significant, * = P<0.05, *** = P<0.001.

source of variation	abundance of cyanobacteria percentage cover			
	df	MS	F	P
Block	7	429.15	6.15	***
Shade	1	309.13	4.43	*
Block x Shade	7	161.55	2.31	*
Error	37	69.80		
Cochran's test:		0.16		
C_{crit}, P0.05 = 0.19				

6.4.6 Grazing Activity

Estimates of grazing intensity were variable indicating that more wax discs should have been used in each sampling area. Discs from both the first and the third two-week period had strong heteroscedacity and would not conform to Cochran's test for differences less than P = 0.01. This restriction meant that two interactions which occurred during the final two week period and were significant at P = 0.05 (grazing x shade, P = 0.02 and grazing x shade x

watering, P = 0.03) were regarded with caution and further testing was not conducted.

The average area of each disc grazed during all three two-week periods was around 30 percent in areas with normal limpet density and around 10 percent where limpets had been removed. This difference was significant, on all three sampling occasions. During the final two-week period, grazing was significantly greater in shaded areas than in non-shaded areas (Table 8).

Table 8) Analyses of variance for treatment effects on limpet feeding activity (measured as area of wax discs scraped during feeding - see text) at the time of manipulation and on two occasions thereafter (data were arcsine transformed). 'Block', 'shade' and 'watering' were treated as fixed factors. n.s. = not significant, ** = P<0.01, *** = P<0.001.

Source of variation	Limpet feeding (percentage area of wax discs scraped)											
	1st & 2nd weeks after manipulation				3rd & 4th weeks after manipulation				5th & 6th weeks after manipulation			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
Block	7	196.4	1.51	n.s.	7	262.0	1.07	n.s.	7	177.4	0.79	n.s.
Grazing (G)	1	8247.4	63.49	***	1	16858.1	68.74	***	1	15175.7	67.35	***
Shade (S)	1	128.7	0.99	n.s.	1	906.5	3.70	n.s.	1	9084.3	40.31	***
Watering (W)	2	71.2	0.55	n.s.	2	18.3	0.07	n.s.	2	384.9	1.71	n.s.
G x S	1	52.9	0.41	n.s.	1	64.0	0.26	n.s.	1	1234.1	5.48	n.s.
G x W	2	11.0	0.09	n.s.	2	149.3	0.61	n.s.	2	4.6	0.02	n.s.
S x W	2	343.8	2.65	n.s.	2	183.0	0.45	n.s.	2	22.5	0.1	n.s.
G x S x W	2	89.0	0.68	n.s.	2	552.8	0.75	n.s.	2	797.3	3.54	n.s.
Error	72	129.9			76	245.2	2.25	n.s.	69	225.3		
Cochran's test:			0.208				0.153				0.19	
C_{crit}, P0.05 = 0.18												
C_{crit}, P0.01 = 0.21												

6.5 DISCUSSION

6.5.1 Analyses of Treatment Effects

Grazing was a major factor regulating microalgal abundance, with areas of reduced grazing having greater microalgal standing stock (in terms of chlorophyll) than areas of normal grazer density. Insolation (irradiance and thermal stress) accounted for a similar proportion of the variation, and at the end of the experiment there was 50 percent more microalgal standing stock and a greater percentage cover of cyanobacteria in shaded areas than in unshaded areas. Stresses during emersion are considerable during the summer and are frequently considered to be responsible for reduced microalgal abundance at that time of year (Aleem, 1950; Castenholz, 1963; Underwood, 1984c). In this study insolation stresses and grazing intensity were both equally important factors regulating microalgal biomass.

Some stress associated with insolation was clearly reducing growth in unshaded areas. Shade canopies considerably reduced not only the ambient light, but also evaporation (desiccation stress) and temperature. Since there was no apparent increase in algal abundance on plots that were watered with seawater during low tide, it appears unlikely that reduced desiccation beneath the shade canopies was the cause of the algal enhancement there.

Achnanthes was the principal genus of diatoms present on the shore at Port St Mary (Chapter 5), and species from this genus are considered to be especially tolerant of extreme environmental conditions (Lamontagne *et al.*, 1989). Cyanobacteria have mechanisms to counteract photoinhibition (e.g. Clarke *et al.*, 1993; Herbert *et al.*, 1995; Oquist *et al.*, 1995). They are renowned for their tolerance of extreme environments and have a better survival rate than green algae (see Fogg *et al.*, for review, 1973; Bewley, 1979). Despite the hardiness of these algae, climatic conditions during the study were clearly sufficiently severe to reduce standing stock compared to shaded areas.

Lamontagne (1989) showed that lower shore microalgae were photo-adapted to low light levels and were photosynthetically most active whilst immersed, but can tolerate low levels of insolation whilst emmersed by photoinhibition. However, high levels of irradiance, in particular exposure to ultra violet 'B' radiation, cause permanent damage. Photoinhibition reduces net productivity and growth, as some of the remaining photosynthetic production is allocated to repair (e.g. Lesser *et al.*, 1994). In this respect, physical and biological stresses directly influence rates of net primary production rather than standing stock and hence primary production would have been a better dependant variable than standing stock (Cubit, 1984; Bustamante *et al.,* 1995). Appropriate techniques for measuring microalgal primary production in manipulative field experiments have yet to be developed.

Extreme temperatures may also reduce net productivity. However, the optimum temperature for photosynthesis by intertidal cyanobacteria is quite high (35°C, Stewart, 1965; Stewart, 1967). Consequently some of the damage caused by higher levels of irradiance may be offset by increased photosynthesis as a result of the simultaneous increase in temperature. It was not possible to distinguish between effects caused by temperature and light intensity in the present work. Laboratory studies would be required of microalgal productivity to determine the relative importance these factors (e.g. Matta and Chapman, 1995). However, for cyanobacteria it seems unlikely that thermal stress is a major factor on temperate shores (Fogg *et al.*, 1973).

Grazing and insolation were also important factors regulating the growth of *Fucus* germlings, with enhanced growth in areas that were shaded and which also had reduced limpet density. Reduced insolation could have been responsible for the enhanced growth of algal germlings since the early stages of brown algae are sensitive to high irradiance (e.g. *Macrocystis pyrifera* sporophytes above 143 µmol s-1m-^{2}, Fain and Murray, 1982;

Sargassum muticum germlings above 88 µmol $s^{-1}m^{-2}$, Hales and Fletcher, 1989). Subsequent examination of the shaded treatment areas showed that the germlings were *Fucus vesiculosus* which had grown below the tide level where they are normally most abundant. Presumably germlings also began to develop in shaded areas of normal limpet density but were removed by grazing.

Colonisation and growth by ephemeral algae were only evident in areas where limpet densities had been reduced and were most apparent on newly exposed rock created where samples had been removed. Why newly exposed surfaces were more attractive than the surrounding rock is not clear, although cultures of diatoms and cyanobacteria have been shown to inhibit settlement and growth of seaweeds (Keating, 1978; Huang and Boney, 1984; Huang and Boney, 1985a). Possibly, the existing microalgal community on the shore, which contained both cyanobacteria and diatoms (Chapter 5), had a similar inhibitory effect on colonisation by opportunistic ephemerals.

Differences in the microalgal community between shaded and open areas were also detected by limpets which selectively grazed the shaded areas when the tide was in. During the last two weeks of the experiment grazing in shaded areas was roughly double that in open areas. Limpets are known to revisit adjacent areas on consecutive days, presumably in response to a plentiful food supply in the selected locality (Chelazzi *et al.*, 1994a). Clearly, this behavioural response confounded the results since differential grazing reduced variations in microalgal abundance between treatments. There was no evidence of limpets moving their home scar site and relocating beneath canopies in order to benefit from reduced stresses during low tide.

Studies of intertidal zonation and community structure have frequently focused on the effects of either physical or biological factors, and until recently interactions between these factors have not been examined in detail

(see Menge, 1992). Buschman (1990) reduced desiccation, and grazing in a factorial study and showed that community structure and succession changed when grazing and desiccation stresses were reduced simultaneously, but not when they were reduced in isolation.

Cubit (1984) manipulated grazers in the high intertidal and demonstrated that seasonal changes in algal abundance were caused by variations in algal productivity rather than by rates of algal loss to herbivory or physical stress. However, increased foraging activity during the summer time coincided with reduced algal abundance and Cubit concluded that a shortage of food during the summer regulated the abundance of grazers on the shore. Consequently, when conditions for algal growth became more favourable during the winter, the rate of algal removal by grazing did not increase as grazer density had been determined by conditions during the previous summers. In the present study grazing intensity and insolation had an additive effect on microalgal biomass and an interactive effect on the abundance of ephemeral algae and *Fucus* germlings.

Interactions between physical and biological factors may have considerable implications for species diversity and community structure. For example, on Manx shores grazing pressure appears to be regulated by temperature and is greatest during the summer when microalgae are less abundant (Chapter 5). If the abundance of grazers is, at least in part, restricted by a shortage of food during the summer, then a temporal refuge for juvenile macroalgae to 'escape' grazing will follow during the cooler seasons. In the present study *Fucus* germlings grew when physical conditions were moderated, but were only able to 'escape' grazing in areas where limpet foraging effort was reduced to that in wintertime.

Typically, researchers have considered that spatial variability in grazing activity created 'windows' for both microalgal growth and macroalgal 'escapes'. The present study indicates that seasonal variation in grazing

activity may also create a temporal refuge for macroalgal 'escapes', which occur at times when conditions for growth are more favourable than those experienced during the summer.

The intimate association of physical and biological factors on this shore is further emphasised by the responses in nutrient enrichment treatments. Here, standing stock was enhanced by increased levels of nutrients only where less stressful conditions were supplied by shading, and where the removal of algae by grazers was also reduced.

6.5.2 Experimental Design

The design used did not include replication of sites, and it is not possible to make general conclusions about other shores (see Hairston, 1989). Increasing the design to encompass two sites could have helped overcome this deficiency, but would have necessitated doubling the already considerable research effort (for the existing design two people every day for two months), halving the number of replicates at each site, or omitting a factor from the design. From an ecological perspective I considered that a complete multifactorial study at one site would be more informative than a simpler, less generalised experiment conducted at two or more sites.

In addition to problems with spatial replication, experiments involving manipulation of climatic conditions should be repeated over several years in order to account for temporal variations in weather conditions. This is now accepted practice for work on yields from agricultural crops (M. Mortimer, Pers. Comm.), but unfortunately was not feasible here.

Average sunlight hours during the study period were greater than normal for the time of year. (monthly averages for June and July: 1995 = 237h., ten yearly average ± 1SE = 191 ± 10h.). Although the relative impact of low tide stresses and grazing may vary from year to year, the seasonal patterns of algal abundance recorded were similar to those in previous years (Chapter,

5; Hill and Hawkins, 1991), suggesting that the results were not simply caused by unusually sunny weather.

In summary, during the summer a combination of adverse physical conditions, especially insolation, and limpet grazing reduce microalgal abundance on the shore. At other times of the year reduced grazing intensity, reduced physical stresses and to a lesser extent increased nutrient availability may provide more favourable conditions for microalgal growth and macroalgal escape.

CHAPTER SEVEN

Influence of Epilithic Microbial Films on the Settlement of *Semibalanus balanoides* Cyprids

7.1 ABSTRACT

Larvae of marine invertebrates respond to settlement-inducing cues at a range of spatial scales. The cypris larvae of barnacles have been studied extensively and show considerable exploratory and selection behaviour prior to settlement. The presence of a microalgal film on surfaces enhances settlement. Additionally, cyprids may be able to distinguish optimal conditions for post-settlement survival on the basis of cues provided by these films. Previous work has mostly been laboratory-based or has used artificial settlement surfaces deployed in the field. These techniques may not accurately represent conditions in the natural environment where cyprids encounter a more diverse array of settlement cues.

In the present study the influence of naturally occurring epilithic fouling communities on the settlement of *Semibalanus balanoides* cyprids (Crustacea: Cirripedia) was examined using a combination of laboratory and field based investigations. In choice chambers, cyprids were able to differentiate between rock surfaces originating from different levels on the shore and preferentially selected rocks from the mid shore which is their usual zone. In the absence of any conspecifics or visible traces of previous barnacle colonisation, cyprids selected rock surfaces with a mature microbial film in preference to either an unfilmed surface or one with a developing film. Settlement in the field was predominantly influenced by the proximity of conspecifics and by traces of previous barnacle colonisation. These factors significantly increased settlement, and seemed to over-rule cues from the film. Some difficulties in the application of laboratory based studies to settlement in the natural environment are discussed.

7.2 INTRODUCTION

Biofilms are of considerable ecological importance to marine communities. They are a major source of primary production in shallow waters (Fielding *et al.*, 1988; Bustamante *et al.*, 1995) and provide food resource for grazers such as molluscs and fishes (Underwood, 1984a; Hawkins *et al.*, 1989; Edgar and Shaw, 1995). In addition, these films form an interface between a substratum and the water column, and in this respect have an important influence on settlement and subsequent succession by benthic invertebrates and macroalgae (Wahl, 1989).

Most benthic organisms have a dispersive stage in their life cycle which ends when the larva or propagule encounters a surface and settles, before finally developing as a sessile adult (for reviews see Santelices, 1990; Norton, 1992 for algae and; Pawlik, 1992; Rodriguez *et al.*, 1993 for invertebrates). The propagules of green and brown algae are motile and able to react to physical and chemical stimuli (Santelices, 1990; Fletcher and Callow, 1992), some settle preferentially on filmed surfaces (e.g. Dillon *et al.*, 1989). However, the swimming speeds of these propagules are extremely slow compared to water currents in the sea (Norton, 1992). Consequently microbial films probably have a greater influence on the survival and growth of young algae than on habitat selection. For example Huang and Boney (1984, 1985a) demonstrated that single species cultures of benthic diatoms facilitated survival and growth of *Ulva lactuca* whilst inhibiting survival and growth of *Fucus vesiculosus, Gigartina stellata* and *Chondrus crispus.* Similar effects have been demonstrated for other marine algae (Norton, 1983).

By contrast, for the larvae of many benthic invertebrates habitat selection is an active process that occurs at a range of spatial scales in response to physical, chemical and biological settlement stimuli (Crisp, 1974; Bourget, 1988). Microbial films have been shown to promote settlement of numerous

marine invertebrates including polychaetes (Knight-Jones, 1951; Wilson, 1955; Gray, 1966; Kirchman *et al.*, 1982; Pawlik and Butman, 1993), bivalves (Tritar *et al.*, 1992), gastropods (Scheltema, 1961), bryozoans (Mihm *et al.*, 1981; Brancato and Woollacott, 1982), echinoderms (Cameron and Hinegardner, 1974; Johnson *et al.*, 1991b), Porifera, Ascidia (Keough and Raimondi, 1995) and barnacle larvae (Le Tourneux and Bourget, 1988; Keough and Raimondi, 1995). Most of these accounts are of preferential settlement on filmed surfaces although some studies describe reduced settlement of bryozoans (Mihm *et al.*, 1981) and barnacles (Maki *et al.*, 1988; Maki *et al.*, 1990; Neal and Yule, 1994).

Most of the settlement studies listed above were based on observations made in laboratory experiments using choice chambers or similar apparatus. Whilst this approach may help to focus on specific questions concerning various cues and settlement behaviour it cannot mimic the complexity of the natural environment and so the relative contribution of microbial films to settlement and colonisation on natural substrata is not clear. In addition, most researchers have examined interactions between a single constituent of the film, such as bacteria (Dillon *et al.*, 1989; Maki *et al.*, 1990; Maki *et al.*, 1992; Tritar *et al.*, 1992) or diatoms (Huang and Boney, 1985a) and a settling organism. Studies which compared the effects of single species and multi-species microbial assemblages on survival of the juvenile stages of macroalgae have shown that deleterious effects of a single species within the film could be alleviated by the presence of other species (Meadows, 1963; Huang and Boney, 1985a).

Where the influence of microbial populations on larval settlement has been examined, almost without exception, the fouling communities were grown on artificial surfaces such as plastic dishes or glass microscope slides. These microbial assemblages were frequently described as 'natural' (e.g. Todd and Keough, 1994). However, the surface properties of artificial surfaces are considerably different to those of natural substrata and this may influence

both settlement and survival (Mihm *et al.*, 1981; Wahl, 1989; Anderson and Underwood, 1994; but see also Henschel *et al.*, 1990; Becker, 1993 for examples of few differences in fouling assemblages between surfaces). Hence, the results of studies using artificial surfaces may be of limited application. Relatively few authors have assessed the influence of microbial films on natural rock surfaces which are typically colonised by marine invertebrates (but see Strathmann *et al.*, 1981; Le Tourneux and Bourget, 1988). In the present study the influence of natural epilithic biofilms on the settlement of marine invertebrates was examined, using larvae of the intertidal barnacle *Semibalanus balanoides* as a model.

Barnacle larvae or 'cyprids' exhibit considerable habitat selection behaviour prior to settlement, they have been extensively studied in both laboratory and field conditions and respond to various settlement cues at a range of spatial scales (Crisp and Meadows, 1963; Crisp, 1974; Crisp, 1984; Bourget, 1988; Neal and Yule, 1994).

Laboratory experiments have shown that cyprids settle preferentially on surfaces which are covered by microbial films (Crisp and Meadows, 1963) and that they are able to distinguish between films of different ages and films grown in different conditions (Neal and Yule, 1994; Wieczorek *et al.*, 1995). Cyprids may therefore be able to use cues in the microbial film to aid habitat selection. Since the life cycle of micro-organisms is very short, it has been suggested that cyprids may gain 'up to date' information about conditions at a particular position on the shore from cues within the film (Neal and Yule, 1994; Wieczorek *et al.*, 1995) or to help determine shore level (Strathmann *et al.*, 1981; Raimondi, 1988b; Johnson and Strathmann, 1989).

Cyprids can also distinguish between different types of rock (Holland *et al.*, 1984; Huxley *et al.*, 1984; Raimondi, 1988a) and usually settle preferentially on rugose surfaces (Yule and Walker, 1984; Bourget, 1988). However, the presence of conspecifics or remnants of previous colonisation provide an

exceptionally strong inducement to settlement (Knight Jones, 1953; Wethey, 1984; Bourget, 1988; Raimondi, 1988b; Crisp, 1990; Raimondi, 1991) and the relative contribution of microbial cues compared to those from conspecifics remains unclear.

In this study a combination of laboratory and field experiments are used to demonstrate the influence of intertidal epilithic biofilms on barnacle settlement and determine the relative importance of these films compared to better known settlement cues.

7.3 METHODS

7.3.1 Study Sites

Study sites were shores in the South of the Isle of Man (4°W., 54°N.) where *Semibalanus balanoides* was the most abundant space occupier on the mid shore (~ 3. 4m above LAT). Samples of rock for laboratory choice chambers were collected from either a moderately wave exposed shore at Port St Mary or from a sheltered shore near Castletown (see: Southward, 1953; Hawkins, 1979 for site descriptions). Field experiments were prepared in areas of dense barnacle cover at Port St Mary.

7.3.2 Choice Chambers

These experiments were conducted at the Port Erin Marine Laboratory, Isle of Man during June 1994 and May 1995, when cyprids were abundant and could easily be collected using a plankton net trawled back and forth along the side of a small jetty nearby. Sub-samples from these trawls were sorted with the aid of a dissecting microscope. Cyprids were removed using a Pasteur pipette, transferred to a beaker of filtered seawater and kept at 10°C until they were introduced into choice chambers, normally within 24h. of collection.

Choice chambers were prepared in a controlled temperature room maintained at similar conditions to those in the sea at that time of year (10°C, 16h. light 8h. dark). Nine replicate chambers were used for each experiment conducted during 1994. However, as there was considerable variability in settlement between chambers this was increased to 20 replicates whenever sufficient cyprids could be obtained (Table 1). Each chamber consisted of a 300 ml Pyrex bowl which had been washed and sterilised in an autoclave. Chambers were filled with 200 ml of filtered (0.22µm) seawater and aerated with a steady stream of bubbles delivered from a Pasteur pipette connected to an air supply (Figure 1). Water movement generated by bubbles rising from the air supply helped to circulate cyprids around the chambers, maximising the likelihood of them encountering each of the settlement surfaces offered. Water movement may also have promoted settlement (Crisp, 1955).

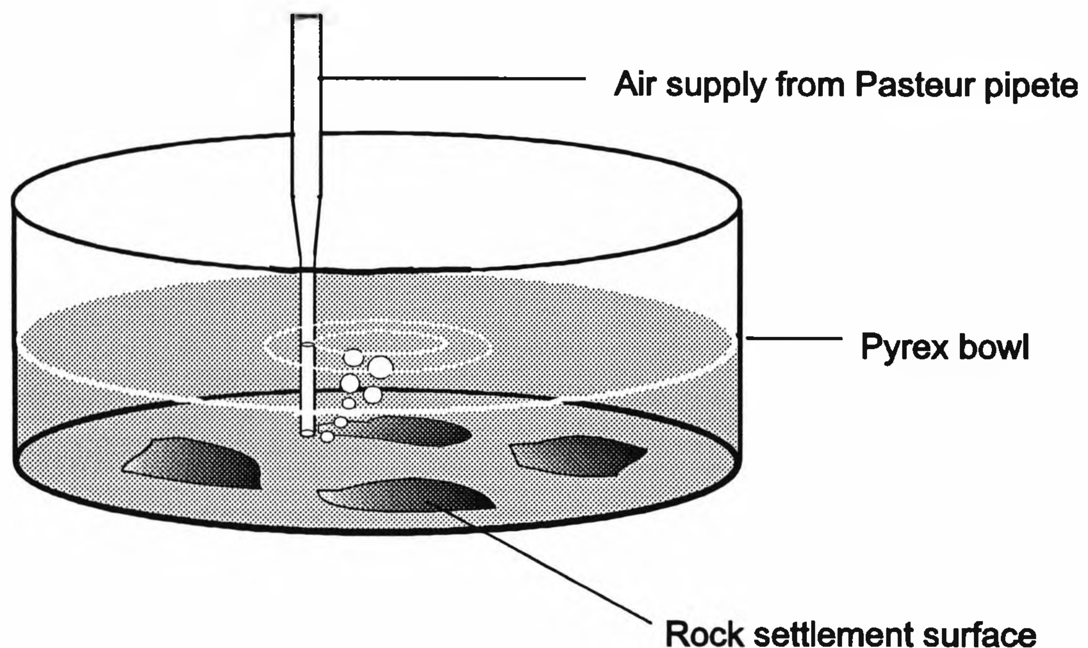


Figure 1) Diagram of a choice chamber with air supply and four settlement surfaces.

Settlement substrata were rocks chips (each approximately 6 cm^2) chiselled from randomly located positions at selected tidal heights on the shore. Depending upon the experiment, three or four different types of rock surface were introduced into each tank using a pair of sterile tweezers. Care was taken not to cross-contaminate samples during collection and handling. Samples were equally spaced around the edges of the basins and were separated by a gap of about one centimetre. In order to ensure an even spatial distribution of each rock type the orientation of samples was rotated in a clockwise direction as each successive tank was prepared.

Approximately 20 cyprids were introduced into each tank using a Pasteur pipette. This number was selected as a compromise, being sufficient to provide adequate data from the number of tanks used, but not large enough to cause problems of cyprid-cyprid interactions (see Rittschof *et al.*, 1992; Clare *et al.*, 1994; Wieczorek *et al.*, 1995). Settlement was recorded daily until all cyprids had either settled or become inactive.

Five choice chamber experiments were conducted and these are summarised in Table 1. The surface types being compared were placed in the chambers face upwards. With the exception of surfaces which had been experimentally manipulated, it was assumed that the rocks used supported a microbial film typical of the part of the shore from which they had been collected. Rock samples were not colonised by barnacles, cyprids nor other macrobiota and had no obvious remnants of a previous barnacle matrix on their surface.

The first experiment, conducted during 1994, was designed to establish 1) whether cyprids could differentiate between naturally filmed and unfilmed rock surfaces and 2) whether cyprids could differentiate between naturally filmed rocks from different shore levels at Port St Mary (moderately wave exposed). For this work, rock chips were collected at random from three shore levels lower, mid and upper (~ 2.0, 3.4 and 4.4 m above LAT

respectively). Mid shore samples were collected from the middle of the *Semibalanus balanoides* zone (~ 50 % cover of adult barnacles) whilst upper and lower shore samples were collected on the extreme edges of this zone (< 5% coverage of adult barnacles). One sample from each level together with a sample of unfilmed rock was placed in each chamber (Table 1).

Table 1) Summary of experiments using choice chambers and *Semibalanus balanoides* cyprids.

Date	Abbrev -iation	Substratum choices available in chamber	No. of replicate chambers	No. of cyprids in each chamber	Hypotheses under consideration
10/6/94	ELS EMS EUS UF	moderately wave exposed lower shore moderately wave exposed mid shore moderately wave exposed upper shore unfilmed rock surface	9	20	1) Do cyprids select filmed surfaces in preference to unfilmed surfaces. 2) Can cyprids differentiate between filmed rocks from different shore levels
14/6/94	SLS SMS1 SMS2 UF	sheltered lower shore sheltered mid shore - low adult barnacle density sheltered mid shore - high adult barnacle density unfilmed rock surface	9	20	1) Is selection of surfaces from the mid shore caused by the proximity of these samples to adult barnacles 2) Do cyprids respond to the mid shore biofilm community
2/5/95	FPC F UF	filmed and previously colonised by adult barnacles (adult barnacles removed 4 months earlier) filmed and not previously colonised (surface 1mm chiselled and burned 4 months earlier) unfilmed rock surface	20	20	1) Do cyprids respond to filmed surfaces or to remnants of barnacles on the surface of these rocks
10/5/95	F FB UF UFB	as above as (F) but with an adult barnacle transplanted on to the rock surface unfilmed rock surface as (UF) but with an adult barnacle transplanted on to the rock surface	20	20	1) Which provides the stronger settlement cue, presence of a microbial film or presence of an adult conspecific.
20/5/95	F PF UF	as above poorly developed film and not previously colonised surface (prepared as (F) but exposed for 2 weeks earlier) unfilmed rock surface	20	20	1) Do cyprids respond to a recently formed microbial film 2) Do cyprids respond to 'footprints'left by other cyprids.

Unfilmed rock was obtained by initially chiselling away the upper surface of the bedrock at mid tide level so that a new rock surface was exposed. Rock chips were then chiselled from this area so that no part of the sample had previously been exposed to seawater.

A second experiment was conducted to establish whether the proximity of adult barnacles was providing a cue which enhanced settlement on the mid shore. Rock surfaces were collected from the lower, mid and upper shore on a sheltered shore near Castletown, where barnacles were much less abundant (Table 1). Two sets of samples were collected from the mid shore, one from areas adjacent to patches of adult barnacles (locally around 25% cover) and the second from areas where the abundance of adults was minimal (<5% cover).

Additional experiments were run during 1995 and these were intended to further investigate the principal cue(s) which enabled cyprids to detect rocks that had originated from the mid shore. In the first experiment unfilmed (UF) surfaces (prepared as described above), filmed surfaces without traces of adult barnacles (F), and filmed surfaces with traces of adult barnacles (FB) were used. Filmed surfaces and filmed surfaces with traces of barnacles were both prepared four months before the start of settlement so that a natural microbial community had time to develop. For filmed (only) surfaces, previous traces of barnacles were removed by chiselling away the top of the rock surface. Filmed surfaces with traces of adult barnacles were prepared by gently scraping barnacles from areas of dense barnacle matrix, on the mid shore, using a paint scraper. Several areas (each ~ $30cm^2$) of both surface types were prepared on the shore. These were used to provide samples for the choice chambers, one sample of each type being placed into each chamber (Table 1).

A second experiment during 1995 used unfilmed and filmed rocks (prepared as above) together with rocks which had been prepared as for the filmed ('F') surfaces but had only been allowed two weeks to acquire a microbial film. Here, it was of interest to see whether cyprids could differentiate between mature and developing microbial communities.

A final experiment was designed to establish the relative importance of cues provided by filmed surfaces, and those provided by the presence of conspecific adult barnacles (Table 1). Here, adult barnacles were carefully removed from intertidal rock using a sharp scalpel and transplanted on to either filmed or unfilmed rock samples. This procedure was easiest with solitary barnacles living on the surface of smooth soft rock. Care was taken not to damage the rostrum or the base. One barnacle was fixed on to a smooth area of each test substratum by gluing the outer edge of the rostrum to the rock with small spots of super-glue. A second rock of each type (filmed and unfilmed) was prepared with two spots of glue, but without a barnacle. These rocks, complete with transplanted barnacle were placed into filtered seawater for 24 hours whilst the glue completely cured. Rocks on which the barnacles had survived transplantation (i.e. observed to be feeding normally) were then used in the choice chambers together with one rock of each type with glue, but no barnacle.

7.3.3 Field Experiments

Three areas of shore were selected which had exceptionally dense barnacle cover (~ 95%). Typically, these were at the edge of horizontal ledges that were exposed to tidal currents. Treatments were prepared at two distances from the adult barnacle population. This was achieved by clearing circular areas of either 30cm or 50cm diameter from the barnacle matrix using a paint scraper. These areas were randomly distributed between the ledges selected. Within each ledge care was taken to choose positions which were heavily covered with barnacles, would be surrounded by at least 20cm of matrix after clearance, and had a relatively smooth underlying rock surface.

Within each circular clearance area three treatments were prepared. These surfaces were identical to those described for choice chamber experiments of 2/5/95 (unfilmed, filmed and filmed with barnacle remnants). However, the method of preparation was refined slightly.

Unfilmed surfaces and filmed surfaces were prepared by removing the upper 1mm of rock using a sharp wood chisel. The newly exposed surface was then heated with a butane blow lamp to denature all traces of arthropodin, a substance released by adult barnacles which promotes cyprid settlement (Knight Jones, 1953; Crisp and Meadows, 1963; Yule and Crisp, 1983). During this procedure care was taken to shield the biofilm on the surrounding rock from thermal damage. These areas became fouled by microbiota during the next four months before the barnacle settlement season commenced. Then, at the start of the barnacle settlement in May 1995, the surface of unfilmed treatments was again removed using a wood chisel, but without further heating with the blow lamp. Filmed surfaces previously colonised by barnacles were simply areas of the scraped barnacle matrix formed when the circular clearance areas were prepared.

Three duplicates of each treatment were prepared in each cleared area. To achieve a uniform spatial distribution of treatment types, these were arranged in a circle (25cm in diameter) located around the centre of the cleared areas (Figure 2). The sequence of treatments was the same in each cleared area (FB, F, UF, FB, F, UF etc. Figure 2). However, as each successive cleared area was prepared, the starting position was rotated by 30° from north.

The corner of each treatment was marked with a small depression made using a battery operated hand drill. These holes were later used to help position a small quadrat for counting settlement (Figure 2).

One shortcoming of this experimental design was the random spatial distribution of the two differing sizes of treatment area, which meant that spatial variation in settlement could not be analysed (as a third factor in the analyses). Pairing small and large treatment areas together as an 'experimental block' would have provided a solution to this problem but would have been difficult to achieve because of constraints on the sizes of

suitable areas of barnacle matrix which were limited by areas of uneven topography, *Fucus* clumps etc.

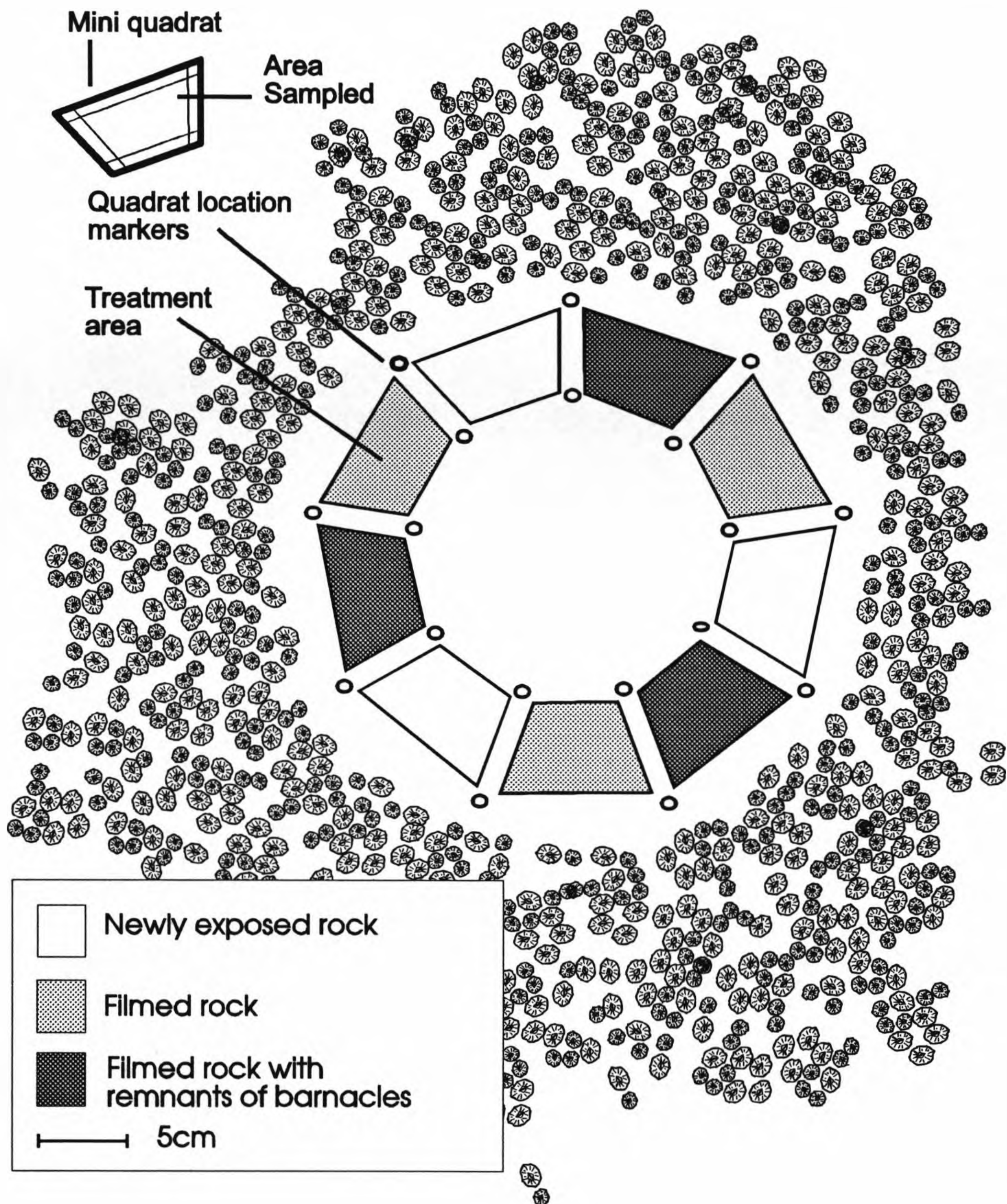


Figure 2) Example of settlement area with differing microbial films prepared approximately 3.5cm away from barnacles in the surrounding population at mid tide level, Port St Mary, Isle of Man. Identical treatments were also prepared 13.5 cm from the surrounding population (see text).

The numbers of cyprids and metamorphosed spat were recorded approximately every two days during the settlement season (25/4/95 - 23/5/95) using a mini quadrat. To minimise the effect of variation at the edge

of treatments, counts were made only from a central 12.5 cm^2 portion of this mini quadrat (Figure 2). Hence the actual area from which settlement was recorded was between 3.5 and 6.5cm from the adult barnacle matrix for small cleared areas and was 13.5 to 16.5cm away in large cleared areas. Within each cleared area settlement was determined by averaging counts across the three duplicates of each treatment.

Direct counts of microbiota in treatments were made on rock chips collected from additional areas of shore prepared in the same way as the field treatments. Six rock chip samples were collected from each surface type (UF, F, FB), air dried and fixed in 2.5% gluteraldehyde in filtered sea water (Hill & Hawkins, 1990). Samples were then viewed at 480x magnification using a Philips XL 30 scanning electron microscope. Six randomly located photographs were taken on the surface of each sample, and these were later examined at six times magnification to provide reasonable resolution for identification with a large field of view (Patterson *et al.* 1986).

7.3.4 Data Analyses

Counts of cyprid settlement from choice chambers showed strong heteroscedasticity and so were analysed using Friedman's test in Minitab (v.10.1) on an IBM personal computer, followed by Tukey-Type multiple comparisons (Zar, 1984 p. 200)

Data from field experiments conformed to Cochran's test for homogeneity of variance (Winer *et al.*, 1971) after square root transformation and were analysed using the GLM command in Minitab. 'Distance' and 'surface type' were both treated as fixed effect factors in the analyses. Plots of residuals were examined after each ANOVA to check that error terms were normally distributed. Comparisons between levels within treatment factors were selected *a priori* and determined using Bonferroni comparisons (Maxwell and Delaney, 1990 p. 190).

Cyprid settlement data were analysed from days when settlement was maximal as these occasions were most likely to reveal differences between treatments. Also, choices made by cyprids during the peak of the settlement period would have a large impact on recruitment to the adult population. Analyses of counts for metamorphosed spat were made towards the end of the settlement season, but before net abundance had started to decline because of post metamorphosis mortality (e.g. Keough and Downes, 1982; Hawkins, 1983; Hoffman, 1987; McGuinness and Davis, 1990). In essence, abundance at this time represented the effect of the treatments on overall settlement for the season.

7.4 RESULTS

7.4.1 Choice Chambers

Cyprids settled on rock surfaces in all choice chambers with significant differences in settlement between treatments in all experiments except experiment 2 (1994) which used rocks from a sheltered shore (Table 2).

Cyprids settled preferentially on rocks from the mid shore. This effect was significant using rocks from the moderately wave exposed shore but not so for rocks from the sheltered shore (Figure 3a, b and Table 2). None of the rocks used had any visible signs of previous barnacle colonisation on them, but rocks from the mid shore were more likely to have been colonised previously than those from the upper or lower shore. Therefore it remained uncertain whether cyprids were responding to traces of adult barnacles or some feature of the microbiota on the mid shore.

Subsequent work showed that settlement was significantly greater on surfaces which had previously been colonised by barnacles than those which had not (Figure 4a, and Table 2). However, in the absence of previously colonised surfaces, cyprids selected surfaces with a developed

biofilm in preference to surfaces with a poorly developed film or an unfilmed surface (Figure 4b, c and Table 2).

Table 2) Summary of results for settlement of *Semibalanus balanoides* cyprids in choice chamber experiments. nt = not tested, n.s. = not significant, * = P<0.05, ** = P<0.01, *** = P<0.001.

Date	Abbreviation	Substratum choices (origin of rock sample in settlement chamber) and outcome of Friedman's Test	Outcome of Tukey-Type multiple comparison test			
10/6/94		$S_{0.001,3}$ = 18.56 , ***				
	ELS	moderately wave exposed lower shore	-			
	EMS	moderately wave exposed mid shore	*	-		
	EUS	moderately wave exposed upper shore	n.s.	*	-	
	UF	unfilmed rock surface	n.s.	*	n.s.	-
			ELS	EMS	EUS	UF
14/6/94		$S_{0.05,3}$ = 0.296 , n.s.				
	SLS	sheltered lower shore	-			
	SMS1	sheltered mid shore - low adult barnacle density	nt	-		
	SMS2	sheltered mid shore - high adult barnacle density	nt	nt	-	
	UF	unfilmed rock surface	nt	nt	nt	-
			SLS	SMS1	SMS2	UF
2/5/95		$S_{0.001,2}$ = 15.17 , **				
	FPC	filmed and previously colonised by adult barnacles (adult barnacles removed by scraping 4 months earlier)	-			
	F	filmed and not previously colonised (surface chiselled and burned 4 months earlier)	n.s.	-		
	UF	unfilmed rock surface	n.s.	*	-	
			FPC	F	UF	
10/5/95		$S_{0.01,3}$ = 14.25, **				
	F	as above	-			
	FB	as (F) but with an adult barnacle transplanted on to the rock surface	n.s.	-		
	UF	unfilmed rock surface	*	*	-	
	UFB	as (UF) but with an adult barnacle transplanted on to the rock surface	n.s.	n.s.	n.s.	-
			F	FB	UF	UF B
20/5/95		$S_{0.01,2}$ = 13.56, **				
	F	as above	-			
	PF	poorly developed film and not previously colonised surface (as (F) but prepared but 2 weeks earlier rather than 4 months)	*	-		
	UF	unfilmed rock surface	*	n.s.	-	
			F	PF	UF	

Transplanting adult barnacles on to filmed surfaces (which had not previously been colonised by barnacles) did not enhance settlement. If barnacles were transplanted on to unfilmed surfaces settlement was enhanced but not significantly so (Figure 4b and Table 2).

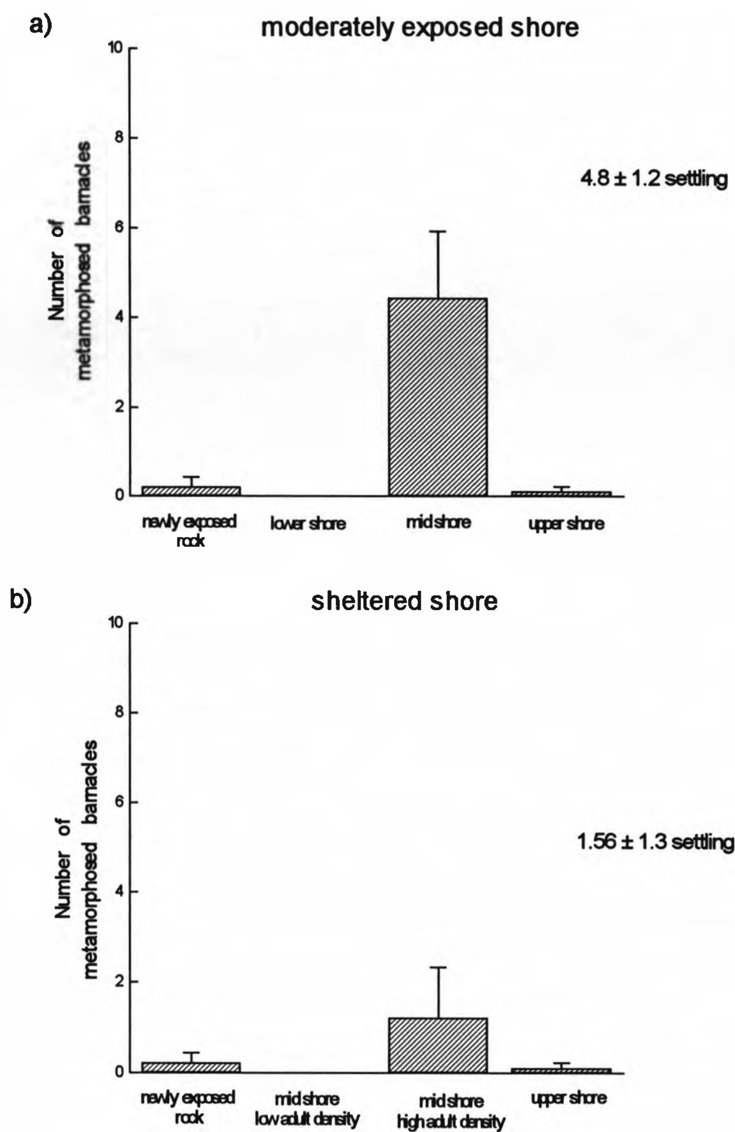


Figure 3) Number of settled and metamorphosed barnacles (mean ± 1SE) in choice chambers on rock surfaces from a) a moderately wave exposed shore b) a sheltered shore. The number of barnacles settling is shown (mean ± 1SE).

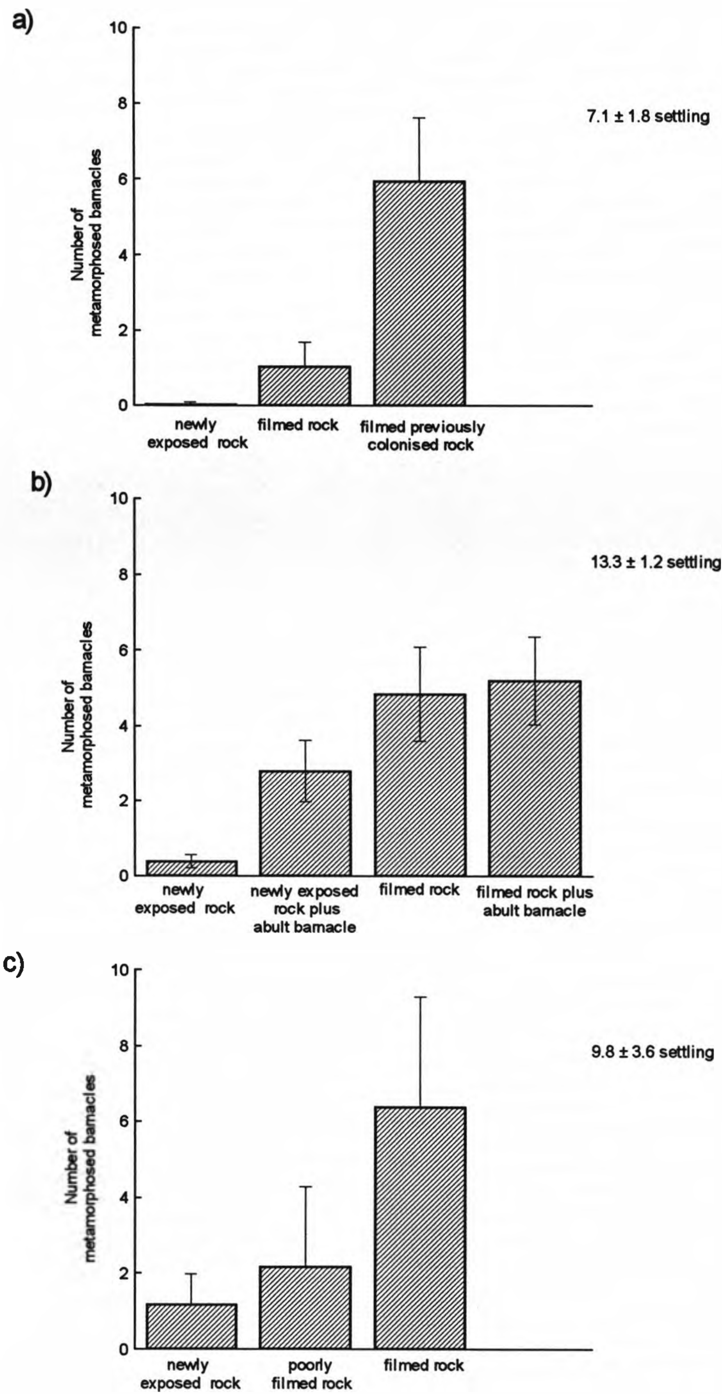


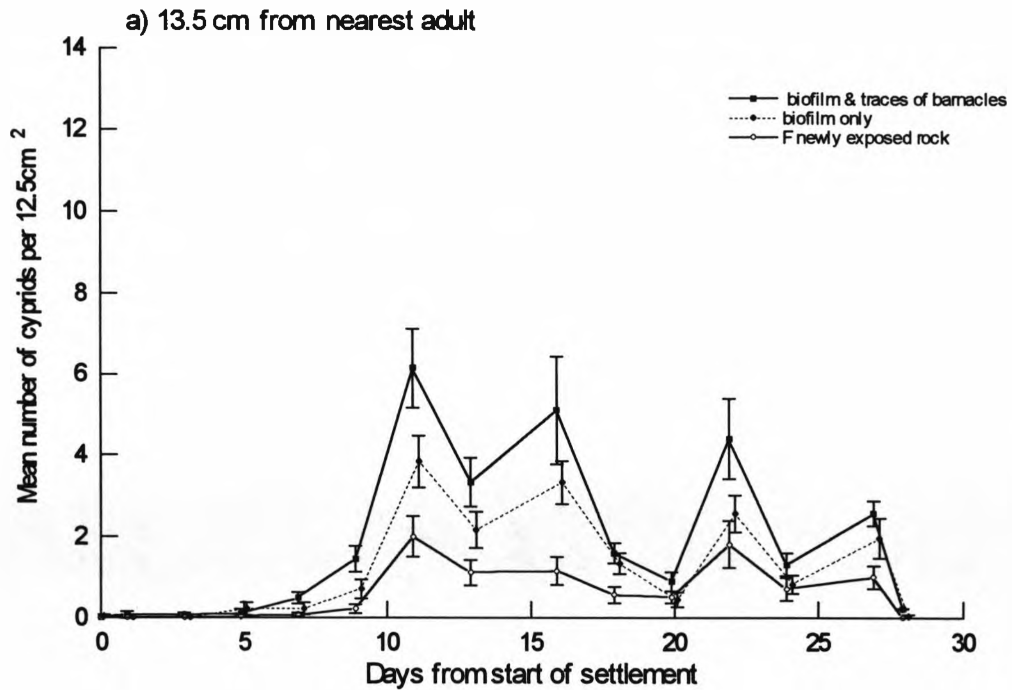
Figure 4) Number of settled and metamorphosed barnacles (mean ± 1SE) in choice chambers on intertidal rocks with different microalgal film / adult barnacle combinations. The number of cyprids settling (mean ± 1S.E.) is shown. a) Comparison between newly exposed rock, filmed rock, and filmed rock which had previously been colonised by barnacles. b) Comparison between newly exposed rock and filmed rock prepared both with and without a live adult barnacle fixed to the rock surface. c) Comparison between newly exposed rock, poorly filmed and filmed rock (see text for details).

7.4.2 Field Experiments

Settlement of cyprids was highly variable between days (Figure 5). As treatment plots were not independent with time differences between sampling dates were not tested. However, the same order of surface selection by cyprids was maintained throughout the settlement period. Areas close to the adult barnacle population received greater settlement than those which were further away. Settlement was greatest on filmed surfaces which had previously been colonised and was least on unfilmed surfaces. These variations were compared statistically for counts made at the peak of settlement on day 11 (6/5/95) and day 13 (8/5/95) when settlement had declined slightly.

Analysis of variance revealed significant differences in the numbers of cyprids between film types on both sampling occasions (Table 3). On both day 11 and 13 settlement was greater in treatments closer to the adult population, but this difference was only significant on day 13. Differences between film types were most evident on day 13 with significant differences between each of the three treatments. Settlement was greatest on previously colonised surfaces and least on unfilmed ones. These two film types were also significantly different on day 11. Clearly, settling cyprids could differentiate between the three surfaces offered, but the relative contribution of either 'films' or 'distance from adults' remained unclear because of high settlement variability.

The patterns of substratum selection observed for cyprids were also reflected in the abundance of metamorphosed spat between treatments (Figure 6). The problem of high variability of cyprid settlement was partially overcome by analysing differences in the abundance of metamorphosed barnacle spat towards the end of the settlement period (19/5/95), but before there was a net loss of spat caused by post-metamorphosis mortality. Surfaces close to the adult population had significantly greater settlement than those further



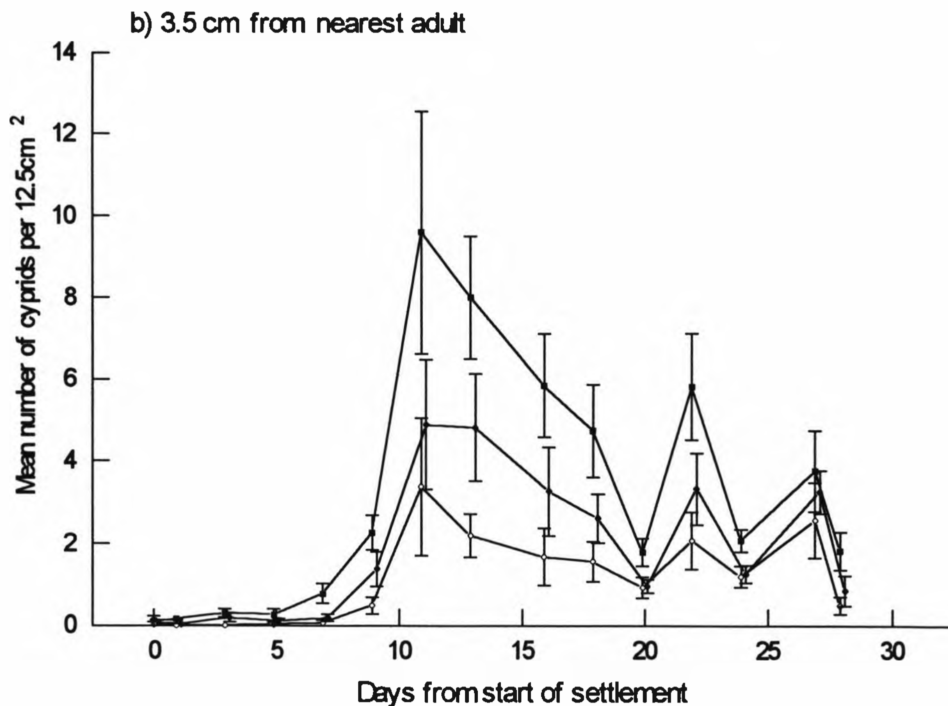
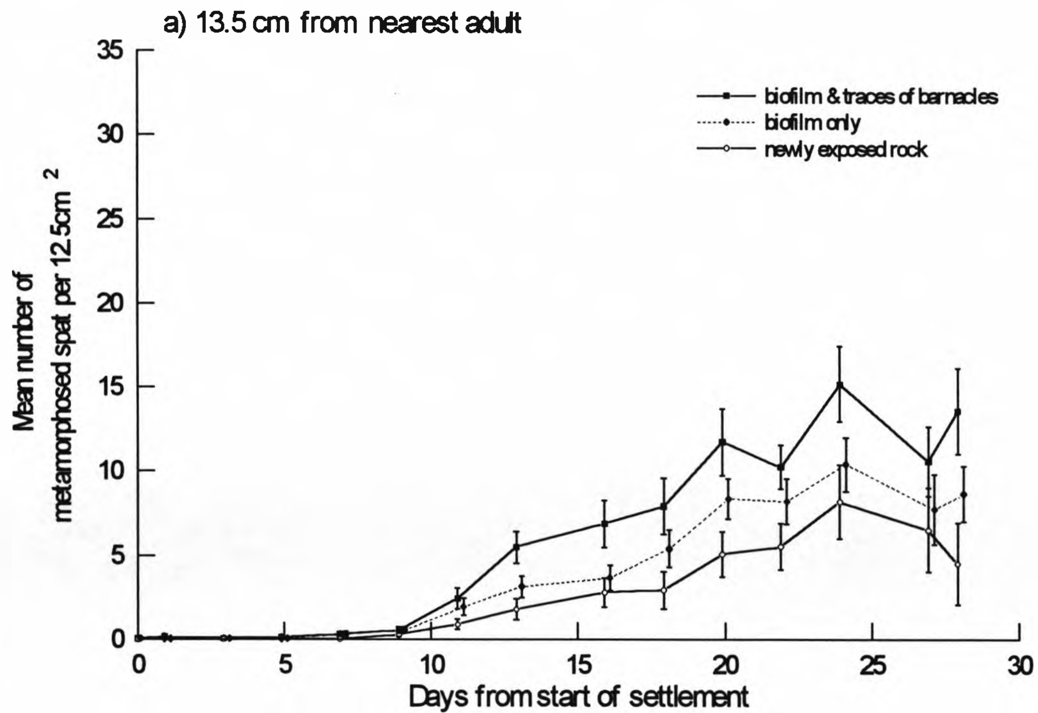


Figure 5) Mean number of barnacle cyprids in treatment areas of differing microbial films, at different distances from the surrounding barnacle population at mid-tide level, Port St Mary, Isle of Man during settlement in April and May 1995. Data points have been offset slightly for clarity (bars = 1SE).



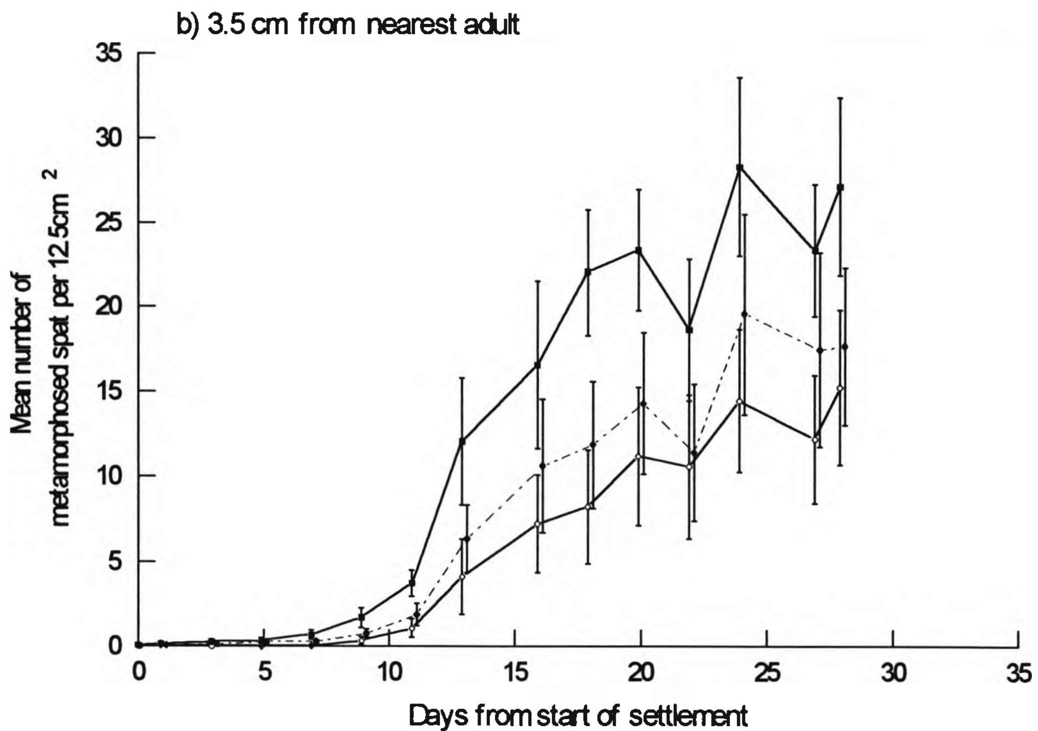


Figure 6) Mean number of metamorphosed barnacle spat in treatment areas of differing microbial films at different distances from the surrounding barnacle population, at mid-tide level, Port St Mary, Isle of Man during settlement in April and May 1995. Data points have been offset slightly for clarity (bars = 1SE).

away (Table 3). This factor accounted for greater settlement variation than that caused by differences between film types. There were also differences between film types with significantly greater settlement on filmed and previously colonised surfaces compared to unfilmed surfaces. Filmed surfaces which had not previously been colonised had intermediate levels of settlement.

Table 3) Analyses of variance for treatment factors on settlement of *Semibalanus balanoides* cyprids at the peak of the settlement season and on metamorphosed spat present towards the end of the settlement season. Data were square root transformed. 'Distance' (from adult barnacles) and 'film' (type of microbial film present) were treated as fixed factors. n.s. = not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

source of variation	df	Cyprid settlement day 11 (6/5/95)			Cyprid settlement day 11 (8/5/95)			Metamorphosed spat day 22 (19/5/95)		
		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Distance	1	1.06	1.20	n.s.	6.78	16.68	***	15.99	9.50	**
Film	2	7.02	7.96	***	5.44	13.37	***	8.76	5.21	**
Distance * Film	2	0.09	0.10	n.s.	0.31	0.77	n.s.	0.32	0.19	n.s.
Error	48	0.88			0.41			1.68		
Cochran's test: C_{crit}, P0.05 = 0.38			$C = 0.32$			$C = 0.30$			$C = 0.35$	
		Bonferroni analysis of differences between treatment effects								
UF = unfilmed surface	UF	-			-			-		
F = filmed surface	F	n.s.	-		*	-		n.s.	-	
FPC = filmed surface previously colonised by barnacles	FPC	*	n.s.	-	*	*	-	*	n.s.	-
		UF	F	FPC	UF	F	FPC	UF	F	FPC

There were also differences in the species composition of microalgae between settlement surfaces (Table 4). Unfilmed surfaces, not surprisingly, were uncolonised. Both filmed surfaces and filmed surfaces with remnants of barnacles had a more diverse fouling community, with diatoms, cyanobacteria and crustose coralline algae. However, microalgae were sparse, possibly reflecting mortality caused by unusually warm weather during June, 1995 (see chapter 6). Where microalgae were present their

abundance was compared between treatment surfaces using a Kruskal-Wallis test followed by Tukey-Type multiple comparisons (Table 4). The analysis did not reveal any differences between the abundance of either diatoms, cyanobacteria or crustose coralline algae between unfilmed and poorly filmed surfaces. However, filmed surfaces which had not previously been colonised by barnacles had significantly more cyanobacteria than either unfilmed or poorly filmed surfaces. Surfaces which had been exposed to seawater for just two weeks (used in one of the choice chamber experiments) were not colonised by either cyanobacteria or crustose coralline algae but had a similar cover of diatoms to surfaces which had been exposed for four months.

Table 4) Comparison of microbiota on rock surfaces (mean ± 1 SE). UF = unfilmed surface, F = filmed and not previously colonised (surface 1mm chiselled and burned 4 months earlier), PF = poorly developed film and not previously colonised surface (prepared as (F) but only 2 weeks earlier rather than 4 months), FPC = filmed and previously colonised by adult barnacles (adult barnacles removed by scraping 4 months earlier). The outcome of the Kruskal-Wallis and Test Tukey-Type multiple comparisons are shown, nt = not tested, * = P <0.05, n.s. = not significant.

	Diatoms no. per mm^2	Cyanobacteria % cover	Lithothamnion % cover
UF	0	0	0
PF	2.84 ± 2.07	0	0
F	2.84 ± 1.33	2.03 ± 0.71	7.5 ± 5.14
FPC	0.71 ± 0.71	1.0 ± 0.49	4.0 ± 2.01
	$H_{0.05,3,3}$ = 4.51, n.s.	$H_{0.05,3,3}$ = 9.77, *.	$H_{0.05,3,3}$ = 7.17, n.s.

Comparisons between microbiota - Tukey-type multiple comparisons after Kruskal-Wallis test

	UF	PF	F	FPC	UF	PF	F	FPC	UF	PF	F	FPC
UF	-				-				-			
PF	nt	-			nt	-			nt	-		
F	nt	nt	-		*	*	-		nt	nt	-	
FPC	nt	nt	nt.	-	nt	nt	n.s.	-	nt	nt	nt.	-

7.5 DISCUSSION

Habitat selection was chiefly influenced by the proximity of adult barnacles and by remnants of previous barnacle colonisation on the settlement surface, with both these factors significantly increasing settlement. In the

laboratory choice chambers, cyprids could distinguish between filmed surfaces which had originated from different shore levels, and selected filmed surfaces in preference to unfilmed surfaces. However, in the field, whilst filmed, but not previously colonised surfaces received greater settlement than unfilmed surfaces, this effect was only significant on one sampling occasion (one out of three tests on cyprids or spat).

In laboratory choice chambers, cyprids distinguished between rock surfaces collected from different levels of the shore, settling preferentially on those from the mid shore. Similar results have been obtained in field conditions by Strathman, *et al.* (1981) who transplanted panels, which had been fouled on the lower shore, to the upper shore, and showed that *Balanus glandula* could be induced to settle above its normal range. They concluded that cyprids were able to differentiate between some feature of the micro-flora which varied between shore levels. Raimondi (1988b) also demonstrated that cyprids were attracted to settle if surfaces were coated in extracts from other shore dwellers typical of the tidal height where the adult barnacle population was most abundant.

The cause of the enhanced settlement on mid shore rocks in the present study is unclear. It may have resulted from either a greater abundance of barnacle remnants at that level, or possibly chemical exudates from neighbouring barnacles, had become absorbed into the microbial film on the mid-shore (e.g. Crisp and Meadows, 1963). Alternatively a particular microbial may have been present at this shore level.

Microscopic examination of treatment surfaces did not reveal any differences in fouling community, between filmed surfaces and filmed surfaces which had previously been colonised by adult barnacles. However, the abundance of microalgae in the intertidal zone does vary with tidal height (Underwood, 1984c; Dye and White, 1991). On Manx shores, these differences are especially distinct during the late spring and early summer when barnacles

are settling (see Chapter 5). A relevant laboratory study would be to compare cyprid settlement between surfaces fouled with microflora which is typical of that found on either the lower, mid or upper shore during the early summer.

Microscopic examination of settlement surfaces showed that crustose coralline algae were an important constituent of these fouling communities. Recent work has shown that bacteria associated with similar encrusting coralline algae promote settlement of some invertebrate larvae (Johnson *et al.*, 1991a; Johnson *et al.*, 1991b). In the intertidal zone at Port St Mary these algae were most abundant in rock pools and on the lower shore where cyprid settlement was minimal. However, detailed examination of the microflora on the shore revealed that microscopic patches of these algae were present throughout the shore and further work on the effects of these patches on cyprid settlement may be worthwhile.

Diatoms were equally abundant on fully and partially filmed surfaces suggesting that they did not form the basis for the difference between these surfaces perceived by the cyprids. A note of caution must be made here as there are considerable difficulties in resolving the microbiota within fouling communities. Scanning electron microscopy as used here only resolves organisms at the surface of the film (MacLulich, 1986; Hill and Hawkins, 1990). Hence differences in species abundance between filmed and partially filmed surfaces may have been much larger than those observed.

In the absence of cues provided by remnants of previous adult populations, cyprids settled preferentially on filmed surfaces in choice chambers. Le Tourneux and Bourget (1988), working in field conditions, showed that constituents of the microbial film such as diatoms were important cues to settlement, but found that on a scale of a few microns the sites actually selected by the cyprids were frequently uncolonised areas of substratum. Houdon and Bourget (1983) and Raimondi (1988a) also concluded that

areas with minimal microbial colonisation or detritus were attractive for the final stages of habitat selection. Whilst these observations indicate that cyprids frequently select a clear area for attachment, the presence of a microbial film is undoubtedly important at an earlier stage in habitat selection (Crisp and Meadows, 1963; Neal and Yule, 1994).

Mature films were more attractive to settlement than developing films. An excellent study by Wieczorek *et al.* (1995) also demonstrated that settlement increased on older films. Other researchers have, however, reported the opposite trend (Maki *et al.*, 1990; Neal and Yule, 1994; Keough and Raimondi, 1995). These observations may at first appear contradictory However, a range of different film types (single species and multi-species) and several barnacle species (*Balanus amphitrite, Balanus perforatus, Elminius modestus, Balanus variagatus*) were used by the different researchers. Clearly, differing results might be expected from such a diverse array of experiments. For barnacle species that usually live in regularly grazed locations such as the intertidal zone (e.g. *Balanus perforatus*), the presence of a developed microalgal film might indicate favourable conditions. However, for a species which usually occupies the subtidal (e.g. *Balanus amphitrite*), a developed microalgal film may indicate conditions which were less attractive for settlement, for example, an area of substratum which is soon to become overgrown by microalgae. Therefore, it seems most appropriate to describe the effects of biofilms on settlement of individual species, rather than effects for barnacles as a whole (see Ramondi, 1988).

Differences between filmed and unfilmed surfaces in the present study could have been caused by cyprid footprints (see Yule and Walker, 1985) acquired prior to sample collection on filmed samples, but not on unfilmed ones. However, this seems unlikely as rocks with poorly developed biofilms would have been equally likely to be encountered by cyprids in the field, as rocks with a fully developed film, but colonisation of filmed rocks was much greater than that on either surfaces with poorly developed films or unfilmed ones.

Somewhat surprisingly, addition of an adult barnacle to filmed surfaces in the choice chambers did not enhance settlement as might have been expected. Numerous previous studies have shown that cyprids frequently settle shortly after encountering an adult barnacle (Knight Jones, 1953; Crisp, 1974; Crisp, 1984; Wethey, 1984; Bourget, 1988; Crisp, 1990). The results indicate that the proximity of conspecifics may be unimportant at a scale of a few centimetres and that at this level of habitat selection the presence of a microbial film provides the greater stimuls.

Habitat selection in the intertidal was clearly influenced by a combination of settlement cues. Filmed surfaces which had previously been colonised by barnacles provided the most attractive surface whilst unfilmed surfaces were least favoured. Settlement was greatest in areas nearest to dense populations of adult barnacles. This observation is supported by numerous early studies on the settlement behaviour of barnacles (Crisp, 1974; Larman and Gabbot, 1975; Larman *et al.*, 1982; Yule and Crisp, 1983).

The study also raises an interesting issue about the circumstances in which either laboratory or field-based experiments are most appropriate for settlement studies (see comments by Bourget, 1988; Pearce and Scheibling, 1991). For example, in field conditions it was not possible to conclusively establish that settlement was enhanced on filmed surfaces (that had not previously been colonised by conspecifics) compared to unfilmed surfaces. However, it was possible to demonstrate this effect in the laboratory, but not when surfaces which had previously been colonised by adult barnacles were also present (a situation more similar to that in the field?). Consequently, whilst experimental conditions may be easier to control in laboratory studies than in the field, the outcome of such studies does not necessarily provide answers relevant to settlement in the natural environment.

CHAPTER EIGHT

General Discussion

8.1 LIMITATIONS OF THE STUDY

8.1.1 Spatial Variability of Epilithic Microalgae

The abundance of microalgae on Manx rocky shores was highly variable within shores, at spatial scales of less than a few centimetres. This variation may have been caused by highly localised differences in topography, aspect, drainage, stochastic recruitment events and grazing. Limpets are known to graze small patches of algae intensively during each foraging excursion (see Figure 4,Chapter 1) and may return to adjacent areas on subsequent occasions (Chelazzi *et al.*, 1994a; Della Santina *et al.*, 1995; Gray and Naylor, 1996; Chapter 4). Their grazing removes some, but not all of the epilithic algae, with about 50% of the algae passing between the teeth of the limpets feeding apparatus and remaining on the shore (R. C. T. unpublished data). Hence limpet grazing creates considerable spatial variation in microalgal abundance, which occurs at a scale of 5-10cm between grazed and ungrazed areas, and at a scale of < 100µm within grazed areas. This natural patchiness adds considerable variance to estimates of microalgal abundance and limits the ability of statistical tests to establish differences between sampling locations or sampling occasions (MacLulich, 1986; Hill and Hawkins, 1990).

8.1.2 Estimating Microalgal Standing Stock

Chlorophyll extractions from algal cells provide a reasonably reliable method for estimating microalgal standing stock (Underwood, 1984c; Hill and Hawkins, 1990, Chapter 3) and depending on the surface area of each sample this technique can integrate small scale variations in microalgal abundance (e.g. 15cm² sample areas used in Chapters 3 and 6). However, there are uncertainties about the accuracy of chlorophyll estimates since the amount of chlorophyll present may vary according to the physiological condition of the cells (Ryther, 1956; Humphrey, 1961; Oquist, 1974; Foy and Gibson, 1982) and therefore it is advisable to confirm estimates by cell counts. Methods for quantifying abundance by direct counts, such as

scanning electron microscopy (SEM) and confocal microscopy, were very time-consuming and it was not possible to validate all of the chlorophyll extraction data. Nevertheless, where comparisons were made these indicated a reasonably reliable relationship between the abundance of algal cells and chlorophyll extracted from adjacent areas of rock (Chapter3).

Counts of algal cells made using the SEM were also subject to inaccuracy as cells in underlying layers of the film were not resolved (Hill and Hawkins, 1990, see Section IV). Confocal microscopy provided a solution to this problem but was even more time-consuming as several layers within the film had to be captured, stored, and then used to reconstruct the film in three dimensions (see Section IV). Transmission electron microscopy has also been used to examine the three dimensional structure of microalgal communities on artificial substrata in rivers (Lock *et al.*, 1984). However, with both electron microscopy and confocal microscopy the scale of resolution is very small < 250 µm. Variability at this scale is considerable as a single field of view could cover an area where virtually all surface algae had been removed by grazing or an area which has passed between the teeth of a limpet radula and remained ungrazed.

Because of uncertainties with chlorophyll determinations and the variability of direct counts of cells on the rock surface, I suspect that future progress may be made by removing algae from the substratum, suspending cells in solution, mixing, and then counting abundance by cytometry. This method of direct counting would not be limited by the necessity to resolve individual cells, and could integrate small scale variation across a larger surface area (i.e. a method comparable to chlorophyll extraction but that assesses the number of cells rather than the amount of pigment).

The present study was further limited by lack of a convenient technique to measure microalgal productivity. Manipulative field experiments were also

somewhat constrained by resources. These two aspects are considered in more detail below.

8.2 STANDING STOCK VERSUS PRIMARY PRODUCTIVITY

Most research on microalgal films has measured standing stock, that is the amount of microalgae present at the time of sampling (e.g. Underwood, 1984c; MacLulich, 1987; Hill and Hawkins, 1991). This technique is suitable if the objective is to determine the amount of microalgal resource present at a given time, either for comparisons between different areas of the shore or between sampling occasions. However, in the present study also I wanted to determine the rate at which the food supply was replenished and to compare the effects of various environmental factors on microalgal growth. In these instances productivity would have provided a better measure, as standing stock gives no indication of the proportion of algae removed from the shore, for example by grazing. Consequently, productivity and standing stock may differ considerably (see 'bottom up' and 'top down' factors below). However, appropriate methods for measuring productivity of these films are yet to be developed.

Productivity has been measured for macrobenthic habitats such as coral reefs (Klumpp and McKinnon, 1989; Klumpp and McKinnon, 1992), kelp forests (Kain, 1977; Smith, 1988) and mangrove forests (Alongi, 1994), but studies of microbiota have focused on planktonic (e.g. Gleitz and Thomas, 1993), soft sediment (e.g. Gilbert, 1991; Cahoon and Cooke, 1992; Yallop *et al.*, 1994) or freshwater epilithic (e.g. Loeb, 1981; Lock, 1993) communities. The importance of benthic microalgae to inshore productivity has only been realised more recently (Fielding *et al.*, 1988; Gilbert, 1991; Kristensen, 1993; Bustamante *et al.*, 1995).

Productivity can be assessed by measuring ^{14}C assimilation (e.g. Loeb, 1981), respirometry (e.g. Varela, 1985; Klumpp *et al.*, 1987; Polunin and Klumpp, 1989; Lock, 1993) or the increase in standing stock on surfaces from which grazers are excluded (Workman, 1983; Dye and White, 1991; Fuji *et al.*, 1991; Bustamante *et al.*, 1995). The first three approaches adequately record productivity whilst the last method is slow to yield results (several weeks in my experience) and measures settlement and productivity rather than productivity alone. Consequently, the assay may include species that are not normally abundant in grazed films (Loeb, 1981). For example, in this study, ephemeral algae and germlings, which grew in areas where limpet density had been reduced (Chapter 6), would have been included had estimates of 'productivity' been made in this way. Because of these limitations this technique should only be used for broad scale geographical comparisons (e.g. Bustamante *et al.*, 1995).

Where seasonal variations in intertidal benthic productivity have been recorded, these frequently differ from estimates of standing stock obtained at the same time (e.g. Workman, 1983; Dye and White, 1991; Fuji *et al.*, 1991). Measurements of microalgal standing stock and grazing obtained in the present study were combined, using the formula below (devised by R. C. T. and M. Johnson, Port Erin Marine Laboratory), to estimate microalgal productivity on the shore at Port St Mary.

$$\text{Productivity } \mu d^{-1} = \frac{Ln\, A_{t+n} - Ln\, A_t + (Gd^{-1} \times n)}{n}$$

Where $Ln\, A_{t+n}$ = natural logarithm of microalgal abundance at time $t+n$ (present sample)

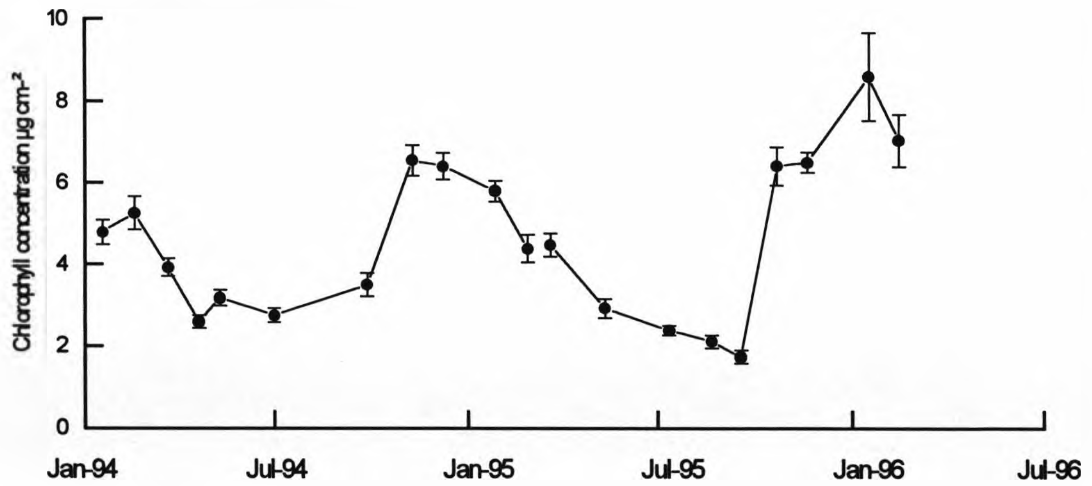
$Ln\, A_t$ = natural logarithm of microalgal abundance at time t (previous sample)

Gd^{-1} = the proportion of shore grazed per day

n = number of days since last sampling

This approach was limited by the frequency of estimates for grazing and microalgal standing stock, and uncertainties about the relationship between the area grazed and the amount of algae removed from the shore.

Nonetheless the results indicate considerable differences between times of maximal standing stock and times of maximal productivity (Figure 1). Standing stock was greatest during the winter, whilst productivity appears to be greater during the spring and autumn. These data are similar to those obtained by Fuji *et al.* (1991), and Workman (1983) who recorded maximum productivity during the spring and summer, respectively, but differ from estimates made by Dye and White (1991) who recorded maximum productivity during the winter. Such differences between productivity estimates highlight the need for further research to develop a standard method for measuring productivity on rocky shores.



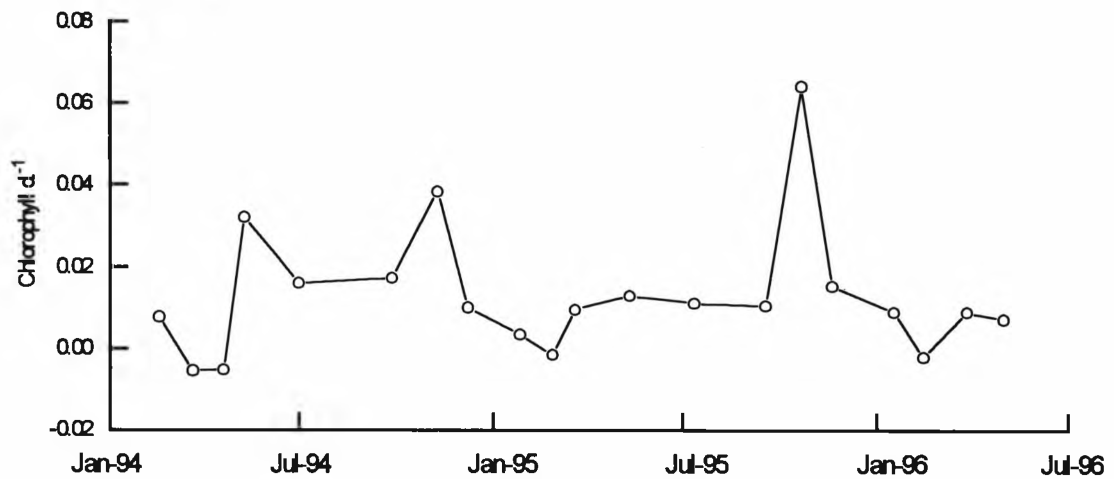


Figure 1a) Microalgal standing stock measured as chlorophyll a concentration and b) an estimate of primary productivity, based on monthly standing stock and grazing intensity, for the mid shore at Port St Mary, Isle of Man.

8.3 RESTRICTIONS OF EXPERIMENTAL DESIGN

Early work on rocky shores was, in general, descriptive (e.g. Orton, 1932; Rees, 1935; see Southward, 1958, for review of many early studies) and, whilst inferences about factors regulating distribution patterns and community structure could be drawn from these studies, experimental investigation was required to confirm the ideas generated. During the last 40 years there has been a considerable increase in use of manipulative field ecology to test hypotheses about community structure. Recently considerable attention has been paid to establishing appropriate experimental design and techniques for analyses of such experiments. The following concerns have been debated: sampling methodology (e.g. Meese and Tomich, 1992), appropriate controls (e.g. Quinn and Keough, 1993), replication and generality (Hurlbert, 1984; McKone, 1993). Ideally, an experimental approach should address all of the above. However, because of constraints of resources, time and budget, compromises are invariably made. There are several critical accounts of inappropriate experimental procedures that may have resulted from such compromise (e.g. Connell, 1974; Underwood, 1981b; Hurlbert, 1984). Some of these papers give excellent advice on the best approach to specific problems of experimental design and analysis (Underwood, 1981b; Hurlbert, 1984; McKone, 1993), but there is a lack of comprehensive work to guide researchers through the experimental design decision making process, or to emphasise the implications of any compromises made (however, see résumé by Hairston, 1989).

For example, consider an experimental design similar to that in chapter 6. This study required a considerable research effort, but was still not replicated between times or sites, and had minimal controls. Given finite resources how should a decision be made to optimise the 'value' of an experimental design for a given deployment of effort. The objectives of Chapter 6 were to determine the relative importance of grazing, insolation

and desiccation on microalgal standing stock. The following table lists some of the experimental approaches that may be considered.

Table 1) Alternative experimental designs for allocation of sampling effort between various control and experimental considerations.

	Allocation of sampling effort						Total sampling units		
Option	sites	sampling dates	blocks	factors	levels	controls	experimental	control	combined
a)	3	3	5	3	2	2	270	540	810
b)	3	3	5	1	2	2	90	180	270
c)	1	3	5	3	2	2	90	180	270
d)	3	1	5	3	2	2	90	180	270
e)	1	1	5	3	2	2	30	60	90
f)	1	1	5	3	2	1	30	30	60

In Table 1 option (a) is the most desirable but is highly impracticable because of the large number of sampling units needed. The problem is partially overcome in option (b) where two factors are removed from the design (grazing and shading for example). This approach seems most likely to generate significant results for the particular factor considered, since the design, preparation and sampling strategy are not compromised by considerations relating to any other factor (for example, see discussion of vertical zonation, Chapter 5). However, even if three separate studies like (b) were conducted, each considering a different factor, little would be learned of interactions between the factors or of the relative importance of each. A more informative outcome may be gained by options c to f in which interactions and the relative importance of each factor are obtained at the expense of generality. In other words detailed information is gained about the effects of all three factors of interest, but it remains uncertain whether or not the results are relevant to other locations or other sampling occasions.

The solution to these difficulties is to ensure that the hypothesis being tested is relevant to the question of interest about the community under investigation. In this thesis I was interested in determining the hierarchy of factors affecting microalgal abundance at one shore, Port St Mary (approaches e or f). Had I wished to investigate the relevance of a single factor on several shores in the locality or on several occasions approach b or c would have been more appropriate. Sampling effort was reduced further by using treatments that would minimise the number of controls required (approach f). For example, removing limpets rather than excluding them with cages which would have necessitated inclusion of cage controls in the design.

Decisions on appropriate experimental design can be considerably assisted by preliminary descriptive studies to determine the spatial and temporal variability within the community and the spatial scales at which this variability operates. Preliminary work will indicate the appropriate number of replicates assigned to each experimental treatment (Hurlbert, 1984), and will provide information on appropriate levels of replication between sites or sampling occasions (Hairston, 1989). For instance, if a system shows greater temporal variability than spatial variability, as was the case for epilithic microalgae studied here, then replicating experiments between different sampling occasions (e.g. different seasons or years) may be more informative than replication between sites.

In conclusion, since 'nature has no stake in our understanding of its interactions it may not be possible to carry out all desirable experiments' (Hairston, 1989), and research will require compromise between design, execution and analyses.

8.4 THE IMPORTANCE OF 'BOTTOM UP' AND 'TOP DOWN' PROCESSES AND REGULATION OF COMMUNITY STRUCTURE ON ROCKY SHORES

Community structure in the intertidal is influenced by a combination of abiotic and biotic factors. These can be categorised into 'bottom up' and 'top down' factors. Bottom up regulation describes a situation where community structure is regulated by factors, largely physical, affecting lower trophic levels, such as availability of space, supply of nutrients or desiccation stress. For example, increased food supply leading to increased diversity or abundance of herbivores (see Karr *et al.*, 1992; Menge, 1992).

Top down regulation describes conditions where lower trophic levels, such as algae, are directly or indirectly controlled by the activities of higher trophic levels. For example, exclusion of limpets from the rocky intertidal leads to increased abundance of diatoms, ephemeral algae and subsequent succession by macroalgae (Jones, 1948; Southward, 1964). Similarly, exclusion of a dominant predator may cause an increase in the abundance of prey species, but can reduce the diversity of the community as a whole as the prey species out-competes other shore dwellers (Paine, 1966; Paine, 1974).

In reality most communities are regulated by a combination, or interaction, of bottom up and top down processes (see Karr *et al.*, 1992; Menge, 1992) and several recent studies support the idea that these processes are intimately linked (Rosemond *et al.*, 1993; Posey *et al.*, 1995).

The present study has emphasised the importance of both types of regulation on Manx shores. Here, microalgal abundance appeared to be regulated directly by physical conditions experienced whilst the tide was out and by grazing whilst the tide was in (Chapters 5 and 6). Spatial and temporal variations in the relative impact of theses factors may influence community structure as a whole. Here, I describe microalgal abundance

using simple models to help illustrate the influence of abiotic and biotic factors on both microalgae and community structure as a whole.

Intertidal microalgae are adapted to low light levels; however, at especially low irradiance growth will be restricted (Stahl *et al.*, 1985; Lamontagne *et al.*, 1989). During the winter and early spring, optimal conditions are found on the upper shore, since at lower levels light becomes limiting (Figure 2 a_i and b_i). With the onset of summer, insolation and desiccation stress render the upper shore less attractive and more favourable conditions for growth are found progressively lower down the shore. The more susceptible algae are eliminated from the upper shore (Figure 2 b_{ii}) whilst more resistant ones remain, but in reduced abundance (Figure 2 a_{ii}). The distribution and abundance of these algae appear to be regulated by stresses experienced whilst emersed ('bottom up' factors). Similar seasonal variations in vertical zonation have been observed for cyanobacteria living in intertidal mud flats (Admiraal and Peletier, 1980), and ephemeral macroalgae which progressively colonise lower shore levels as emersion stresses increase (Knight and Parke, 1931; Rees, 1935). However, for microalgae on rocky shores, the situation is complicated by seasonal variations in grazing intensity ('top down' factors).

As the more favourable tidal level for the algae moves from the upper to the mid shore, during the early summer, grazing intensity also increases. This change in feeding activity has its greatest impact on the mid shore because limpet density is greatest there, and so microalgal abundance in this region becomes considerably reduced. By combining these effects it is apparent that in the summer, cyanobacteria, which have a broad range of stress tolerance (Fogg *et al.*, 1973), are most abundant on the lower shore (Figure 2 a_{ii}) whilst diatoms which have more specific requirements are effectively eliminated from the shore (Figure 2 b_{ii}).

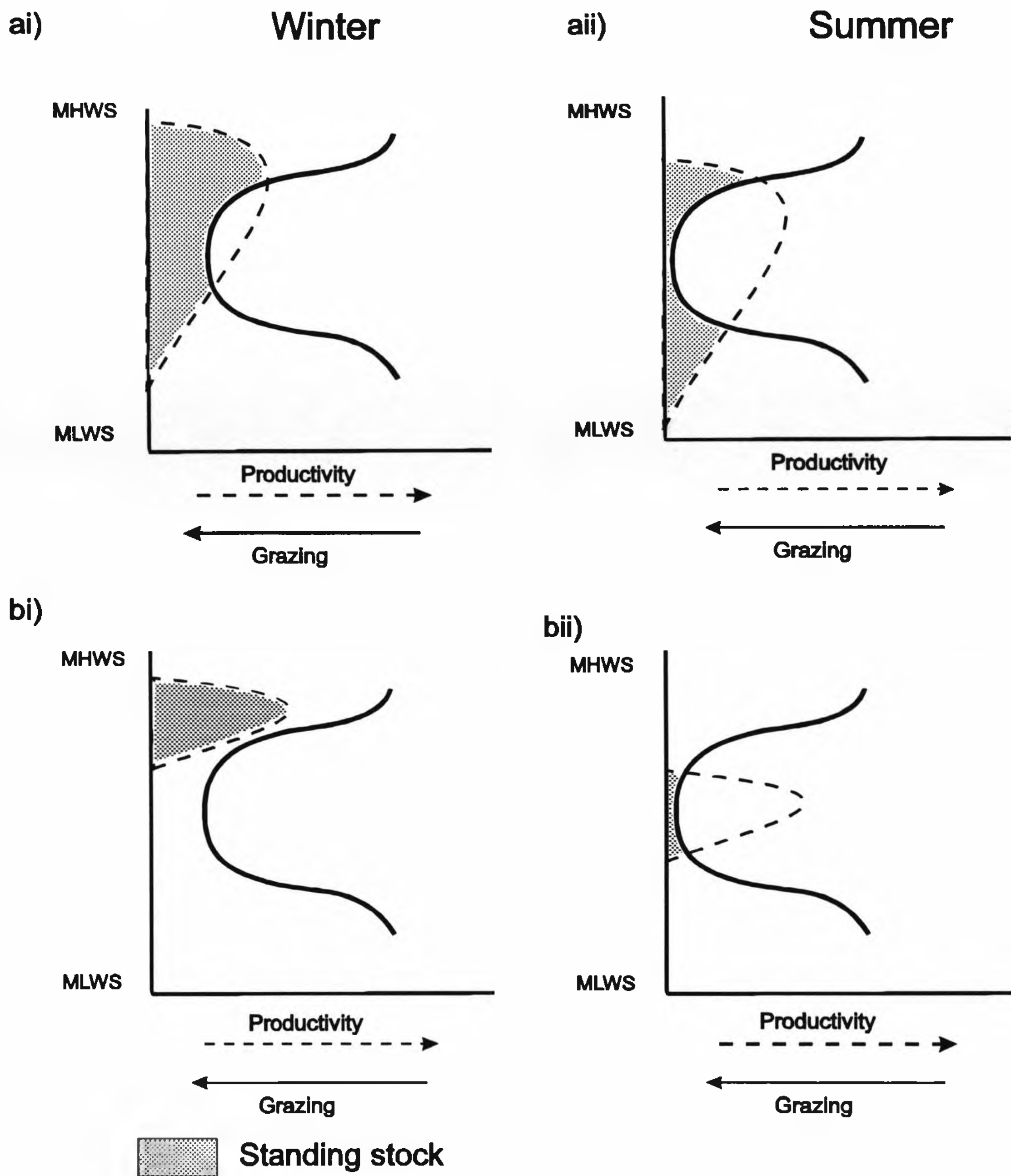


Figure 2) Seasonal changes in microalgal productivity, standing stock and consumption by limpets, on a moderately wave exposed shore, for ai, ii) a resistant alga and bi, ii) a less resistant alga. Note, that for both types of algae the tidal height where conditions are most favourable is lower on the shore during the summer.

Clearly then, this community is regulated by both bottom up and top down factors. At a first glance the system may seem similar to that of plankton communities, where increased numbers of zooplankton reduce the abundance of phytoplankton during the early summer (Parsons *et al.*, 1977). Zooplankton and phytoplankton have similar generation times, and an increase in phytoplankton leads to a relatively rapid (one to two months) increase in the abundance of grazers (Parsons *et al.*, 1977). However, on Manx shores the principal grazers, limpets, are much longer-lived than the microalgae on which they feed. The decline in microalgal abundance during the early summer appears to be predominantly caused by increased emersion stresses (Chapters 5 and 6). Increased demand for food by the limpets, further reduces microalgal standing stock creating a shortfall in the food supply for the limpets during the summer.

Limpets have sufficient food reserves to survive for short periods of time (Santini and Chelazzi, 1995), but beyond this they will starve and their abundance will decline. Hence, microalgal food resources may limit growth and cause mortality of intertidal grazers (Underwood, 1979; Branch, 1981; Hawkins and Hartnoll, 1983; Cubit, 1984; Underwood, 1984b). During the summer, thermal and desiccation stresses will be considerable and these factors may also regulate limpet abundance (Davies, 1969; Davies, 1970; Underwood, 1979). Some molluscs migrate to lower shore levels when resources become limiting, but this is less likely for homing species such as limpets (Branch and Branch, 1981; Branch, 1981). Consequently, limpet density on the mid and upper shore may be set by stressful conditions during the summer. Similar effects have been observed, over various time scales, for fishes in temperate streams and birds in forests. For temperate river fishes growth was limited by a summer depression in resources, whilst for forest birds, population density was regulated by food shortages which lasted for up to 20 years (see Karr *et al.*, 1992).

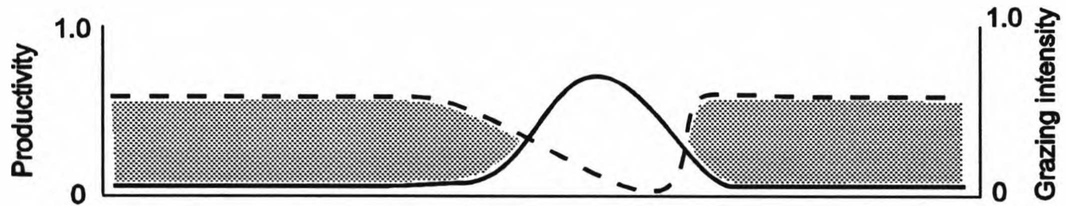
Macroalgal abundance on moderately wave exposed shores could also be influenced by the seasonal restriction to limpet abundance. If limpet density is limited by conditions during the summer, then during cooler months of the year, microalgal productivity will considerably exceed the rate at which it is consumed by the limpets, so creating an opportunity for macroalgal escapes (Figure 3 a).

Variations in microalgal standing stock on sheltered shores (Chapter 5) were generally similar to those on wave exposed shores, but microalgae on these shores were protected from emersion stresses by the dense macroalgal canopy. These shores supported a greater density of grazing molluscs, and whilst I do not have any experimental observations to confirm this, I suspect that production and consumption on sheltered shores may be more closely synchronised (Figure 3b).

Microalgal standing stock was less on sheltered shores, during the winter, than on wave exposed shores, possibly because light levels were reduced by dense macroalgal canopy on the sheltered shores (Chapter 5). Limpet feeding intensity appears to be regulated by temperature (Chapter 5) and so, on both sheltered and wave exposed shores, will increase during the summer. However, unlike wave exposed shores, conditions for microalgae will be favourable on sheltered shores during the summer, since whilst the tide is in, and the macroalgal canopy is supported, irradiance on the rock surface below the canopy will be favourable for microalgal growth. However, when the tide is out microalgae will be protected from excess insolation and desiccation by macroalgae which, when not supported by seawater, will almost completely cover the shore. Hence, seasonal variations in microalgal productivity and grazing intensity will be more synchronous (Figure 3 b). Therefore, despite having lower overall productivity, sheltered shores can support a greater density of grazers (see chapter 5) than wave exposed shores. As a consequence of the greater grazing intensity, recruitment of

macroalgae or invertebrates to areas of bare rock beneath the canopy will be minimal.

a) Moderately wave exposed shore



b) Sheltered shore

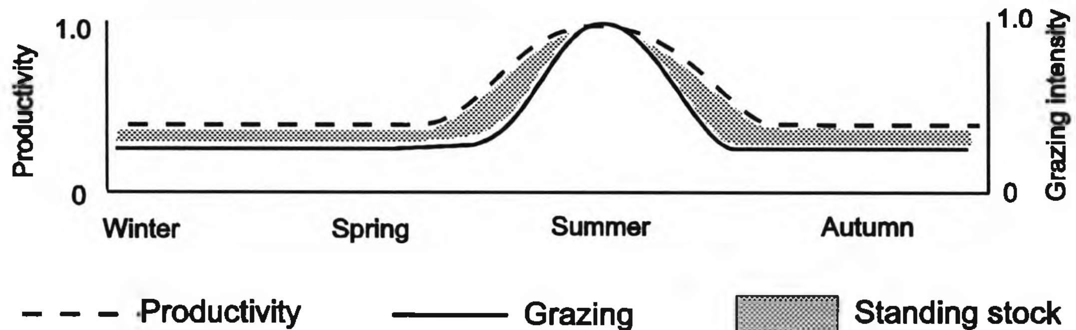


Figure 3) Speculative diagram showing seasonal variations in microalgal productivity and grazing intensity (arbitrary units). On a) a moderately wave exposed shore and b) a sheltered shore.

A combination of the 'bottom up' and 'top down' factors described may help to stabilise intertidal community structure on moderately wave exposed shores. For example, consider a situation where macroalgal cover is abundant (Figure 4a). Conditions at their most limiting, during the summer, will be ameliorated by the presence of the algal canopy which shades both the microalgae and the limpets. Limpets are abundant as food is not scarce and emersion stresses are moderated. On the shore studied here macroalgal abundance varies in a cycle lasting several years (Hawkins and Hartnoll, 1983). Reduced abundance of canopy occurs when the algae age

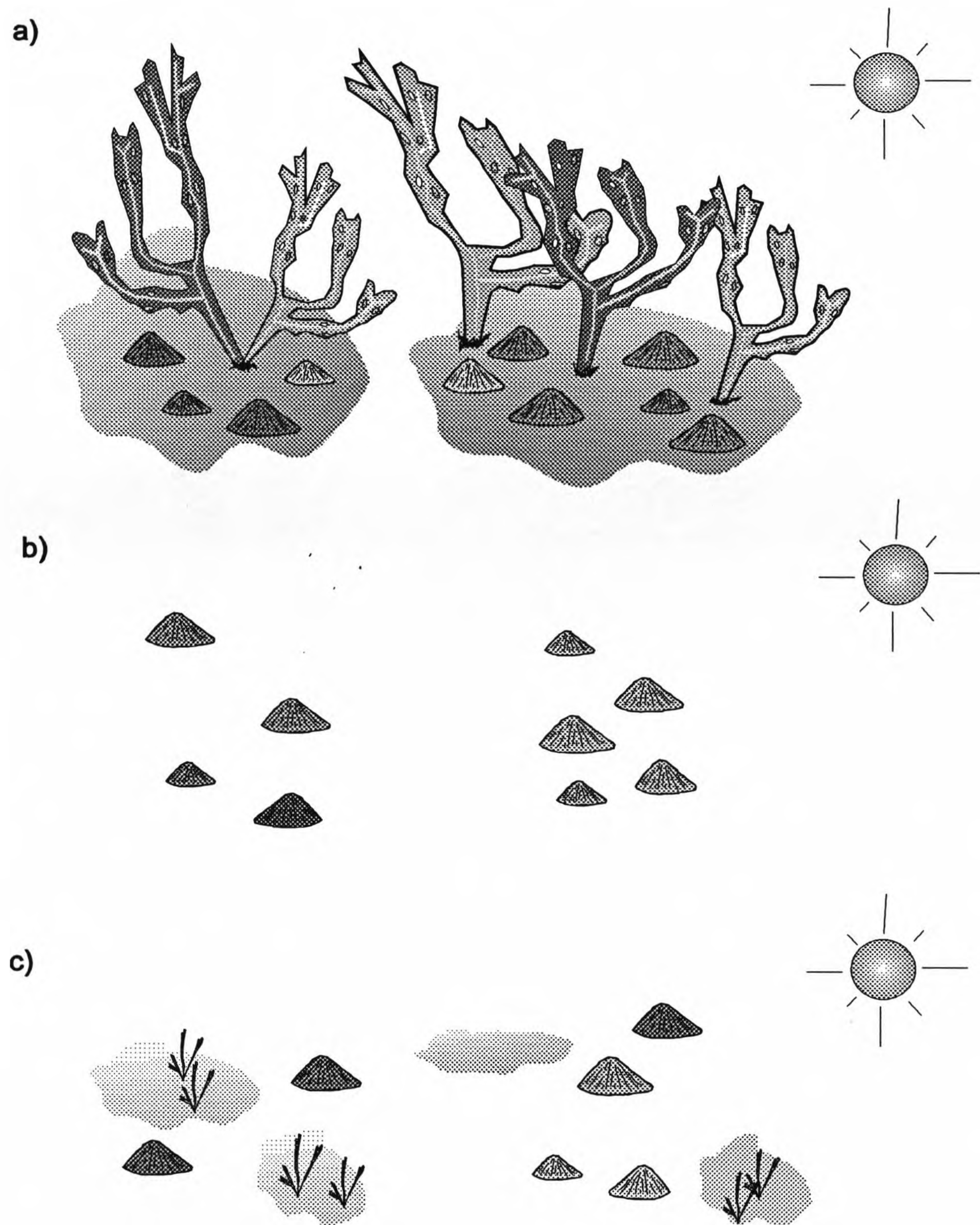


Figure 4) Interactions between macrobiota and microbial film during summertime emersion. a) Mature *Fucus* provides shade which protects microalgae and limpets from desiccation and insolation stress. b) Macroalgal canopy dies or is removed by storms , microalgal productivity reduced by insolation stress. Grazers suffer from insolation stress and shortage of food. c) Limpet density declines and consequently grazing becomes more patchy. Microalgae and juvenile *Fucus* plants start to grow in less grazed areas. Eventually *Fucus* plants mature and their canopy provides shade for limpets and microalgae, on the rock beneath. Microalgal growth and limpet abundance increase, and the shore returns to the situation described in (a).

and die, or are dislodged by storms. At this time limpets and microalgae will become exposed to increased emersion stresses (Figure 4b). Microalgal productivity during the summer will be reduced as, in the absence of shade from the macroalgae, microalgae are photoinhibited (e.g. Stahl *et al.*, 1985; Lamontagne *et al.*, 1989). A combination of reduced food supply and increased emersion stresses causes limpet abundance to decline (Figure 4b). As the limpet density decreases the possibility of recruitment to the macroalgal population increases, since germlings can escape grazing (Figure 4c) and grow in to mature plants. Conditions on the rock surface below, become more favourable for both microalgal productivity and limpets as the *Fucus* canopy becomes re-established. Hence, limpet abundance increases once again (Figure 4a).

8.5 FURTHER WORK

There is a clear need to develop rapid, relatively non-destructive methods for directly sampling and quantifying epilithic microalgae at a scale which can integrate localised variations in abundance. Possibly this could be achieved by brushing the substratum, suspending the algae in solution, and counting by cytometry, for example.

Methods for estimating primary productivity should be compared in field conditions and a standard method developed. Seasonal differences in primary productivity, standing stock and removal of algae by grazers could then be determined in parallel to gain a more complete understanding of the factors regulating the abundance of both the microalgae and the grazers. Similar studies have been conducted for microalgae and herbivorous reef fishes (e.g. Polunin and Klumpp, 1992). Seasonal variations in limpet foraging activity have been identified but the causes of these patterns need to be clarified. There are considerable taxonomic uncertainties over some of the principle constituents of microalgal films; diatoms, cyanobacteria and

Protozoa, (e.g. Underwood, 1984c; Castenholz, 1992); when these are resolved species interactions within the film can be examined more fully. For example, a greater understanding of the species which cause facilitation (e.g. enhancing colonisation of filmed rock by barnacle cyprids, Chapter 7) or inhibition (e.g. reduced colonisation of filmed rock by ephemeral algae, Chapter 6) within these films.

8.6 CONCLUSIONS

This study has refined methods for estimating microalgal abundance and has developed a system for recording limpet feeding activity in the field. In general, microalgal abundance was similar between shores, zonation with tidal height was apparent and was predominately influenced by the distribution of grazers and by physical conditions during emersion. The most striking feature of variability in these communities was the extent to which abundance was regulated by seasonal changes in both grazing activity and conditions experienced during low tide emersion. As a result of increased emersion stresses microalgal abundance declined considerably during the summer, and possibly limited the abundance of limpets which graze on these algae.

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APPENDICES

Appendix 1. *Summary of Methods Used to Extract Chlorophyll from Epilithic Microalgae.*

Existing protocols were followed as far as possible and references to these are given. However, for consistency, some extraction durations and temperatures were modified as described.

Hot methanol 100% or 95%: Heat to boiling (approx. 65°C), boil for 2 min, cool for 3h. (see H.M.S.O., 1983; Marker, 1994). Samples were acidified by adding 0.2ml hydrochloric acid (0.3M) to 20.0ml of extract and mixing well. Solutions were allowed to stand for 5min., then neutralised by adding small amounts (0.05ml) of dimethylaniline (0.3M) from a micro-pipette until pH increased to between 2.6 - 2.8 (Moed and Hallegraeff, 1978).

Cold methanol 100% or 95%: Room temperature (20°C) for 5h. (Adapted from Strain and Manning, 1942). Acidification as for hot methanol.

Cold chloroform : methanol 2:1: Room temperature (20°C) for 5h (See Dye and White, 1991).

Hot ethanol 90%: Heat to approx. 65°C leave for 2 min (ethanol did not boil), cool for 3h. (adapted from Nusch, 1980; Marker, 1994). Samples were acidified by adding small amounts (0.05ml) of hydrochloric acid (0.3M) from a Gilson pipette until pH was within the range 2.6 - 2.8.

Cold ethanol 90%: 4°C for 24h. (Marker, 1994). Acidification as for hot ethanol.

Hot acetone 90%: Heat to boiling (approx. 65°C), boil for 2 min, cool for 3h. (adapted from hot methanol method, H.M.S.O., 1983). Samples were acidified by adding 0.06ml hydrochloric acid (1% v/v) to 20ml of extract and mixing well. Solutions were allowed to stand for 5min (H.M.S.O., 1983).

Cold acetone 90%: 4°C for 24h. (H.M.S.O., 1983). Acidification as for hot acetone

Appendix 2: *Chlorophyll a Determination*

Chlorophyll a Determination - Uncorrected

Chlorophyll a concentrations were determined using the formula shown in the standard method (Appendix 3). Specific absorbance coefficients (SAC) were: methanol, 13.0 ; Chloroform : methanol, 13.0 (methanol value used as a coefficient was not available for the mixture); Ethanol 12.2; Acetone 11.2 (Marker, 1994).

Chlorophyll a Estimate Corrected for Degradation Products (Quantitative)

$$= \frac{13.0\,[3.0 \times (Å_{665} - Aj)] \times v}{d \times V}$$

Where:

13.0 is the SAC for methanol (SAC's listed were substituted as appropriate)

Ai = $Å_{665}$ after acidification

$$Aj = \frac{(v + 0.3 + \text{vol. acid added} + \text{vol. base added}) \times Ai}{v}$$

Chlorophyll a Estimate Corrected for Degradation Products (rapid qualitative method)

Degree of degradation $= \frac{Å_{430}}{A_{410}}$

Values >1 degradation probably <50%; values < 1 degradation probably > 50%.

Chlorophyll a Estimate Corrected for Chlorophylls b and c =

$$\frac{(11.6 \times Å_{665} - 1.31 \times Å_{645} - 0.14 \times Å_{630}) \times v}{d \times V}$$

This formula (from H.M.S.O., 1983) was used for methanol, ethanol and acetone.

3.0 is a constant for methanol (2.43 was used with acetone and ethanol)

$Å_{665}$ = net absorbance of solution at 665nm

$Å_{645}$ = net absorbance of solution at 645nm

$Å_{630}$ = net absorbance of solution at 630nm

$Å_{430}$ = absorbance 430nm

$Å_{410}$ = absorbance 410nm

v = final volume of solution

d = path length of cell

V = surface area of sample

Appendix 3

Standard Method for Chlorophyll Extraction from Epilithic Microalgae

A) Sample Collection

Where possible collect microalgae by chiselling rock chips (each approximately 2cm x 2cm) from the substratum. Scraping algae from the rock surface is less satisfactory but may be the only option on friable substrata such as sandstone (see Hill and Hawkins, 1990). Samples should be of similar rugosity and size in order to minimise variation. Ideally a pilot study should be conducted to determine the minimum number of samples need to overcome natural spatial variation. Seal samples in separate plastic bags and transport in cool dark conditions.

b) Sample Pre-treatment

Scrutinise samples for small macrobiota (especially macroalgal germlings and barnacles) and reject these. Rinse in filtered seawater (filter should be fine enough to remove algal cells from the water) to remove dirt and loose rock fragments. Immerse samples in filtered seawater for 30 min. to rehydrate. Blot to remove surplus water leaving the rock moist and damp. Proceed to (c) or (d) as quickly as possible to minimise drying.

c) Storage

Ideally work with fresh material. If necessary individually bagged hydrated samples may be stored in darkness at 4ºC for a maximum of one week.

d) Extraction Procedure

Reagents: 95% methanol gives the best extraction but is toxic. Follow appropriate precautions. 90% ethanol is less toxic but also less efficient, especially for extractions from cyanobacteria. Substitute where operating requirements for methanol cannot be met.

Place each sample into a separate wide mouth screw top jar. These should have a similar diameter to the rock chips in order to allow complete immersion using a small volume of solvent (e.g. BDH 60ml glass Ref. 215/0345/01, or 240ml LDPE Ref. 215/0360/13). Weigh each jar complete with lid and sample (weight 1). Add solvent, the exact volume is not critical but should be similar for each sample and sufficient to give a final Å665 of between 0.05 and 0.7 (H.M.S.O., 1983). For example, 5ml per $1cm^2$ of rock surface. Addition of solvent may be aided by using a large volume plastic syringe or filling all jars to a pre marked level. Cold extractions are preferable since they require less monitoring and produce stable chlorophyll extracts. Hot extractions (ii) may be used where more rapid determinations are required.

i) Cold Extraction

Tighten jar lids to prevent evaporation and leave in a cool (room temperature) dark place for 5 - 24 hours.

ii) Hot Extraction

Leave jar lids loose. Place jars in a water bath and heat to boiling (1 hour approx.). Boil for 1 minute (steady stream of bubbles) then remove from waterbath tighten lids to prevent further evaporation, leave in dark at room temperature and cool (3 hours max.).

After extraction wipe the outside of the jars dry and weigh again (weight 2). Take care not to shake the jars or stir up any fine particles which may have settled out of solution

e) Centrifuging

Centrifuging to remove suspended material is not necessary unless Å750 (see f below) exceeds 0.005 per 1cm path length of the optical cell.

f) Spectrophotometric Determination

For checks on spectrophotometer calibration see Marker (1994). Use two high quality optical cells. Check these initially with the pure extraction solvent to ensure they are evenly matched. Record any differences and use these to adjust readings made. Always use cells in the same orientation. Remove a portion of the solvent/chlorophyll mixture from the middle of the solution taking care not to disturb any particulate matter which may have settled on the bottom of the jar. Use one cell to read Å665 and Å750 for each sample. Fill the second cell with pure solvent and use to zero the spectrophotometer between sample readings.

G) Measuring Sample Surface Area

Measure the surface area of rock from each jar e.g. using video and image analysis software. This method will only give an estimate of surface area in two dimensions. Rugosity may add considerable area to samples, if this cannot be standardised between samples then total surface area should be measured using a profilometer.

H) Determine Chlorophyll Concentration

Calculate the net absorbance of each chlorophyll solution (Å665 - Å750). Calculate the final volume of extraction solvent by subtracting the initial weight (weight 1, section d) from the final weight (weight 2, section d) and multiply by the weight of the solvent g ml^{-1}.

$$\text{chlorophyll concentration } \mu g\ mm^{-2} = \frac{13.0 \times Å_{665} \times v}{d \times V}$$

Where:

13.0 is a constant for methanol (if ethanol is used substitute 12.2)

A_{665} = net absorbance of solution at 665nm

v = final volume of solution

d = path length of cell

V = surface area of sample

I) *Correction for Presence of Degradation Products or Chlorophylls b and c*

Spectrophotometric methods (e.g. H.M.S.O., 1983) are problematic with methanol. The determination can be made using acetone but chlorophyll extraction is very poor. HPLC is suggested as an alternative (see Mantoura and Llewellyn, 1983; Wright and Shearer, 1984.

SECTION IV

Collaborative Work

The following manuscripts, which resulted from collaborative work with colleagues at Port Erin Marine Laboratory and at the Department of Foetal and Infant Pathology at Liverpool University, are included in a pocket on the inside back cover.

Thompson, R. C., Wilson, B. J., Tobin, M. L., & Hawkins, S. J., 1996. Biologically generated habitat provision and diversity of rocky shore organisms at an hierarchy of spatial scales. *Journal of Experimental Marine Biology and Ecology*, **202**, 73-84.

Pope, J., Thompson, R. C., Hawkins, S. J., Norton, T. A., Veltkamp, C. J. and Howard, C. V. in prep. The use of confocal laser scanning microscopy, scanning electron microscopy and phase contrast light microscopy to visualise marine biofilms.

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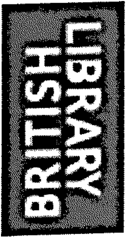

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The use of confocal laser scanning microscopy, scanning electron microscopy and phase contrast light microscopy to visualise marine biofilms.

by

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ABSTRACT: Two of the traditional methods of examining natural biofilms - Phase Contrast Light microscopy and Scanning Electron microscopy (SEM) - are compared to the results obtained with the much newer technique of Confocal Laser Scanning microscopy (CLSM). This last technique is shown to be vastly superior in its ability to produce images of live microorganisms embedded beneath at least 90μm of mucilage, and layers of overlying organisms or non-living debris. Although further work will be required before the full potential of the technique can be realised, it is a valuable new tool for the study of living organisms in hydrated, undisturbed biofilms on both natural and fouled substrata.

We have also developed a simple method for relocating particular sites on slides or natural substrata so that the same cells can be repeatedly observed.

KEYWORDS: Confocal Microscopy, biofilm, microalgae, S.E.M., self-registration

INTRODUCTION

Biofilms are ubiquitous in the natural environment and cover most hard objects in aquatic habitats. They consist largely of water held by highly hydrated extracellular polymers (Flemming 1993). Embedded in the mucus matrix are bacteria, fungi, bluegreen algae, diatoms, flagellates and protozoans, the proportions of each varying seasonally and in different habitats. They may be very abundant, with estimates of 3 $x10^{-7}$ bacteria (Hendy 1951) and 2.7×10^{-5} diatoms cm^{-2} (Edyvean & Moss 1986) in marine biofilms. Dry matter values range from 10-50 mg cm^{-3} of film (Wimpenny & Peters 1987).

Their ecological importance can hardly be exaggerated. Biofilm slime and the microbes it contains are readily consumed, rendering both cells and recently absorbed dissolved organic matter available to protozoan and larger browsers (Hill & Hawkins 1991, Bernhard & Bowser 1992, Decho & Lopez 1993). In freshwater habitats organisms inhabiting biofilms may become highly mineralized. These biofilms dominate the reactivity of the rock/water interface and may determine the type of minerals that become part of the riverbed sediments (Konhauser et al. 1994). Films also play an important role in the natural biodegradation of pollutants which are absorbed, immobilized, or transformed to less toxic compounds (Russell et al. 1991, Schultze-Lam et al. 1993; Hintelmann et al. 1993, Takada et al. 1994),. Films are considered "fundamental to the the ability [of wetlands] to degrade complex organic contaminants" (Hamilton et al. 1993). These abilities have been harnessed by Man for the purification of water (Takasaki et al. 1988, Bovendeur et al.1990, Flemming,1993), mine waste detoxification (Whitlock 1990) and metal recovery (Green & Bedell1990). Films in freshwater may also, however, act as reservoirs for pathogenic

microorganisms including *Legionella* (Marrao et al. 1993, Szewzyk et al. 1994).

In the sea, biofilms influence the settlement of the larvae of sessile invertebrates (Holmstroem et al.1992, Tritar et al. 1992, Tamburri et al. 1992) and the ability of rock dwellers such as seaweeds and barnacles to adhere (Norton1983, Neal & Yule 1994)

Biofilms impinge on human activities directly as they are a major contaminants of pipes, heat exchangers and filters (Hutson et al.1989, Afanas et al. 1992). The reducing conditions beneath the film encourage corrosion in both metals and concrete (Terry & Edyvean 1986, Callow & Edyvean 1990) and reduce the efficacy of cathodic protection (Dexter & Linn 1992). Biofilms on ships' hulls greatly increase skin friction, a 1mm thick film reducing a vessel's speed by 15% (Lewthwaite et al. 1985). They also encourage fouling by larger organisms (Rao 1990).

Despite the importance of biofilms and the intensive work on them, surprisingly little is known about the structure and distribution of the communities within the film (Hall 1992), largely because of technical difficulties in their study. Scanning Electron Microscopy (SEM) and Cryo-SEM, have been used to visualise the surface of intact biofilms (Callow 1986, Maclulich 1986, Hill & Hawkins 1990, Burkholder et al. 1990, Blenkinsop & Loch 1994), but often the image is obscured by a superficial coating of mucilage. Also, the specimen is killed by preparation and cannot therefore be used repeatedly to determine rates of cell division, movement or growth of the embedded organisms.

Preparation for standard SEM Involves complete desiccation of the specimen resulting in a loss of the three dimensional structure of the film, and also, perhaps, exaggerating the importance of diatoms whose frustules are unaffected by drying. Such problems are avoided if the biofilms can be viewed wet. Unfortunately, the abundant slime blurrs the image for light

microscopy. Phase contrast or Interference contrast have been used (Austin 1983, Evans 1988, Leff et al.1993), but for high resolution require thin preparations not more than a few micrometers thick (Grimstone & Skaer 1972). Thus the film must be sectioned or squashed and loses its structure.

Marine films may be up to 2mm thick (Callow & Edyvean 1990) although in turbulent water rarely exceed 1mm and are usually much thinner (Wimpenny & Peters1987). As the microorganisms are distributed throughout the mucilage matrix, a major problem is to view the underlying organisms through the overlying layers of embedded cells and debris. Environmental SEM has been used recently to visualise the topography of hydrated biofilms, with some degree of success (Little et al. 1991, Lavoie et al. 1995), but three dimensional imaging is not possible.

Confocal laser scanning microscopy has a number of advantages over these methods. The ability of the technique to optically section material non-invasively allows fully hydrated specimens to be examined. The resolving power of the confocal microscope greatly exceeds that of the conventional light microscope; the theoretical maximal resolution mean is about 1.4 times better. Thus, particularly in epifluorescence imaging mode, objects down to about a size of 250 nm can be seen. Any highly fluorescing object smaller than this may also be detectable, although its size will dilate up to the point spread function. Confocal imaging can also be used in reflectance mode, although with biological specimens this is generally less satisfactory than flourescence techniques. The ability of confocal microscopes to scan an optical section of about 0.5μm thickness allows for the reconstruction of these perfectly registered sections to be performed to produce three dimensional images (Wilson 1990, Lichtman 1994).

The aim of this paper is to assess the use of confocal microscopy to view biofilms and to compare the results obtained with those from Phase Contrast light microscopy and Scanning Electron microscopy.

MATERIALS AND METHODS

Materials. Our test material was marine biofilms cultured on glass microscope slides placed on purpose built perspex slide racks in the brackish waters (mean salinity 28.7% , Zheng 1995) of the Princes Dock, Liverpool at a depth of 0.3 metres. Slides were removed randomly at intervals and fixed in 0.5% glutaraldehyde in filtered sea water.

Natural substrata, (rock chips, mussel and limpet shells) were collected from the intertidal zone at Port St Mary in the Isle of Man and were despatched in moist packaging to the Department of Foetal and Infant Pathology at the University of Liverpool. On receipt, all samples were fixed in glutaraldehyde and stored in filtered sea water.

All the films were stained for one hour in 0.3% sodium fluorescein solution made up in filtered sea water. The slides were then rinsed in sea water and stored in glutaraldehyde and filtered sea water for the best preservation of cells.

Self-registration. In order to visualise identical areas under both CLSM and SEM, a method of self-registration was required so that specific areas could be relocated for repeated viewing. The registration needed to be in the form of a raised grid for the purposes of SEM (which visualises only surfaces), made from a material that could be seen under CLSM using the filters necessary for visualising the biofilm itself.

The first procedure tested was to lay strands of glue mixed with fluorescent dye over the specimens in a grid pattern. Epoxy resin worked better than standard multi-purpose glues and super glues, but even this was not entirely satisfactory. Often strands moved when being viewed (even under CLSM) and sometimes the entire grid worked loose, or dissolved when being immersed in alcohol (a step in the preparation procedure for SEM).

We also tried wrapping thin fuse wire around the slides and specimens. This had the advantages of producing a grid that was raised and irregular, making self registration easier, but there was a problem with visualisation under CLSM. Even the smallest diameter fuse wire was raised so high above the biofilm surface that obtaining images showing both the wire and the biofilm greatly reduced the clarity of the resultant pictures.

The method finally adopted made use of copper Transmission Electron Microscopy grids. Prior to staining, 300μm mesh size copper grids were glued to the surface of the slides or natural substrata with epoxy resin and allowed to dry. The grids, being small (3.05 mm in diameter), lay close to the specimen surface and, when surrounded generously with epoxy resin, they adhered securely to the surfaces even after SEM preparation procedures.

Images were taken from areas within the grids and registered by putting their position on drawings of both the specimen and the grid. Multiple images per grid and per specimen were taken.

Confocal Laser Scanning Microscopy. Specimens were viewed using a Biorad 600 Confocal laser scanning microscope(CLSM). A x60 1.4 NA lens was used with water as a visualising medium. Simultaneous non-confocal phase contrast imaging was also performed, using the second channel of the CLSM. Slides immersed in the dock for 40-50 days were used to compare CLSM and SEM, since by this time several well developed layers of organisms were present.

Scanning Electron Microscopy. Specimens were prepared using a method of freeze dehydration (Veltkamp *et al*, 1994) and were viewed in a Philips XL 30 scanning electron microscope at accelerating voltages of 10-15 kV. Precise registered areas were obtained by using the drawings prepared from the CLSM procedure and by use of the confocal pictures themselves. Even though widely different from the surface seen under SEM, some of the confocal pictures enabled confirmation of the registration by

means of relocation of particular diatoms and microalgal colonies. Where there were no distinctive organisms, registration depended soley on the position within the grid.

RESULTS

The Confocal microscope had the facility to take both phase contrast and CLSM images from the same specimen at the same time, The use of superimposed SEM grids allowed identical locations on a sample to be readily refound for repeated examination, even after the specimen had been removed from the microscope, thus allowing identical fields to be examined under SEM.

The increased resolution and optical sectioning ability of the confocal microscope allow the identification of some microalgae to generic level or better (Figures A & B). The image was also 'cleaned up' under CLSM as non-living debris was effectively 'blacked out' (Figure Ci & Cii cf. to Ciii). The diatom seen as frustules in Bi and Bii did not register under CLSM, presumably because they were non-living, but a cyanobacterial strand previously obscured was now apparent. Fluorescing cells are also much easier to count than phase images (Figures Cii cf. to Ciii).

As the films were composed mostly of water (70-95% of the wet weight), the desiccation required prior to SEM collapsed the structure of the film (Figure E), whereas under CLSM the film remained hydrated and in its natural state . Even a very thin layer of mucilage obscured underlying organisms under SEM, and debris greatly confused the picture (Figures Di & Fi),. These obstacles did not effect CLSM and much clearer, highly resolute images were obtained (Figures Dii & Fii).

Where there were large amounts of mucilage cover, visualising organisms deep in the film produced an extremely poor image with phase

contrast light microscopy (Figure Gi), but the CLSM was able to penetrate it and produce clear images of the underlying organisms (Figures Gii).

Layers of overlapping organisms also greatly lowered the resolution of the images obtained with both phase contrast and SEM , but with CLSM it was possible to produce optical sections down through a film with little interference from the layers of cells above (Figure Hi cf. to Hii). This also allows it to be used to survey communities on natural substrata with uneven microtopography (Figures H - J).

The laser used in the CLSM could not only penetrate mucilage cover and overlying organisms, in many cases it was even able to observe organisms beneath the copper grid lines (Figures Hi cf. to Hii).

DISCUSSION

Marine biofilms were considered a good test of the various microscopical techniques as they contained a variety of organisms including solitary and colonial diatoms, bluegreen algal filaments, bacteria, seaweed sporelings and debris.

Where the film was very thin and there was no superficial mucilage cover, it was possible to identify many of the organisms with SEM As SEM views the surfaces of objects, the silicon frustules of diatoms lying on top of the film were clearly visualised, but beneath mucilage little could be distinguished. Under CLSM however, the entire depth of the biofilm could be readily visualised as there was no masking of the organisms by mucilage cover up to 90μm thick, or the overlying organisms. Whether a thicker slime containing additional layers of cells would prove more of an obstacle was not tested, but CLSM can resolve images over 100μm within solid bone (Howard et al. 1985).

There were problems identifying some of the organisms under CLSM as it only images fluorescence. The majority of individuals found were diatoms, which can constitute over 90% of the cells in marine biofilms (Rao 1990), and we know of no fluorescent stains for silicon, which forms their outer 'shell.' This made some diatoms difficult to identify in some pictures, although the confocal microscopes' ability to switch to phase contrast sometimes overcame this problem. As the pigments of different microalgal groups are very different, the use of filters, although not aiding specific identification, may allow the distinctive fluorescence of each group to be readily distinguished for rapid survey purposes. Biofilms from other environments where diatoms do not predominate might be less problematical, but no doubt bacteria will present their own difficulties.

CLSM is an extremely versatile method for visualising living biofilms both on natural substrata and glass slides, using either the natural fluorescence of the embedded organisms, or fluorescent stains. The principle benefit of the technique is the ability to view living material through thick hydrated mucilage without disturbing surface layers, and to monitor the organisms inhabiting different layers within the film. Moreover, the film can be viewed at intervals whilst being 'cultured' in the natural environment. It offers a facility to follow the movement, growth and development of individual organisms and whole communities within the film.

The technique should greatly benefit recent attempts to use changes in natural microbial biofilm communities to monitor the effects of pollutants in freshwater (Niederlehner et al. 1990, Cairns et al. 1990, Melendez et al. 1993), and as Liu et al. (1993) have said: "Understanding biofilm growth is critical in predicting the ultimate fate of chemicals in the aquatic environment."

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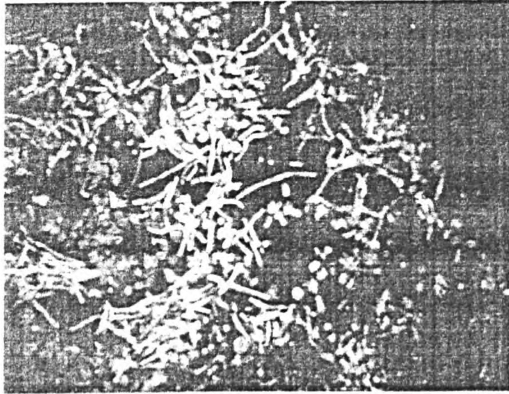

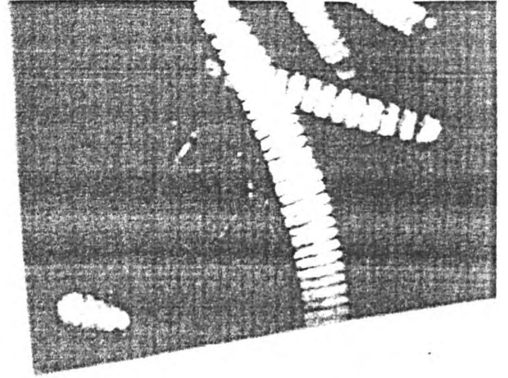

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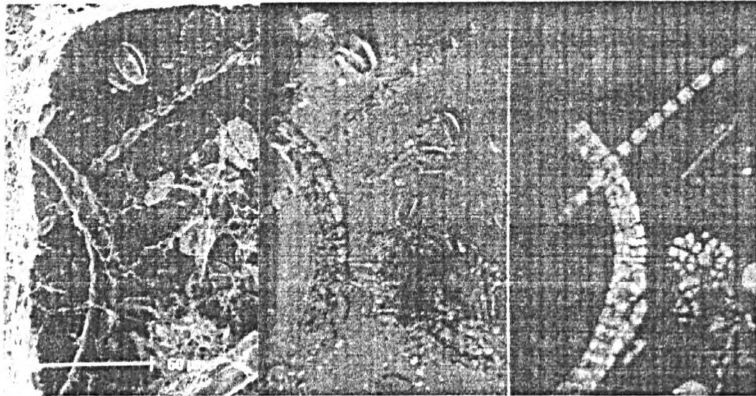

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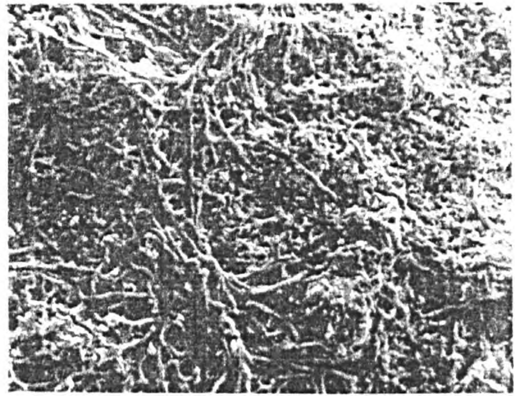

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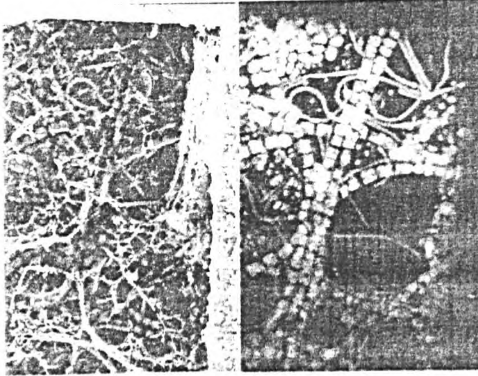

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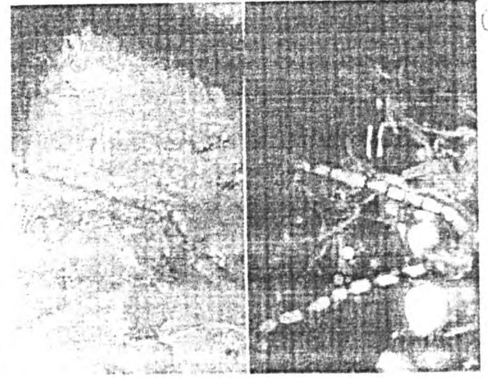

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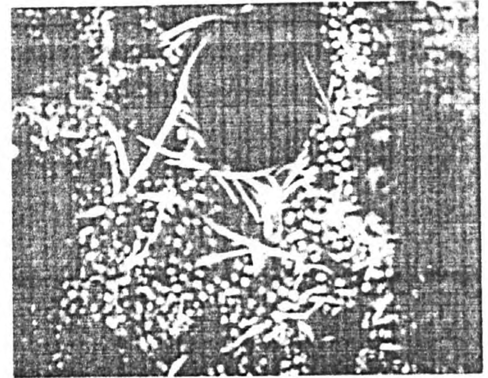

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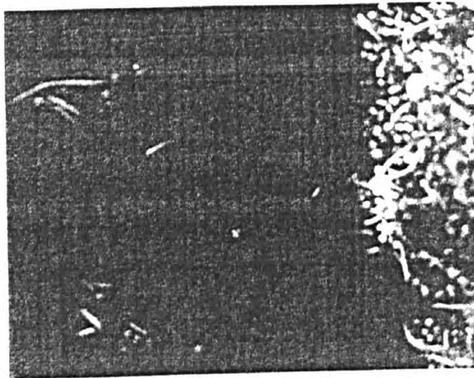

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