ASPECTS OF THE BIOLOGY OF *PORPHYRA* (BANGIALES, RHODOPHYTA) OF THE ISLE OF MAN

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Philosophy

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Every person,

all the events in your life
are there because you have
drawn them there.

What you choose
to do with them is
up to you.

R. Bach

To Mum and Dad

I owe you everything

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ABSTRACT

Various aspects of the biology of intertidal *Porphyra* species present in the Isle of Man were investigated. This involved: observational ecology of four species linked with a detailed seasonality study of a population of *Porphyra laciniata*; assessments of protein levels of four species; the use of electrophoretic techniques as an aid to a study of the taxonomy of Manx *Porphyra* species, and aspects of the culture of the conchocelis phase.

Ecological observations demonstrated a marked seasonality of occurrence with the species *P. linearis* occurring in winter and *P. leucosticta* in summer to autumn. *P. umbilicalis* and *P. laciniata* were present all year round although peaks in occurrence and biomass were observed in spring. The detailed study of the population of *P. laciniata* showed a significant biomass increase in March to April for two consecutive years. The area of shore where *P. laciniata* was studied was shown to be dynamic with dramatic changes at times in the substratum due to the movement of sand in and out of these areas. Mapping techniques proved to be successful in demonstrating the seasonal changes in patch cover and the dynamic nature of the population of *P. laciniata* studied.

P. linearis patches were mapped for two consecutive years and found to occur in almost exactly the same positions from year to year. This is indicative of a population of conchocelis (the previous stage in the life history) being in close proximity or even occurring within the area of the patches of the blade phase. The conchocelis phase (not identified to species level) was found growing in intertidal barnacles close to these *P. linearis* patches and also within the populations of *P. laciniata* in Port Erin.

P. linearis had the highest protein level of all the species measured with a peak level in January of 42.63 % and a mean seasonal level of 35.95 %. *P. leucosticta* had the lowest recorded mean seasonal level (25.07 %).

Only one specimen of the subtidal species *P. miniata* was found during the course of the study and so this species was not involved in the course of the research.

Enzyme electrophoretic techniques were successful in distinguishing between the four species investigated and also demonstrated the presence of a fifth unknown species similar in morphology to *P. laciniata*. Further work using electrophoretic techniques coupled with other morphological observations clearly separated these two species. It is believed (although is not yet conclusive) from comparisons with work carried out by Kornmann (1991) that this unknown species is *P. purpureo-violacea*. Electrophoresis also demonstrated that three morphotypes of *P. umbilicalis* were the same species.

Haploid chromosome numbers were found to agree with those in previous studies. *P. laciniata* had 5 chromosomes and *P. umbilicalis*, *P. linearis* and *P. leucosticta* had 4.

This part of the study demonstrated that the taxonomy of this genus in Britain requires further research in order to be certain of species identity and also of the total number of species present around British coasts.

The culture of the conchocelis phase of *P. laciniata* and *P. linearis* was successful in both free cultures and cultures grown in shells. Some free living cultures were grown for up to three years. The production of conchosporangia did not appear to be a result of a photoperiodic response. Attempts to produce conchospores from *P. laciniata* in both shell and free living cultures was unsuccessful in all conditions tested.

As a result of contamination difficulties the removal of algal contaminants from cultures of conchosporangia of *P. laciniata* using various pH treatments with acidified seawater was tested. This was successful in some treatments and also demonstrated the ability of the conchosporangia to survive acidic conditions (10 % survival in pH 2.0 for 8 minutes).

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The genus *Porphyra* is widely distributed and represented in many different habitats. The research on *Porphyra* species is equally varied, ranging from ecological and taxonomic studies (e.g. Conway, 1964a; Conway, 1964b; Lindstrom & Cole, 1990; Kornmann & Sahling, 1991; Lindstrom & Cole, 1992a) to applied research in the areas of cultivation or culture of economically important species (Tseng & Chang, 1955; Iwasaki & Matsudaira, 1958; Matsumoto, 1959; Suto, 1960; Fujiwara, 1961; Iwasaki, 1961; Yoshida *et al.*, 1964; Iwasaki, 1965; Terumoto, 1965; Dring, 1967; Miura, 1978; Miura *et al.*, 1979; Ji *et al.*, 1981; Merrill *et al.*, 1983; Kato & Aruga, 1984; Araki *et al.*, 1985; Araki *et al.*, 1987; Miura & Shin, 1989; Araki & Morishita, 1990). With studies into detailed aspects of the cultivation of species as a human food crop (Iwasaki & Matsudaira, 1954a; Kurake, 1969; Oohusa, 1984; Waaland *et al.*, 1986; Chen, 1989; Chen *et al.*, 1990; Polne-fuller & Gibor, 1990).

This present study examines some aspects of the ecology, taxonomy and culture of British *Porphyra* species.

Species of *Porphyra* generally have an annual, biphasic, heteromorphic life cycle which alternates between a gametophytic blade phase (haploid - n) and a sporophytic filamentous conchocelis phase (diploid - 2n). However, there are exceptions shown by a few species of this genus. Some of the current knowledge of the life history of *Porphyra* is summarised by West & Hommersand (1981). Kain (1991) also summarised the life history noting that the haploid blade can produce haploid monospores which germinate into other blades. Alternatively, changing environmental conditions causes the blade phase to become sexual, producing non motile male gametes which fertilise the female gametes *in situ* on the blades. The resulting zygote then divides into a variable number of diploid spores (carpospores). These spores develop into the conchocelis filaments which normally live within dead mollusc shells (Drew, 1949; Drew & Richards, 1953;

Ogata, 1955; Campbell & Cole, 1984). Under certain conditions the diploid conchocelis can produce diploid monospores which in turn produce more conchocelis. Alternatively an environmental trigger causes the conchocelis to produce conchosporangia in rows which release conchospores. These conchospores develop into the haploid blade phase.

Blades can be genetic chimeras having two separate sets of cells which differ genetically from each other as a result of the reduction division not occurring until the conchospore germinates (Ma & Miura, 1984; Burzyki & Waaland, 1987; Tseng & Sun, 1989).

The description of the life history of *Porphyra* species brings to light the problem of different terminology used in past literature to describe some of the various types of spores and fertile material (Dring, 1967). Names frequently used for spores are monospore and conchospore. The term monospore was used by early Japanese workers (e.g. Kurogi, 1953; Nakazawa, 1958; Kurogi, 1959; Iwasaki, 1961) to describe the spore released by the conchocelis that gave rise to the leafy thallus. Most other workers used the term conchospore (e.g. Tseng & Chang, 1956; Hollenburg, 1958; Conway, 1964b; Bird, 1973; Waaland *et al.*, 1987; Waaland *et al.*, 1990). The latter terminology will be used in this study, applying the term monospore to regenerative spores of the conchocelis or blade phase. The term carpospore applies to spores from a blade which give rise to conchocelis filaments. Conchosporangia and conchosporangial branches describe the structures in which conchospores are formed. These were originally described as 'fertile cell rows' by Drew (1954).

The Japanese and Chinese have utilised seaweeds as food for many centuries. In China in the 6th century B.C. Sze Tu wrote that 'sea vegetables are a delicacy fit for the most honoured guest' (Bradford & Bradford, 1985).

The Japanese first cultivated *Porphyra* in either 1640 (Miura, 1975) or 1736 (Okazaki, 1971) in an estuary in Tokyo Bay. The Chinese started cultivation using

a simple rock cleaning method about 200 years ago (Tseng, 1981). However, it was not until the early 1960's that cultivation and commercial production of *Porphyra* utilised scientific techniques.

Cultivated *Porphyra* was prepared as dried sheets called 'hoshi-nori', a name which had already been applied to laver collected from intertidal habitats. The cultivated form of *Porphyra* is generally described as 'nori' (in the form of dried sheets) and 'laver' (that which is eaten) in Europe and is otherwise known in other regions or countries as zakai, kim, karengo, sloke or slukos.

In North America the cultivation of species of *Porphyra* for human consumption has been in development for approximately 10 years. The growth of a nori crop was shown to be technically feasible in Puget Sound, Washington (Merrill, 1981). The Japanese species *P. yezoensis* was introduced in order to improve the potential viability of a North American seaweed industry (Mumford, 1990). A greater amount of research has been carried out in North America than in Europe so far, as a result of recognising the potential of mariculture (Mumford & Melvin, 1984; Waaland *et al.*, 1986; Mumford, 1990). Problems experienced in the development of a viable seaweed industry appear to be mainly institutional and political rather than scientific (Mumford, 1987; Merrill pers. comm.).

In Europe the use of seaweeds as foods for human consumption by the indigenous population is very limited. Their use generally occurs as a result of remnants of long traditions in some areas, as additives to foodstuffs or by people with special tastes and interests, as in healthfoods (Indergaard & Minsaas, 1991). The use of *Porphyra* by the indigenous population of Europe is mainly in the form of laverbread [see Indergaard & Minsaas (1991) for information on how this is produced], this being particularly popular in Wales.

By far the greatest consumption of seaweeds as food is in the Far East, particularly in Japan, China and Korea resulting in a wealth of literature on species cultivated in these areas (e.g. Iwasaki & Matsudaira, 1954a; Iwasaki &

Matsudaira, 1958; Kurogi, 1961, 1963; Okazaki, 1971; Chihara, 1974; Miura, 1975, 1978; Xia & Abbott, 1987; Miura & Shin, 1989). The use of seaweeds for food in Japan is still expanding and more seaweeds are now eaten than ever before (Arasaki & Arasaki, 1983; Nisizawa *et al.*, 1987). In 1982/83 the total crop of *Porphyra* in Japan was 360,000 tonnes fresh weight per year with a value of $1800 x 10^6 (Indergaard & Minsaas, 1991).

Porphyra has a great potential as a food for human nutrition in the western world as already realised in the Far East, if the problems of acceptability and tradition can be overcome. In terms of amino acids, seaweed protein is similar to that of egg whites and legumes. Sea vegetables are low in fats and when compared to terrestrial vegetables, have a competitive content of vitamins and minerals (Indergaard & Minsaas, 1991). The protein content was noted to be 1.7 times higher than beef with 75 % of the protein and carbohydrate available to humans with a vitamin C content 1.5 times higher than that of oranges (Xia & Abbott, 1987). Clearly, *Porphyra* is a valuable food item; therefore the utilisation of sea vegetables in the same way as other commonly used vegetables would appear to be a natural way to progress. However, this requires further changes in attitudes and the use of appropriate marketing in order for seaweeds to achieve their just place as vegetables in human nutrition (Indergaard & Minsaas, 1991).

There are 60 to 70 species of *Porphyra* world-wide (Mumford & Cole, 1977) with the greatest number being found in the North Pacific (Kain, 1991). Eleven species have been recorded from Europe of which five widely distributed species have potential for cultivation (Kain, 1991).

In the British Isles there have until recently been five recognised species of *Porphyra* : *P. linearis* Grev.; *P. umbilicalis* (L.) J.Ag.; *P. laciniata* (Roth) C.Ag.; *P. leucosticta* Thur. in le Jol. and *P. miniata* C.Ag.

Initially in this study the names and descriptions of the species found in Britain made by Conway (1964a, c) were used to describe the species found around the Isle of Man. However, during the course of the present work research on *Porphyra* species in Helgoland (Kornmann & Sahling, 1991) shed further light on the nomenclature and descriptions of some of these species and revealed an error made by himself and Conway in earlier work (Kornmann, 1961b; Conway, 1964a). Consequently the nomenclature used in this study follows that of Kornmann (1991).

P. linearis is a winter annual found in abundance in the upper tidal and spray zones at exposed sites. It is easily recognised by its linear form and high shore position (Plate 1.1) (Conway, 1964a, c). Kornmann & Sahling (1991) noted that in Helgoland *P. linearis* occurred in an asexual form.

P. umbilicalis is found mostly on exposed coasts and is characterised by a central holdfast and a thicker 'polythene like' thallus [see Conway (1964b) for a detailed description of this species]. As noted by Conway in Scotland, the species found on the Isle of Man appears to have three morphotypes dependant on the degree of exposure (Conway, 1964b):

i) a moderately exposed mid shore type (Plate 1.2),

ii) a sheltered mid shore type (Plate 1.3) and

iii) an exposed higher shore type (Plate 1.2).

P. laciniata, referred to as *P. purpurea* by Conway (1964a) and Kornmann (1961b), is an annual species found primarily in the mid-tide or low littoral zones, particularly on rocks or boulders on sandy shores with some degree of shelter. It has a peak in growth in spring. Spores of asexual blades and carpospores of this species develop into conchocelis. The identity of this species is still not clear cut. There is possible confusion with the erroneously synonymised *P. purpureo-violacea* (Kornmann & Sahling, 1991).

Plate 1.1 *P. linearis* from the exposed breakwater, Port Erin. Showing the linear form of this species. (largest plant = 18 cm)

Plate 1.2 *P. umbilicalis*, the larger flattened plants are from a mid shore moderatley exposed site and the smaller crumpled form from a high shore exposed site. (larger plants approx. 8 cm diameter)

Plate 1.3 *P. umbilicalis* a sheltered mid shore type. (approx 16 cm diameter)

P. leucosticta, a summer annual, is found growing epiphytically (Conway, 1964a) in the lower littoral zone. It is generally much more delicate in texture and thickness than the other species.

The subtidal species *P. miniata* is a summer annual and is the only distromatic species. It has a distinctive cinnabar red colour. It would appear however, that the Isle of Man is close to the southern limit of this species as during the course of this study only one specimen, growing on a rope in the subtidal, was recorded (Plate 1.4).

The taxonomy of the genus *Porphyra* may initially appear to be uncomplicated as a consequence of the simple appearance of the blade phase. However, it is the very nature of this apparent simplicity that can trap the unwary. Different species can have very similar morphological characteristics, rendering it very difficult to separate some species from each other. The eminent and experienced phycologist (Conway, 1964a) reviewed the species of Britain and did not record a species very similar to the species she referred to as *P. purpurea* [equivalent to *P. laciniata* (Kornmann & Sahling, 1991)]. This may have been an oversight but it is also possible that the former species, described by Kornmann & Sahling (1991) as *P. purpureo-violacea*, may not have been present at the sites of Conway's study. Clearly the description and naming of species in this genus requires great care.

The use of electrophoretic techniques has proved successful in separating species of seaweeds (Cheney & Babbel, 1978; Lindstrom & South, 1989) including *Porphyra* (Miura *et al.*, 1979; Lindstrom & Cole, 1990). Recent work by Lindstrom & Cole (1992a) demonstrates that even after much work on the classification of North American Pacific coast and Canadian species (Krishnamurthy, 1972; Conway *et al.*, 1976; Garbary *et al.*, 1981) the genus *Porphyra* still produced taxonomic surprises. Using electrophoresis combined with traditional

Plate 1.4 *P. miniata* found growing on a rope in the subtidal.

techniques of morphology, chromosome count and ecological distribution, the separation of the *P. lanceolata* - *P. pseudolanceolata* complex into five separate species was possible (Lindstrom & Cole, 1992a).

The use of protoplast isolation and cultivation is currently of great interest to investigators seeking to improve the propagation and genetic qualities of strains of commercially valuable plant species (Chen, 1987). Several authors have addressed the problem of protoplast isolation and culture in *Porphyra* (e.g. Zhao & Zhan, 1981; Tang, 1982; Polne-Fuller *et al.*, 1984; Polne-Fuller & Gibor, 1984; Saga & Sakai, 1984; Fujita & Migita, 1985; Araki *et al.*, 1987; Araki & Morishita, 1990; Chen *et al.*, 1990; Polne-fuller & Gibor, 1990; Liu & Kloareg, 1991) and the application of these techniques whether they be by fusion, transformation or genetic manipulation (van de Meer, 1986; van der Meer, 1987) to the cultivation of *Porphyra* species. The use of protoplasts as an alternative method of propagation in the cultivation process is clearly one of great potential, particularly when considering the cultivation of a species which does not produce monospores (Chen, 1989) (important for self seeding of nets). From the literature it is clear that this is an important area of research in the cultivation of *Porphyra* species although it will not be covered under the constraints of the present study.

It is important when considering the potential of any algal species for cultivation that background knowledge of the general biology and ecology is understood in order to generate further cultivation related research on a given species from a sound basis.

The main aims of this study are to look into some aspects of the biology and ecology of the species found around the coast of the Isle of Man in order to gain insight into their biology and the consequent potential for cultivation.

The aims of this research were:

1. The study of the ecology and seasonality and growth of *P. laciniata* using field techniques.

2. Assessment of protein levels of the species as an aid to the evaluation of the most viable species for cultivation of a valuable crop.

3. The taxonomy of Manx species using the technique of starch gel electrophoresis combined with more traditional techniques.

4. Culture of various stages of the life history of *P. laciniata* and *P. linearis* with particular reference to the culture of the conchocelis phase and the production of conchosporangia and conchospores.

CHAPTER 2

SOME ASPECTS OF THE ECOLOGY AND SEASONALITY OF MANX *PORPHYRA* SPECIES

INTRODUCTION

In spite of the large volume of research in the cultivation of *Porphyra* worldwide there is still relatively little known about the general ecology and seasonality of endemic species in Britain (Grubb, 1924; Drew, 1949, 1954; Lewis, 1954, 1957; Conway, 1964a, b, c, 1967; van Tussenbroek, 1984).

The thallus phase of *Porphyra* species often shows a distinct pattern of seasonal occurrence (Conway, 1964a, b; Mumford, 1975; Boney, 1978) and there can be great phenological differences within a species as a result of gradients of physical factors and variation over time (age and maturation). The extent of phenological variation and increased knowledge of the ecology of a species cannot be determined without seasonal studies and year round sampling and observations of populations (Mumford, 1975).

The study of the seasonality, biomass, zonation, population structure and the protein content yield useful information which leads to a better basic understanding of endemic species which can be utilised in further detailed laboratory studies of aspects of the biology of British *Porphyra* species.

If the thallus phase of *Porphyra* has no monospores or method of self propagation then it is dependant solely on the production of conchospores from the conchocelis phase for thallus production. Even if the thallus phase does produce monospores (Cole & Conway, 1980) it is likely that the production of conchospores will still be necessary at some stage.

The conchocelis phase of *Porphyra* has long been known to grow in mollusc shells since the identification of *Conchocelis rosea* as belonging to the species *Porphyra* by Drew (1949); Drew & Richards, (1953) and Drew (1954). Conchocelis has been found in the subtidal (Clokie & Boney, 1980; Clokie *et al.*, 1981; Akpan & Farrow, 1984) but these studies were not directly related to the rest of the life history of *Porphyra*.

Conchocelis has also been observed and studied in the intertidal (Dixon & Richardson, 1969; Mumford, 1975; Martinez, 1990) [discussed by Boney (1978) and Conway & Cole (1977)]. Growth of conchocelis has been noted in intertidal barnacles by Dixon & Richardson (1969); Mumford, (1975); Matamala *et al.* (1985); Martinez (1990), in limestone rock itself by Drew (1954) and possibly occurring endophytically in some intertidal algae (Boney, 1978). Matamala *et al.* (1985) used a simple technique to study the abundance of conchocelis of *Porphyra columbina* Montagne in the intertidal of central Chile.

The research carried out so far lends greater and greater support to the existence and growth of the conchocelis phase of *Porphyra* in the intertidal zone. The intertidal is a much harsher environment (excessive light, wave action and desiccation) than the subtidal but the ability of conchocelis to bore into shells may provide greater protection from the environment (Martinez, 1990). The presence of the conchocelis phase is an important factor in the ecology of the mainly intertidal thallus phase of *Porphyra* species.

The seasonality of biomass and protein levels of various species of *Porphyra* has been studied by Munda (1972), Zavodnik (1987) and McLachlan *et al.* (1972). The level of protein in *Porphyra* gives a good indication of the quality of any nori products produced if a species were cultivated and consequently the value of a species as a viable food crop (Noda *et al.*, 1975; Noda *et al.*, 1981).

The species studied in this thesis are those that occur around the coast of the Isle of Man. The Isle of Man is situated in the middle of the Irish Sea between approximately Lat. 54° 00' 00" and 54° 30' 00". Fig. 2.1a shows how the average sea temperature and mean light period vary over a 12 month cycle. The highest temperatures occurred in August (15.1 °C) and lowest in February (5.6 °C) in 1991. Figure 2.1b shows the change in nitrate levels as means per month with the

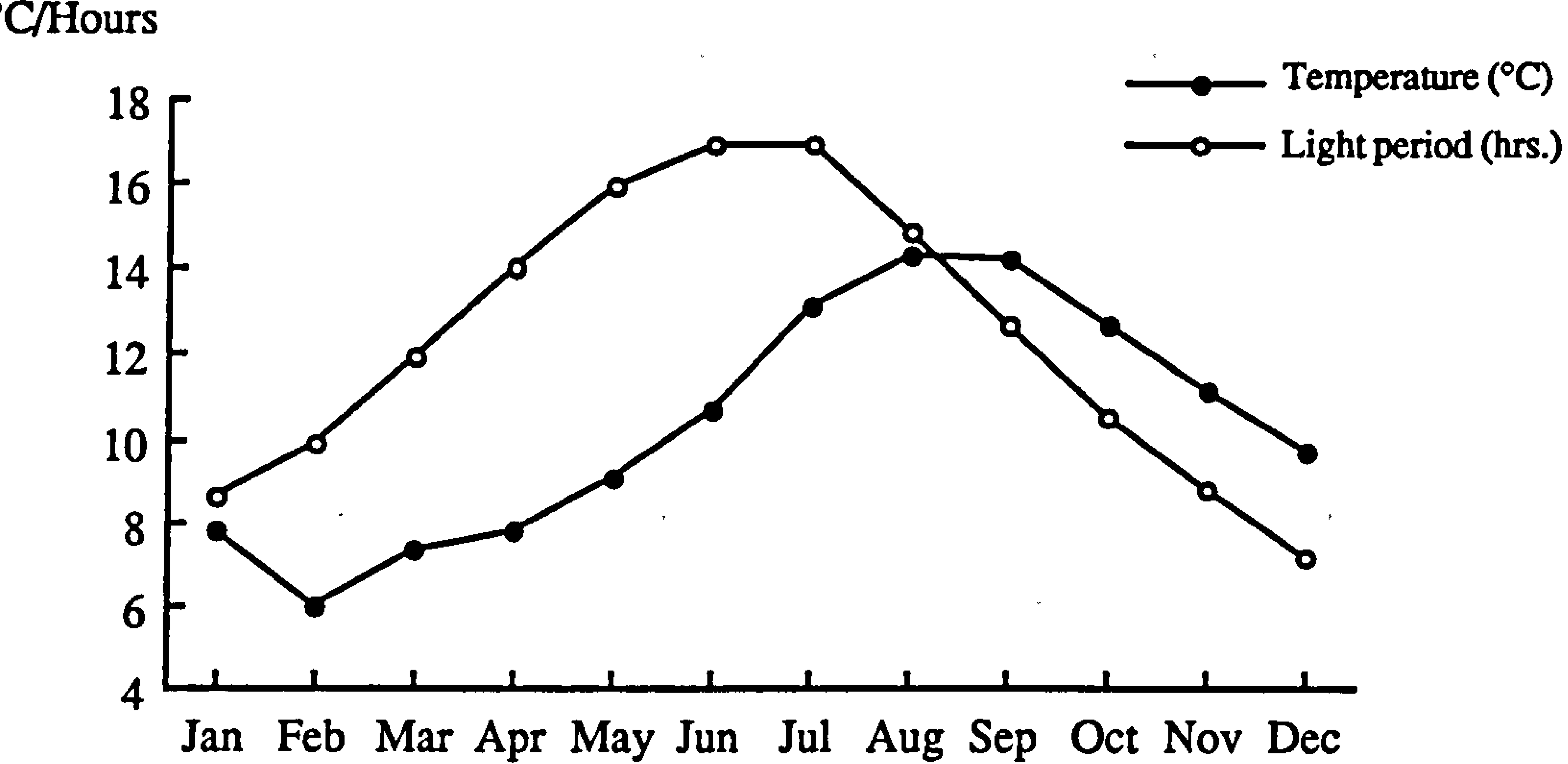


Fig. 2.1a Monthly mean lightperiod(hours) and temperature (C).
mean monthly light period data calculated from from tidetables
Temperature is surface temperatures at Port Erin Breakwater
from Slinn (unpublished data)

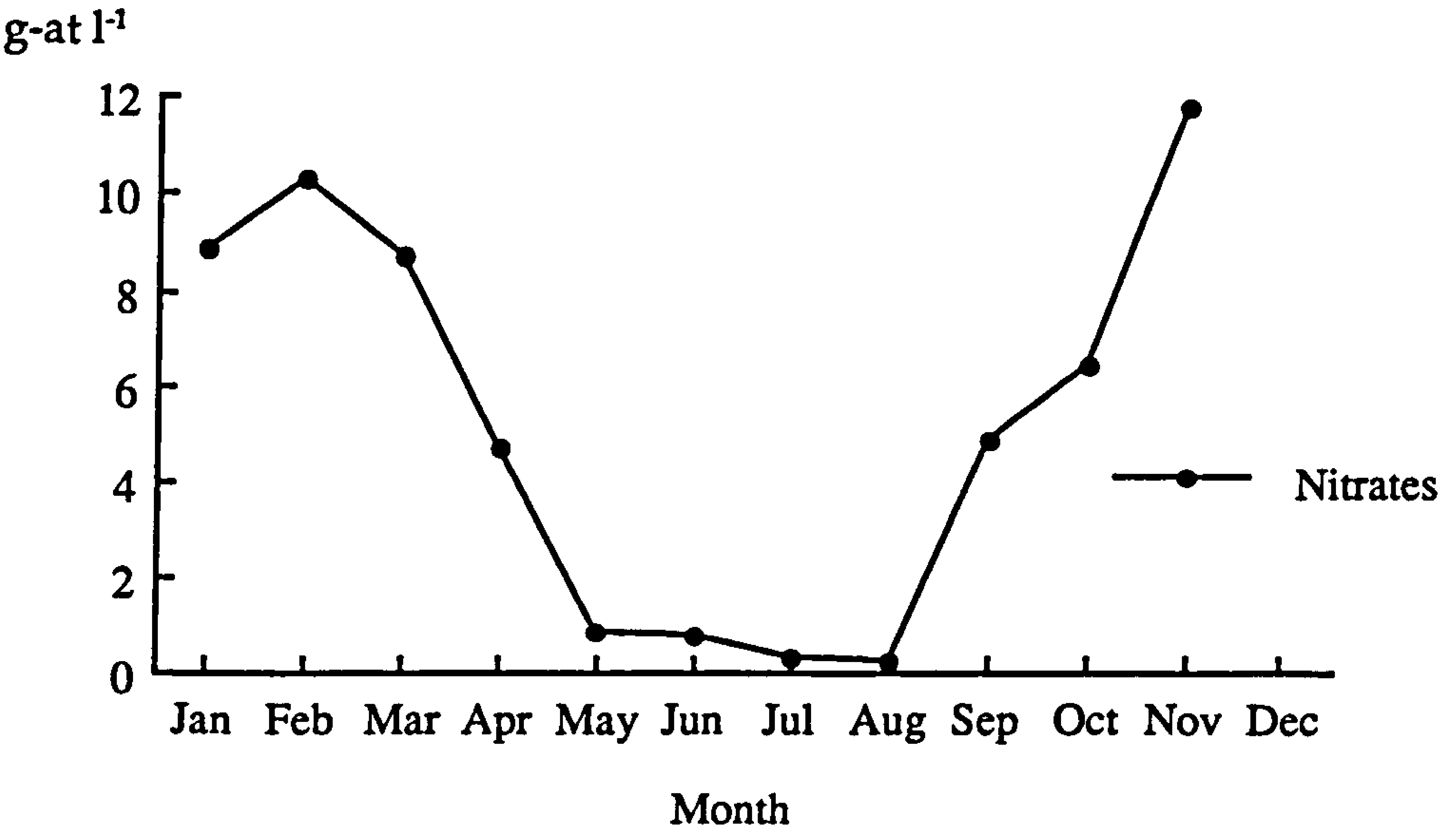


Fig 2.1b Monthly nitrate levels from the Cypris surface station
Data is combined data from 1991/1992 for a complete year cycle

highest nitrate levels occurring in November and the lowest in August. These figures provide information on the main environmental conditions experienced seasonally by species of *Porphyra* that occur around the coast of the Isle of Man.

It is important to understand the general ecology of the species present around British coasts if any further work into the suitability of *Porphyra* species for cultivation is to be done and to enable the utilisation of these species in further cultivation experiments to move in the right directions.

METHODS

Observations around the Isle of Man

General observations were made around the island for study of the seasonal patterns of the *Porphyra* species. At sites around the island (Fig. 2.2, 2.3a) observational notes were made on the general ecology including the occurrence, abundance, fertility and general health of *Porphyra* species present.

At Port St. Mary ledges (Fig. 2.3b) the position of patches of *P. linearis* within a set area were mapped for two consecutive years to determine whether these grew in the same place from one year to the next.

At the same site samples of barnacles from close to the position of these *P. linearis* patches were taken in late October (1991) to see whether any conchocelis filaments were present. Barnacles were also sampled from within the detailed study site, close to positions of the patches of *P. laciniata* on Port Erin beach. The method used followed a similar protocol as that used by Martinez (1990).

Detailed sampling and monitoring of a population

From preliminary surveys and observations an intertidal shore area where a population of *Porphyra* was known to occur were selected at the north end of Port Erin Beach (54°05'20" ; 4°45'30", Fig. 2.3a) and on the west end of Brewery beach near Port St. Mary (54°05'00" ; 4°45'00", Fig 2.3b). These areas contained a part of the substratum transition zone where the sand of the beach meets an area of rocky shore. Unfortunately due to major sea wall constructional work and consequent large scale beach alteration the site at Brewery beach had to be abandoned.

Having selected the sampling sites a sampling technique was designed. This proved difficult since the patchy nature of the *Porphyra* populations and the

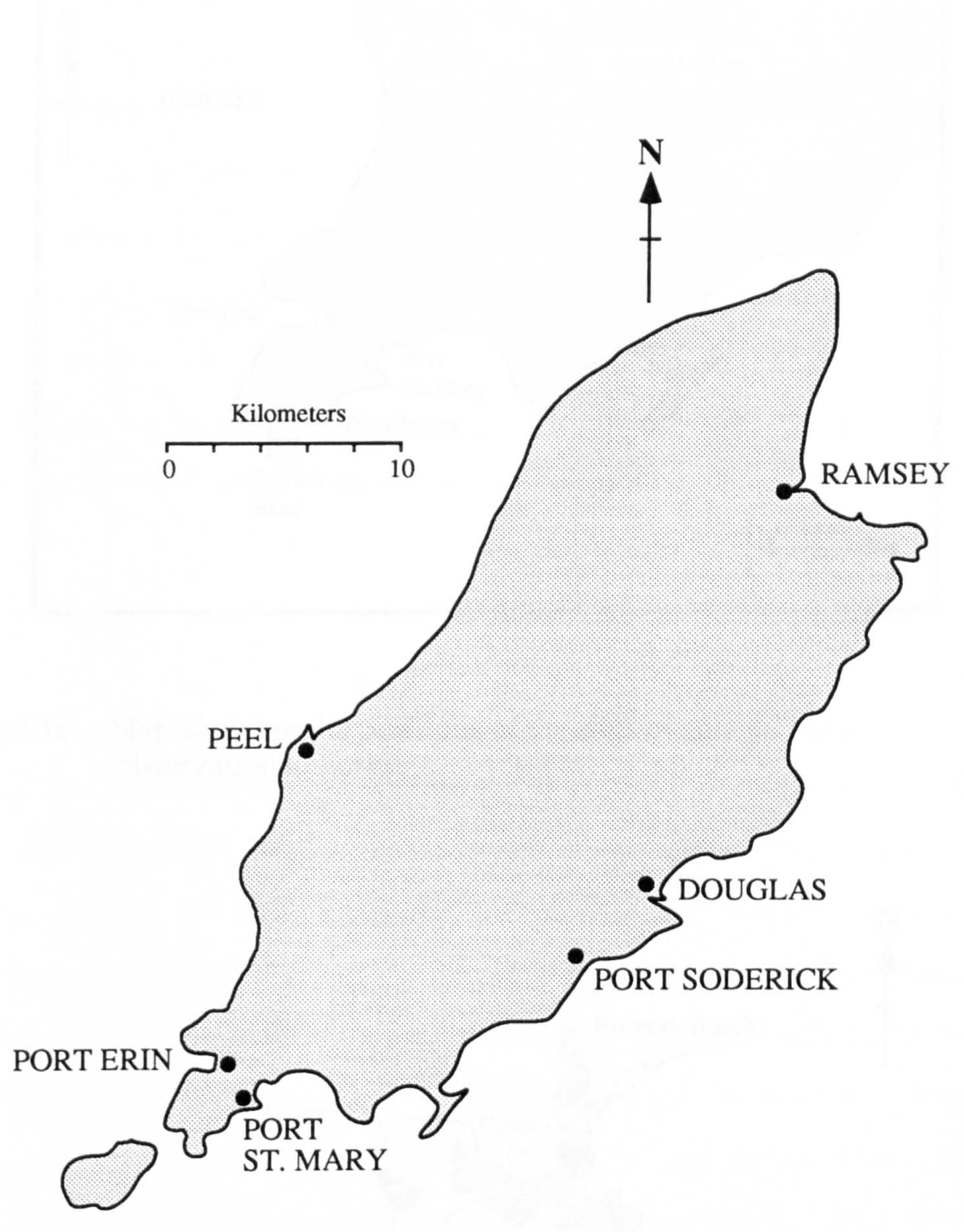


Fig 2.2 Map of the Isle of Man showing main sample site (Port Erin) and sites used for observational work.

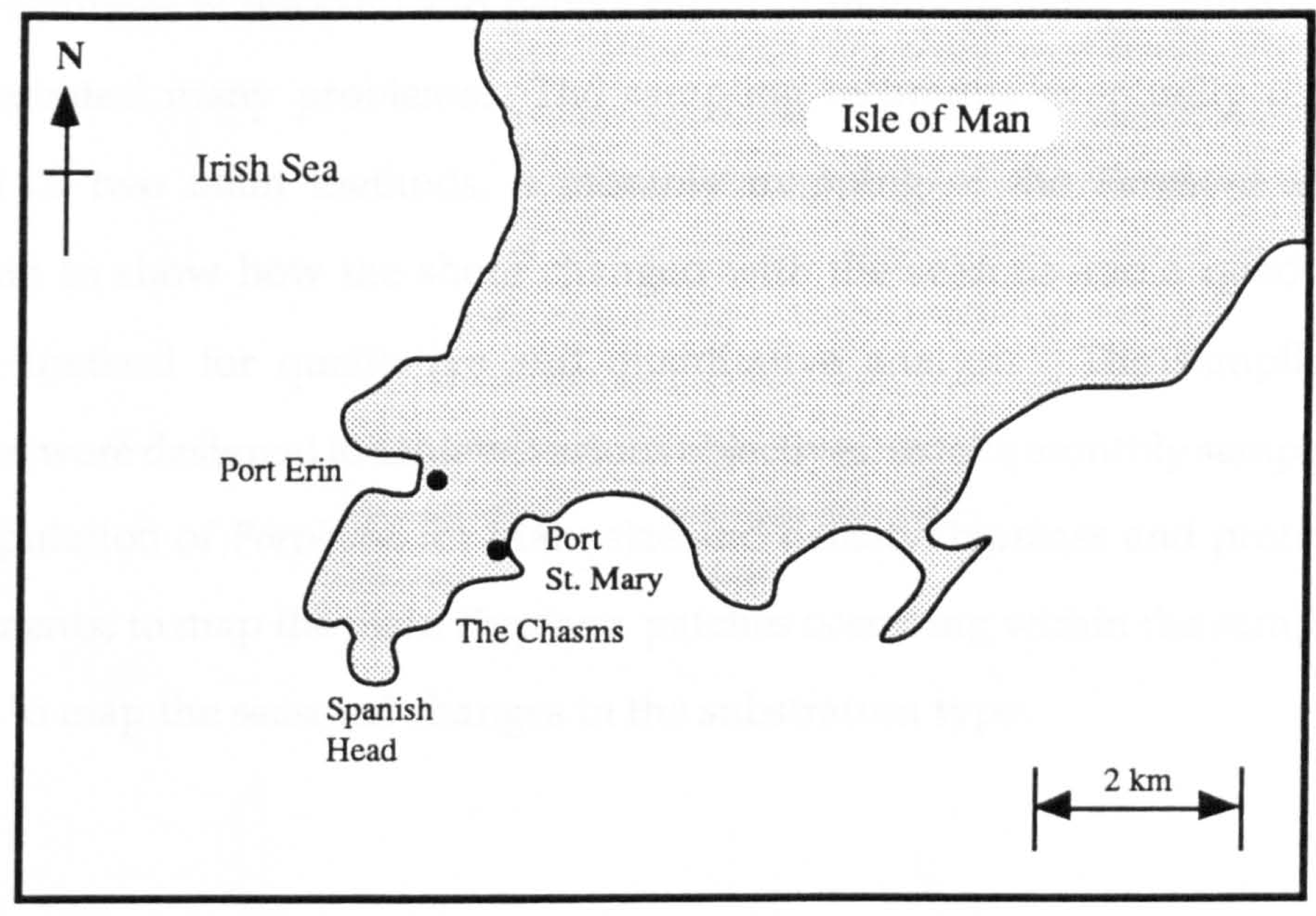


Fig 2.3a Map showing the positions of the sites of general observational work

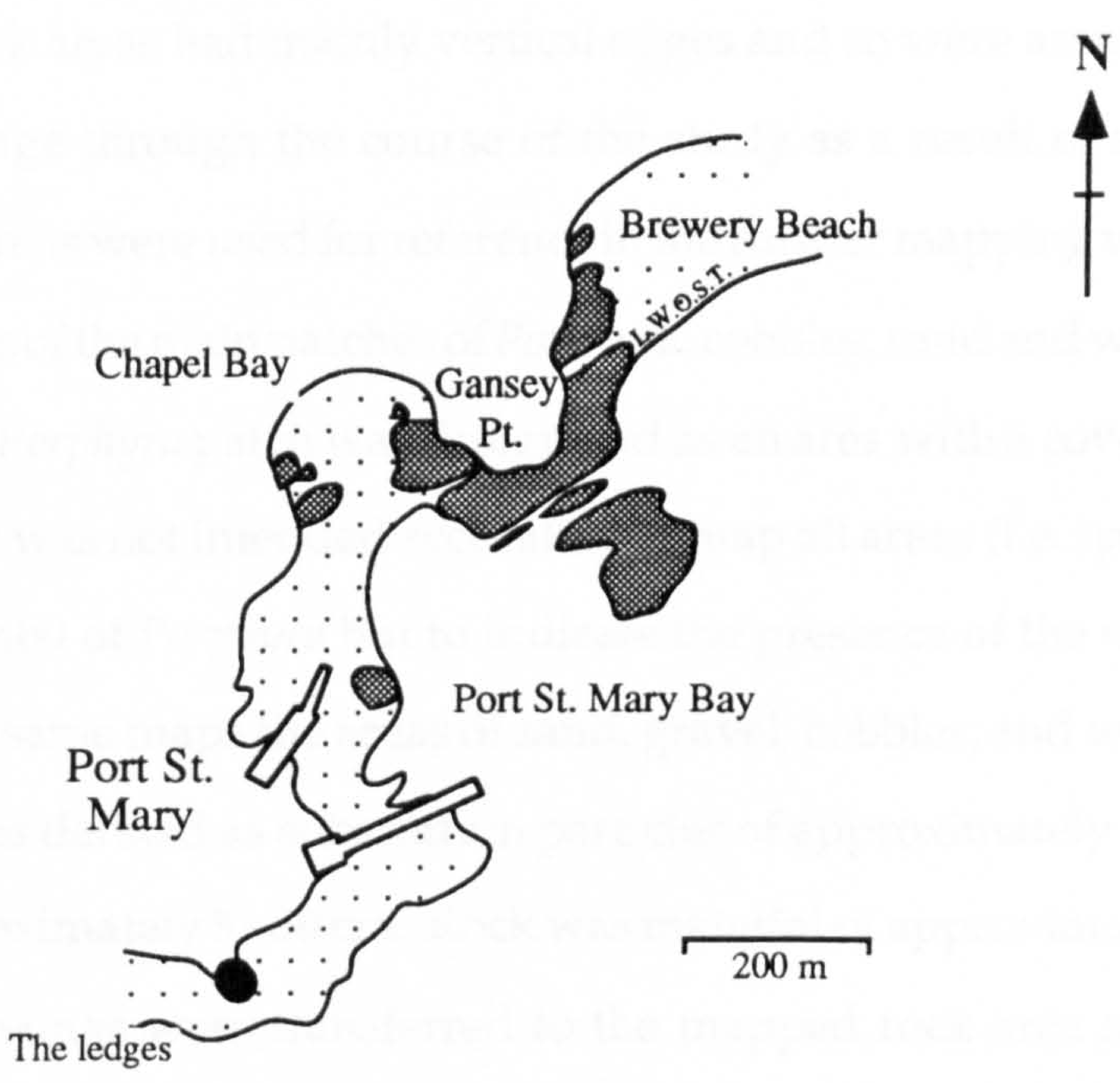
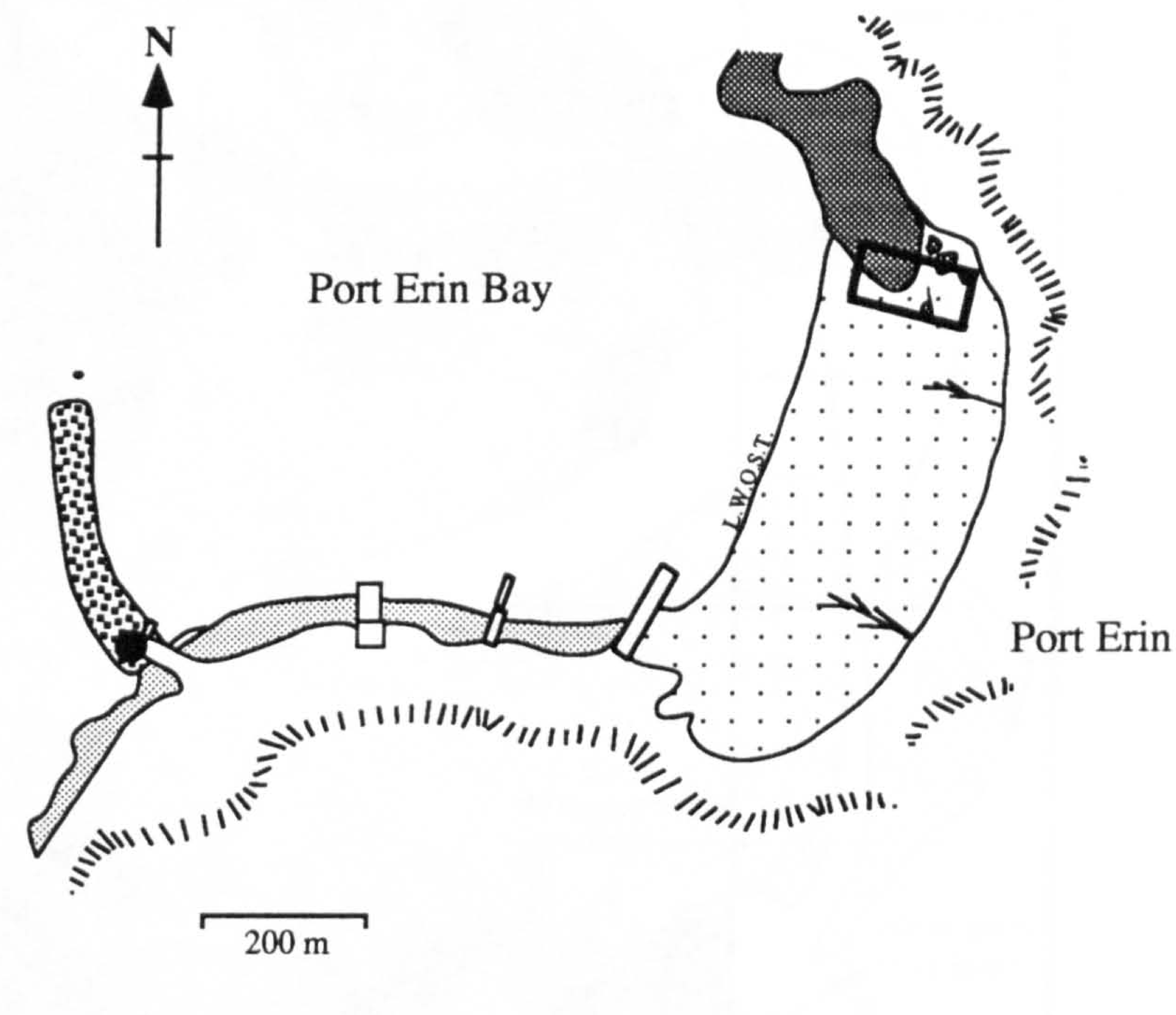


Fig. 2.3b The sites at Port St. Mary ledges including the site where the position maps of *P. linearis* were drawn (●).

dynamic activities of the sand and pebble substratum where these populations occur presented many problems. The sampling technique eventually used consisted of two main methods, a monthly mapping of the *Porphyra* and substratum to show how the shore changed with the seasons and a quadrat sampling method for qualitative and quantitative analysis. The sampling techniques were designed to achieve various objectives: to take monthly samples of the population of *Porphyra;* for plant size and density, biomass and protein measurements, to map the main *Porphyra* patches occurring within the sample area, and to map the seasonal changes in the substratum type.

Mapping

The area used for the mapping study was on the north end of Port Erin beach (Fig. 2.4). A fixed transect and other fixed bolts (see next section) were used for measuring the position of the main bedrock areas within the whole mapping areas (Fig. 2.5). The rock areas had mainly vertical edges and so were assumed to have negligible change through the course of the study as a result of sand encroachment. These areas were used for reference in the further mapping work when drawing positions of the main patches of *Porphyra,* cobbles, sand and water (streams and pools). A *Porphyra* patch was designated as an area with a cover of approximately 100%. It was not intended accurately to map all areas (i.e. sparse areas of individual plants) of *Porphyra* but to indicate the presence of the main denser patches. On the same maps the areas of sand, gravel, cobbles, and water were drawn. Gravel was defined as substratum particles of approximately 1 - 3 cm and cobbles as approximately 3 - 30 cm. Rock was material of approximately 30 cm and above. Drawings were transferred to the mapped rock area scale drawing from the shore slate and then to a drawing package (Canvas™) using a graphics tablet. This package was then used to reproduce the maps and to calculate approximate areas of the patches of *Porphyra.*



Site of the detailed study of *P. laciniata*

Site where *P. linearis* occurs during the winter

Fig 2.4 Map of Port Erin bay showing the site of the detailed study of *Porphyra laciniata* and the site where *P. linearis* occurs.

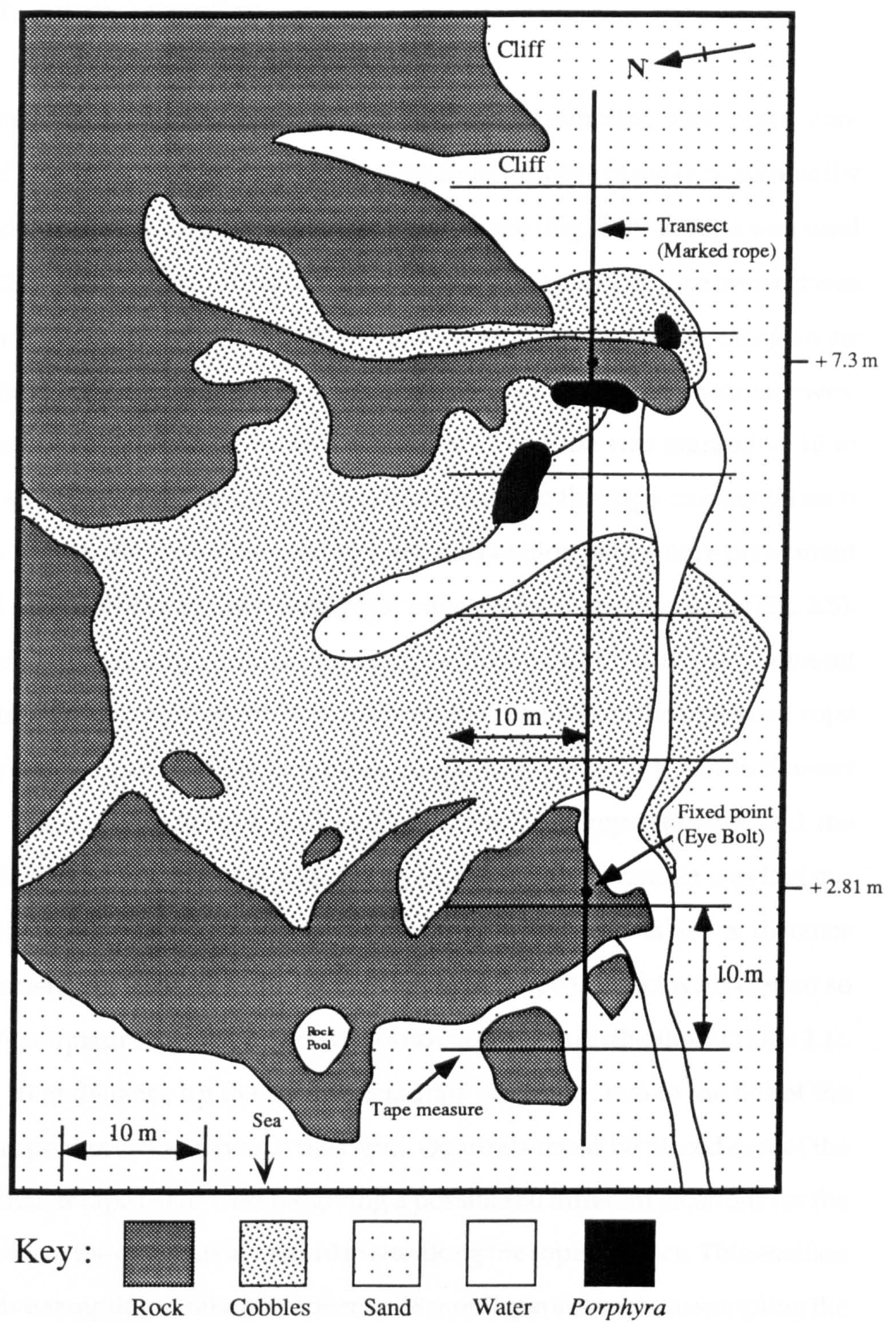


Fig. 2.5 Mapped area at the north end of Port Erin beach showing the position of the transects layed out for quadrat sampling. Tidal heights of the two fixed eye bolts are given as a guide to approximate tidal heights for the rest of the mapped area.

Monthly quadrat sampling

The sampling area, within which destructive sampling took place, consisted of an 80 x 20 m strip of shore, running from the base of the rock zone at the low-mid shore level to the upper supralittoral zone (Fig. 2.5). A rope was used within this area to create a fixed (but easily removable) transect. The transect was laid by fixing two ring bolts into the rock at either end of the shore, using an air drill. The rope was then laid out running up and down the shore (in an east-west direction) passing through the two ring bolts. The rope was marked in 10 m lengths so that a tape measure laid perpendicularly to the rope transect (at each of these 10 m marks) could be used to mark the position of the more permanent areas of rock (relative to the central rope transect) for mapping work (Fig. 2.5). Fixed points were also put into the rock perpendicular (north) to the rope transect approximately opposite every 10 m mark on the rope. Once the east-west rope transect was positioned a 50 m tape measure was used as the north-south transect thereby creating an effective grid using just the fixed rope transect and the perpendicular 10 m interval marks. Every month the rope transect was laid out in exactly the same position and using random number tables a distance measurement between 0 and 10 m from the central rope transect was selected so that a 25 cm^2 quadrat could be placed at a known position on the shore (Table 2.1). At each 10 m distance up the shore 4 quadrats were laid, two to the left of the vertical rope transect and two to the right. Quadrats could be placed east of the perpendicular tape (+) or west (-), giving a possible 80 different locations for the positioning of the quadrats at each 10 m site along the rope transect. This enabled destructive sampling to take place over an 18 month period without sampling the same position twice.

Once a quadrat position was located a 25 cm^2 quadrat was laid and the percentage cover of the major algal species, cover of rock, cobbles, gravel and

Month (m)	Quadrat positions	No. of Quadrats with *Porphyra*
Dec (1989)	3+ & 7+	7
Jan (1990)	1+ & 9+	0
Feb	NS	NS
Mar	0– & 8+	7
Apr	2– & 6+	11
May	2.5– & 5–	10
Jun	1.5+ & 6–	5
Jul	3.5– & 8.5+	3
Aug	1.5– & 4.5–	6
Sep	3.5+ & 9.5–	4
Oct	0.5– & 4.5+	5
Nov	4– & 5.5+	4
Dec	6.5– & 1.5+	3
Jan (1991)	0.5+ & 7.5–	3
Feb	2+ & 10–	3
Mar	7.5+ & 3–	7
Apr	0+ & 4+	10
May	1– & 5+	10

+ = quadrat placed east of the tape measure

– = quadrat placed west of the tape measure

NS = Not Sampled

Table 2.1: Position of quadrats (m) from rope transect, number of quadrats in which *Porphyra* occurred (max. possible = 28).

sand were recorded. Any *Porphyra* present was destructively sampled (after its position had been noted) and placed in a marked plastic bag for later analysis in the laboratory.

Laboratory analysis

The samples of *Porphyra* were first cleaned in freshly filtered seawater to remove sand, gravel and detritus and left in filtered seawater for 15 minutes to rehydrate any desiccated plants.

Each individual plant length was measured by taking the holdfast and running the whole plant down the length of the measuring board so that the length from holdfast to the furthest point laid out along the ruler was recorded as the plant length. All plants in each quadrat sample were measured in this way.

Wet weight was measured for each quadrat sample by rewashing the *Porphyra* in fresh seawater, followed by a standardised shaking and squeezing in a cooking sieve to remove excess water, and then weighing in a foil dish. This process was repeated three times for each of the quadrat samples. Each foil dish was marked with the quadrat position and then placed in an oven at 90 °C for approximately 12 hours for dry weight measurements.

Protein measurement

Total nitrogen content was determined using the Kjeldahl procedure [see Diamond & Denman (1966) for a description of the technique], thereby calculating the protein content. After dry weight measurements the dried thallus was homogenised into approximately 1 mm^2 flakes (using a liquidizer) for protein analysis. The protein content was determined by taking three replicate

samples (of known dry weight) from the homogenised material and running through the Kjeldahl procedure. The total protein content of the *Porphyra* samples was then calculated by the following equation:

$$\% N = \frac{(\text{ml HCl for titration} - \text{ml HCl for blank})(100)}{(\text{sample mass in g})(1000)}$$

To convert % N to protein for *Porphyra* multiply by the following factor: 6.52 [Ryu *et al.* (1982) in Indergaard & Minsaas (1991)]. It would appear from other literature that this value of Ryu *et al* (1982) may be somewhat high. Gnaiger & Bitterlich (1984) used a value of 5.8 N to calculate protein values for plants.

RESULTS

General ecological observations

Figure 2.6 summarises information on the seasonal occurrence of the species studied, with indications of when species are most abundant.

The winter species *P. linearis* was observed at various sites around the Isle of Man. The main populations occurred high on the shore from around high water neaps to high in the splash zone. Populations were found particularly in areas of high wave action, for example on the southern concrete section of Port Erin breakwater (Fig 2.4), on the smooth high shore limestone of Port St. Mary ledges (Fig 2.3b) and on the sea walls at Douglas and Peel (Fig 2.2). In Port Erin plants first appeared on the sides of the solid section of the breakwater during October and as winter progressed the plants appeared higher onto the top of the breakwater as wave action and wave backwash increased. Storms in January and February were followed by an increase in the population. The populations were healthiest around late January to March, disappearing during April to early May. Thalli were fertile within a few weeks of their appearance and individuals were both monoecious and dioecious with spermatia occurring around the edges of the blade.

In Port St. Mary the patches of *P. linearis* that occurred around the top of the shore grew in the same places from year to year. An area where the patches occurred (Fig 2.3b) was mapped for two consecutive years and it was found that they occurred in almost the same position in the second year (Fig 2.7). This phenomenon had been observed in the winter prior to this mapping work. The patches that occurred here were extremely dense during January to early March and formed an almost pure stand of this species. Patches were clearly defined, particularly around the period of greatest biomass. Within the same area as the patches of *P. linearis* samples of the barnacles close to patch positions taken in

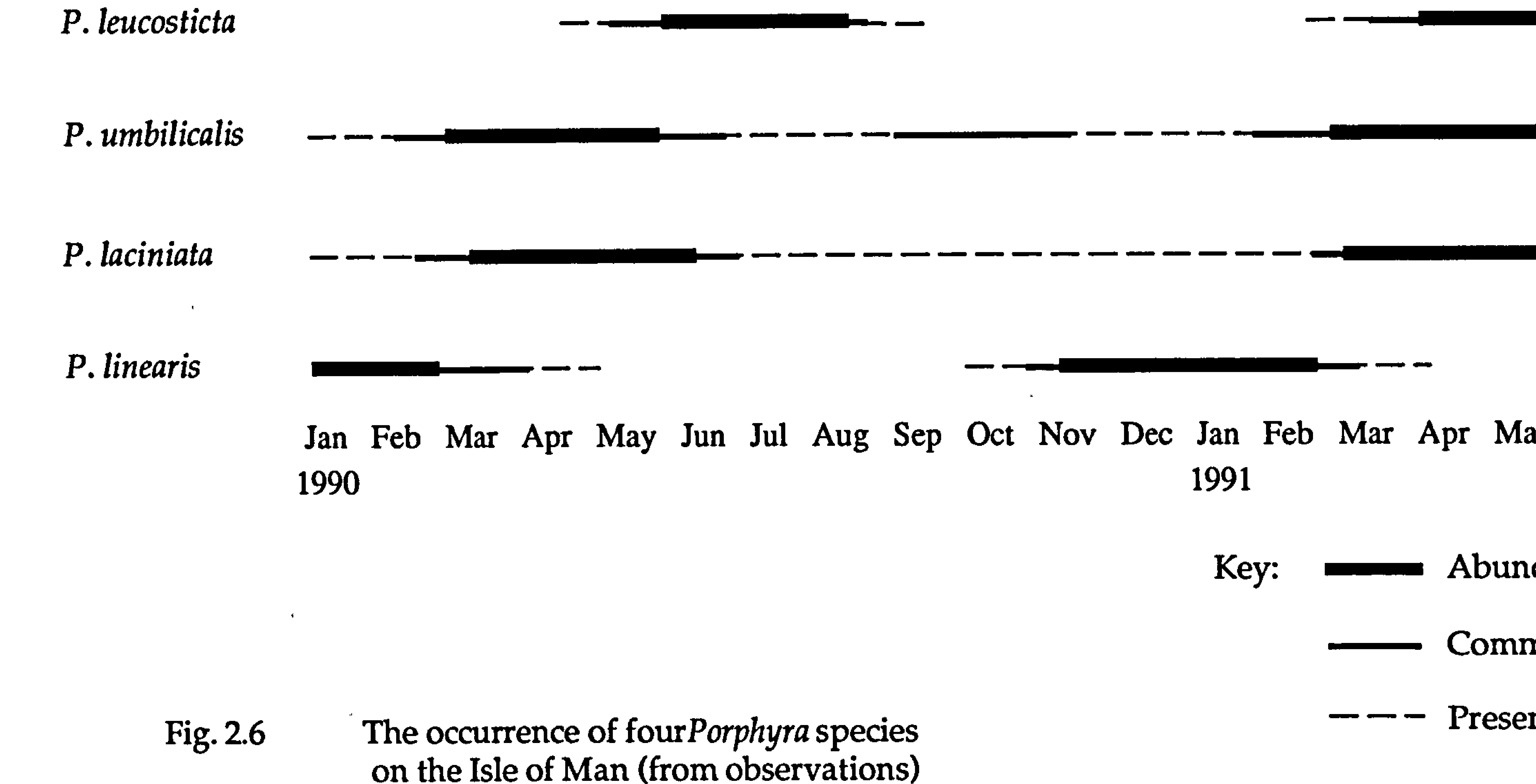
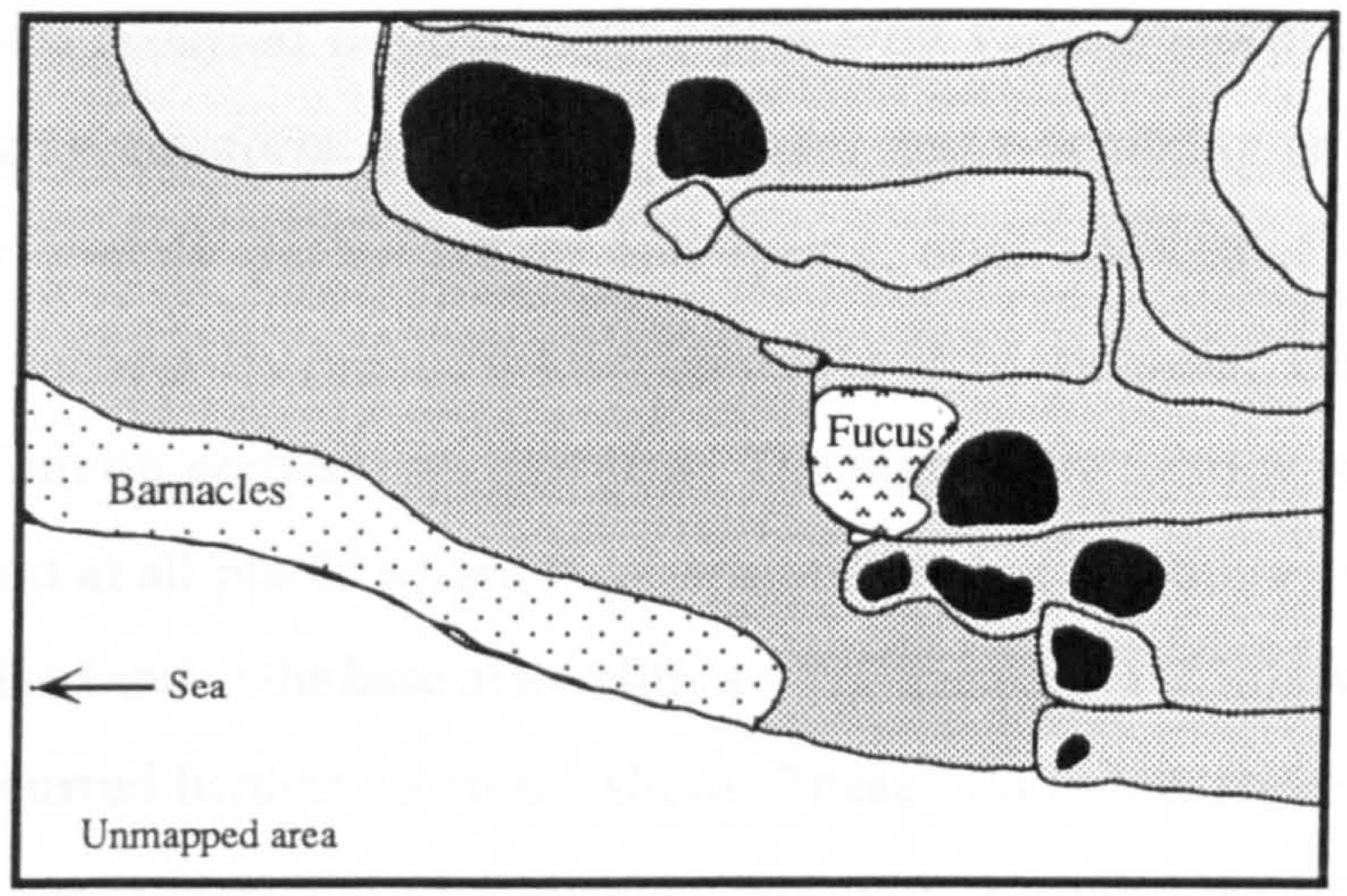


Fig. 2.6 The occurrence of four *Porphyra* species on the Isle of Man (from observations)

P. linearis patches April 1990



P. linearis patches April 1991

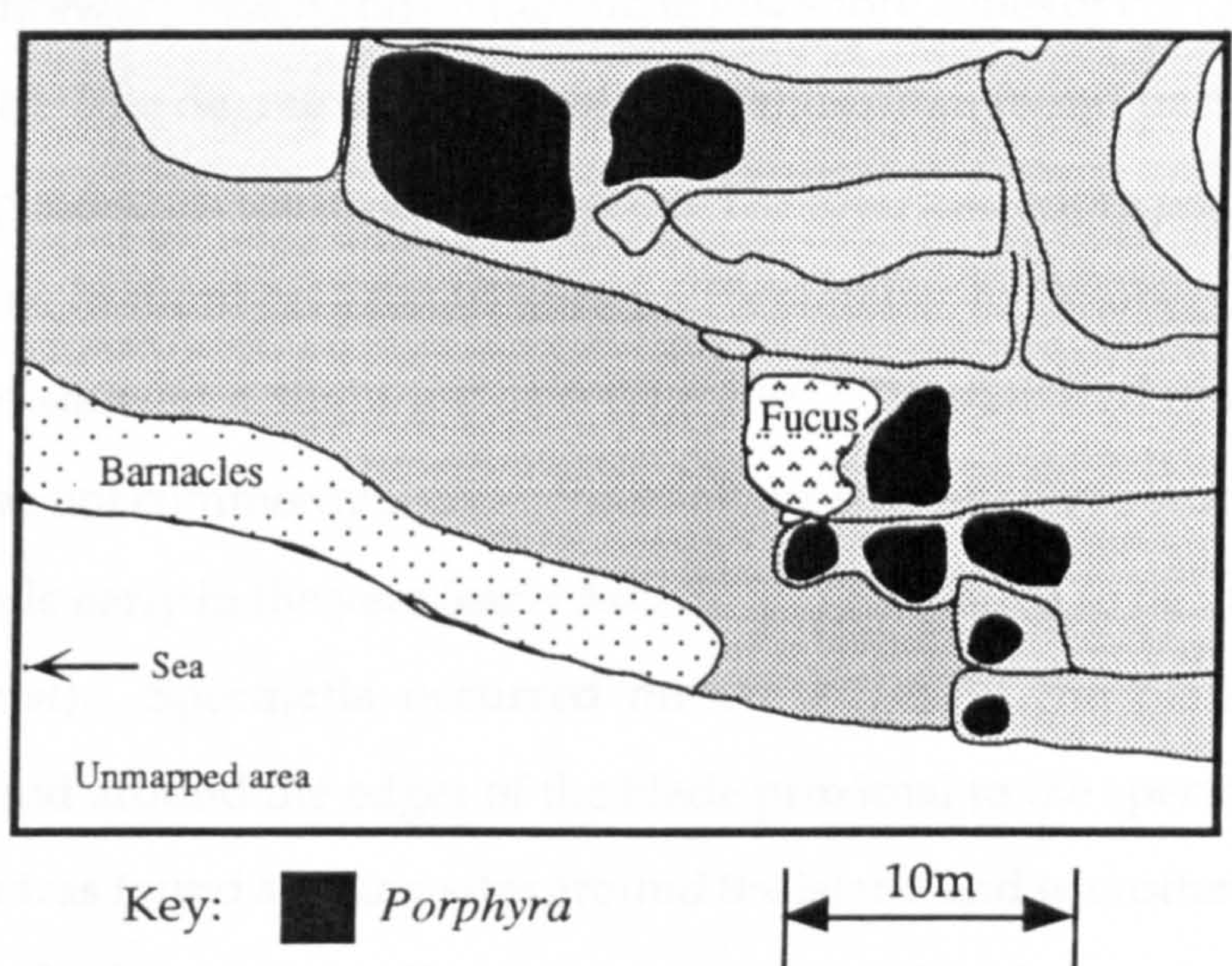


Fig 2.7 The mapped areas of *P. linearis* at Port St. Mary showing how the patches of *Porphyra* occur in almost the same positions from one year to the next. (All grey areas are rock)

autumn (October, 1991) were found to contain conchocelis filaments. Unfortunately photographs of these samples proved unsuccessful and time did not allow for more detailed quantitative work, as carried out by Martinez (1990).

P. umbilicalis occurred throughout the year with various morphological forms. The high shore form, small, highly folded and resembling crumpled paper, occurred mainly during winter and spring, often associated with *P. linearis* patches particularly in the Port Erin and Port St. Mary areas. This form was also prevalent on vertical rock surfaces. The high shore form was found around the island at all places where *P. linearis* occurred and at other exposed sites (Spanish Head and at the base of the cliffs at the Chasms, Fig. 2.3a). Another morphotype occurred further down the shore. These mid to low shore plants were more like the typical large umbilicate form with a central holdfast and larger flat blade. Examples of this mid and low shore form were found on the western end of Brewery Beach and on the mid to low shore zones of Port St. Mary ledges (Fig. 2.3b). The degree of folding of the thallus often found particularly at the edges depended on the level of exposure. The low/mid shore population showed a decline in numbers around autumn (September to October) but the high shore type showed a slight increase (Fig. 2.6). The sheltered mid shore morphotype was not commonly seen in observations made at these study sites. Plants were fertile early in the year (early March) and continued to be until late summer (August). Spermatia occurred on the edges of the blade with carpospores found around the edges of the blade proximal to the spermatia.

P. laciniata was found at many sites around the island and was often found associated with freshwater runoff and in areas of nutrient input, i.e. sewer outfalls (Port Soderick, Fig. 2.2). Plants frequently grew on large boulders and rocks in the mid tide region that were exposed to sand abrasion (Plate 2.1). Often boulders with large populations of *P. laciniata* were completely covered with sand leaving the blades poking through. This species appeared to be able to tolerate this sand cover for periods of a number of weeks with little visible harm.

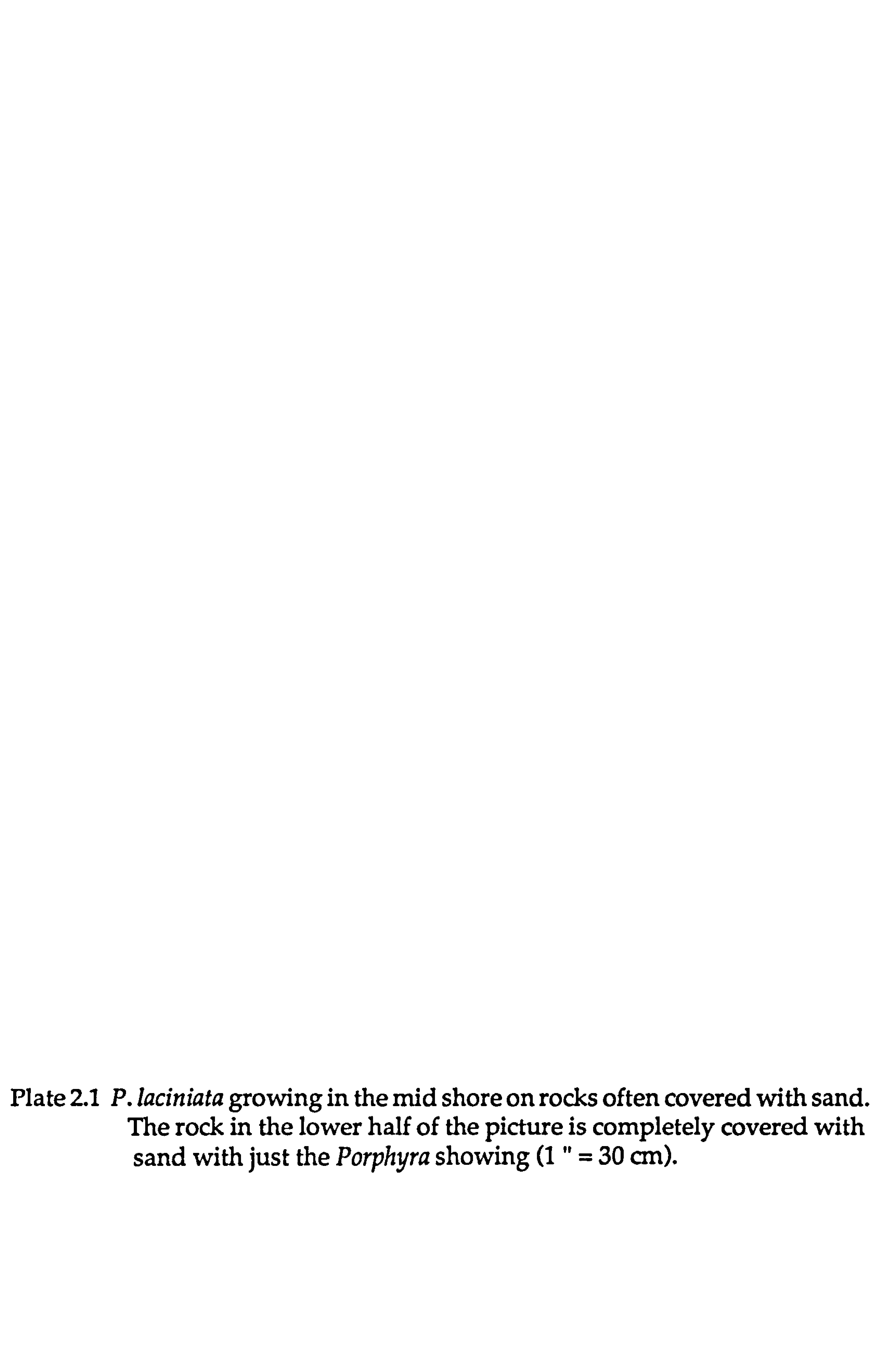


Plate 2.1 *P. laciniata* growing in the mid shore on rocks often covered with sand. The rock in the lower half of the picture is completely covered with sand with just the *Porphyra* showing (1 " = 30 cm).

Plate 2.1 *P. laciniata* growing in the mid shore on rocks often covered with sand. The rock in the lower half of the picture is completley covered with sand with just the *Porphyra* showing (1 " = 30 cm).

Plants appeared in great numbers around March with a peak around April-May. During these periods of optimum growth plants were very dark (almost black) in colour and only later turned to a lighter dark brown colour during times of high insolation. Sometimes plants were completely bleached to a pale straw colour. *P. laciniata* was the largest species observed around the island. Plants up to and sometimes over 1 metre were not uncommon during periods of the greatest biomass. The first fertile thalli appeared a few weeks after onset of rapid growth in late March to April with carpospores appearing first around the apex and edges of the blade often with spermatia occurring on the edges of the thallus. Barnacle samples taken from close to the populations of *P. laciniata* at the detailed study site in Port Erin (Fig. 2.4) were found to contain conchocelis filaments.

The summer species *P. leucosticta* first appeared in mid April from the mid shore to very low shore region and disappeared around September. It was found at various sites around the island: on the top of the collapsed breakwater in Port Erin in the mean low tide level; on the low shore on Brewery beach and on the low shore zone of Port St. Mary ledges (Fig 2.3b). The plants were almost always epiphytic, found growing on *Mastocarpus stellatus* (Stackh. in With.) Guiry in Port Erin and *Fucus serratus* L., *F. vesiculosus* L., *Ascophyllum nodosum* Stackh. (L.) le Jol. and *Enteromorpha intestinalis* (L.) Link at Port St. Mary and Brewery Beach. At Port St. Mary ledges plants were also found growing on the backs of limpets with occasional individuals found growing on rock. This species is recognisable by the colour and form of the blade. The blade has a distinctive mahogany red brown colour when healthy and is much more delicate than the blades of *P. umbilicalis* and *P. laciniata*. Plants were fertile from late April to early August with carpospores occurring in patches around the thallus with spermatia occurring around the edges of the thallus.

Mapping work at Port Erin beach

The mapping work demonstrated clearly how the population of *P. laciniata* developed on the shore area. From December 1989 to March 1990 (Fig. 2.8a) the number of plants and patch size gradually increased. Patches appeared higher up the zone close to permanent rock areas and as the sand moved from on top of the underlying cobbles the *Porphyra* appeared further down the shore. Patches also appeared and grew in some cases without the movement of sand. By April and May 1990 (Fig. 2.8b) the patches were very extensive and the main areas were associated with areas of cobbles. Patches did occur on sand but it was noted that individuals were attached to underlying cobbles. The sand on this area of the beach seemed particularly mobile with dramatic changes taking place within a matter of weeks or sometimes in the case of an onshore storm within a matter of days. In June 1990 (Fig. 2.8b) the sand began to move back across the main area of cobbles and consequently the main patches of *Porphyra* became reduced. During June, July and August 1990 (Fig. 2.8 b,c) plants were affected by high insolation illustrated by the bleaching of some individuals and were also affected by the covering of some plants by sand (illustrated by abrasion damaged individuals). By October and November 1990 (Fig. 2.8c) more sand had moved to cover the area of cobbles. During the period of December 1990 to February 1991 (Fig. 2.8d) only a few small patches of *Porphyra* were present at the top of the shore. The following map for March 1991 shows an increase in both the patches of *Porphyra* and the area of cobbles leading to a further increase during April and May 1991 (a repeat of the previous year's increase) (Fig 2.8e) where extensive areas of the shore were covered by large dark healthy plants. Patches were similar but not identical in the second year of sampling. The area of mapped shore covered with cobbles was at its highest during these months of greatest cover of *Porphyra* as was the case for the previous year.

December 1989

January 1990

February 1990

Not Sampled

March 1990

Key :

Rock Cobble

Sand Water *Porphyra*

Fig. 2.8a Maps of the sample area on the north end of Port Erin beach showing patches of *P. laciniata* and changes in substratum

April 1990

May 1990

June 1990

July 1990

Fig. 2.8b Maps of the sample area on the north end of Port Erin beach showing patches of *P. laciniata* and changes in substratum (see Fig. 2.8a for key)

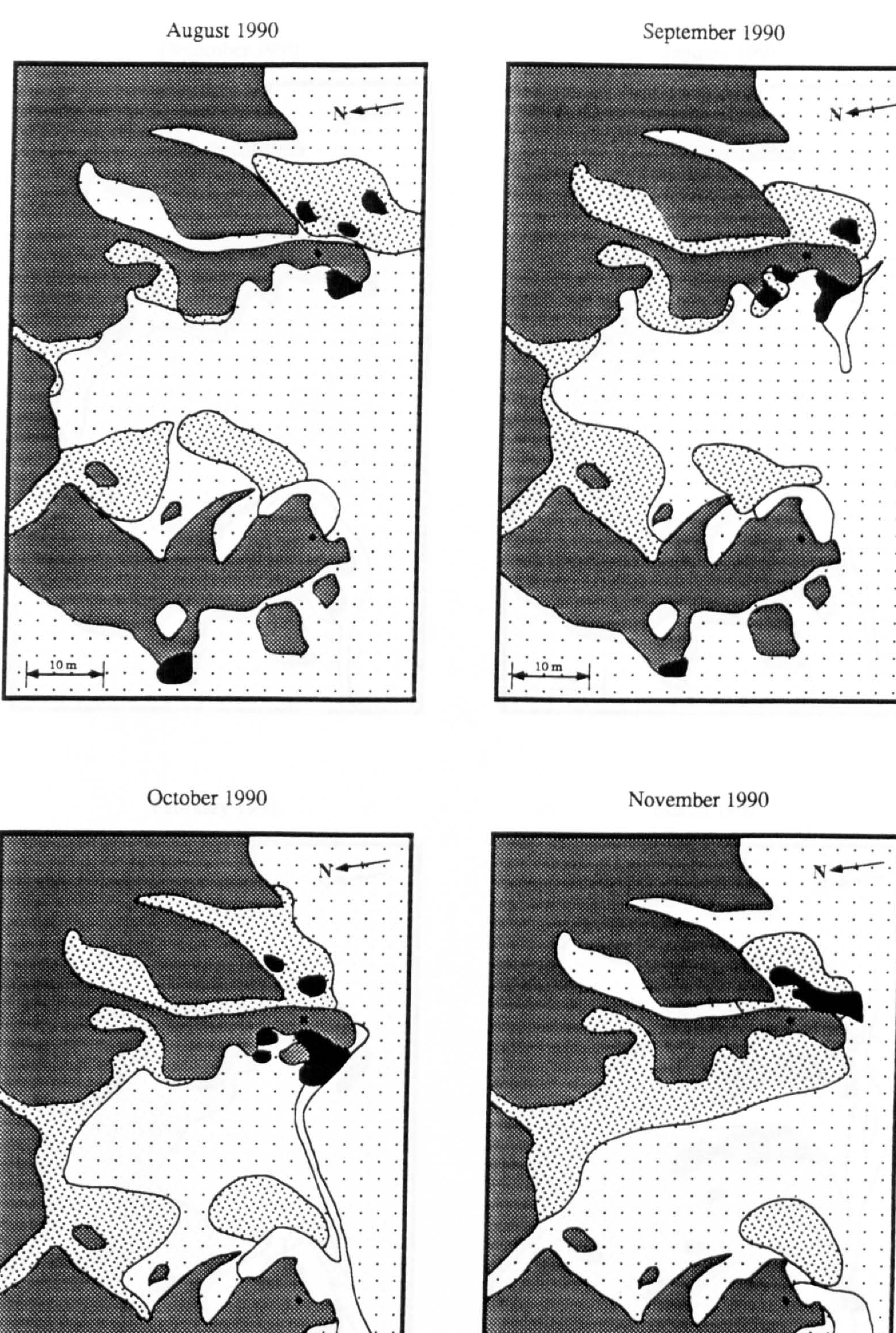


Fig. 2.8c Maps of the sample area on the north end of Port Erin beach showing patches of *P. laciniata* and changes in substratum (see Fig. 2.8a for key)

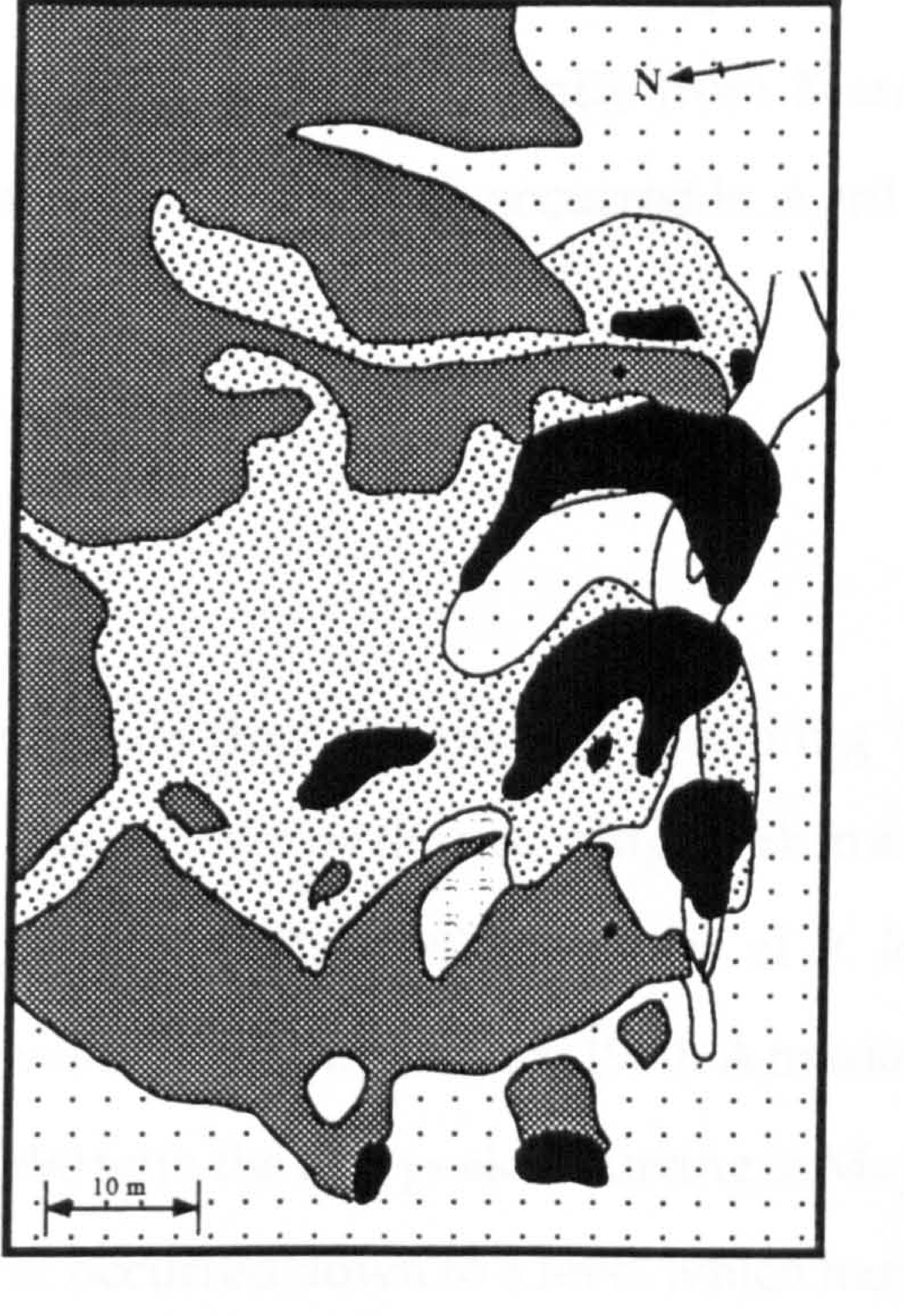
December 1990

January 1991

February 1991

March 1991

Fig. 2.8d Maps of the sample area on the north end of Port Erin beach showing patches of *P. laciniata* and changes in substratum (see Fig. 2.8a for key)



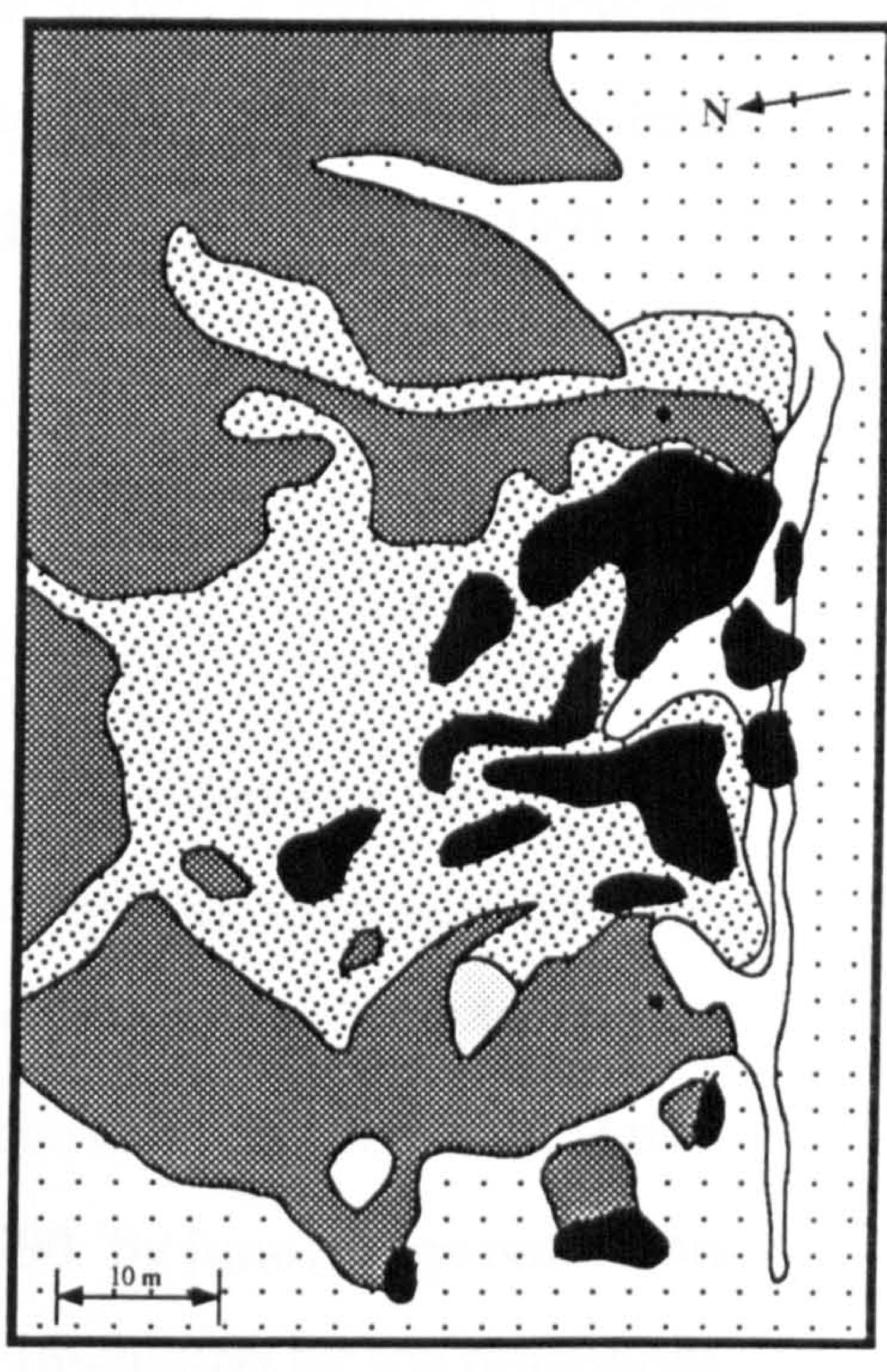


Fig. 2.8e Maps of the sample area on the north end of Port Erin beach showing patches of *P. laciniata* and changes in substratum (see Fig. 2.8a for key)

Using a drawing package (Canvas™) it was possible to calculate areas (m^2) covered by the *Porphyra* patches (Fig. 2.9). This shows a trend in how the population increased dramatically from March to April 1990 when the total approached 400 m^2. A peak occurred in April in 1990 and in May in 1991.

Quadrat sampling

Cover

The results from the quadrat sampling technique demonstrated similar trends to those shown by the mapping work in a more quantitative way. Initially, in December 1989 (Fig 2.10) the cover of *P. laciniata* was low and in fact no *Porphyra* was recorded in January 1990. A marked rise was recorded from March to April 1990 with the first peak occurring in May 1990. By June a large reduction in the cover occurred down to a level which hardly fluctuated for the remainder of the year apart from a slight rise in September. The following year the spring trend was repeated although the cover recorded in March 1991 was significantly lower than that found the previous year. April, however, showed a large increase in cover again with a further increase occurring in May (the highest for the whole study).

When the mean cover of *Porphyra* and cobbles were plotted together (Fig. 2.11a) it can be seen that percentage cover followed a similar seasonal trend with a few anomalies (as in the case of the cover of cobbles in August, 1990). When these data were regressed (Fig. 2.11b) they showed a highly significant result with $p << 0.001$ indicating, that the levels of *Porphyra* were high when the levels of cobbles were high. A similar plot for the cover of sand and cobbles (Fig. 2.12a) shows a negative relationship. This is not surprising since as the cover of one increases the other will decrease accordingly and visa versa. This figure shows though how sand was the major contributor to the percentage substratum cover. The regression (Fig. 2.12b) is highly significant with $p << 0.001$.

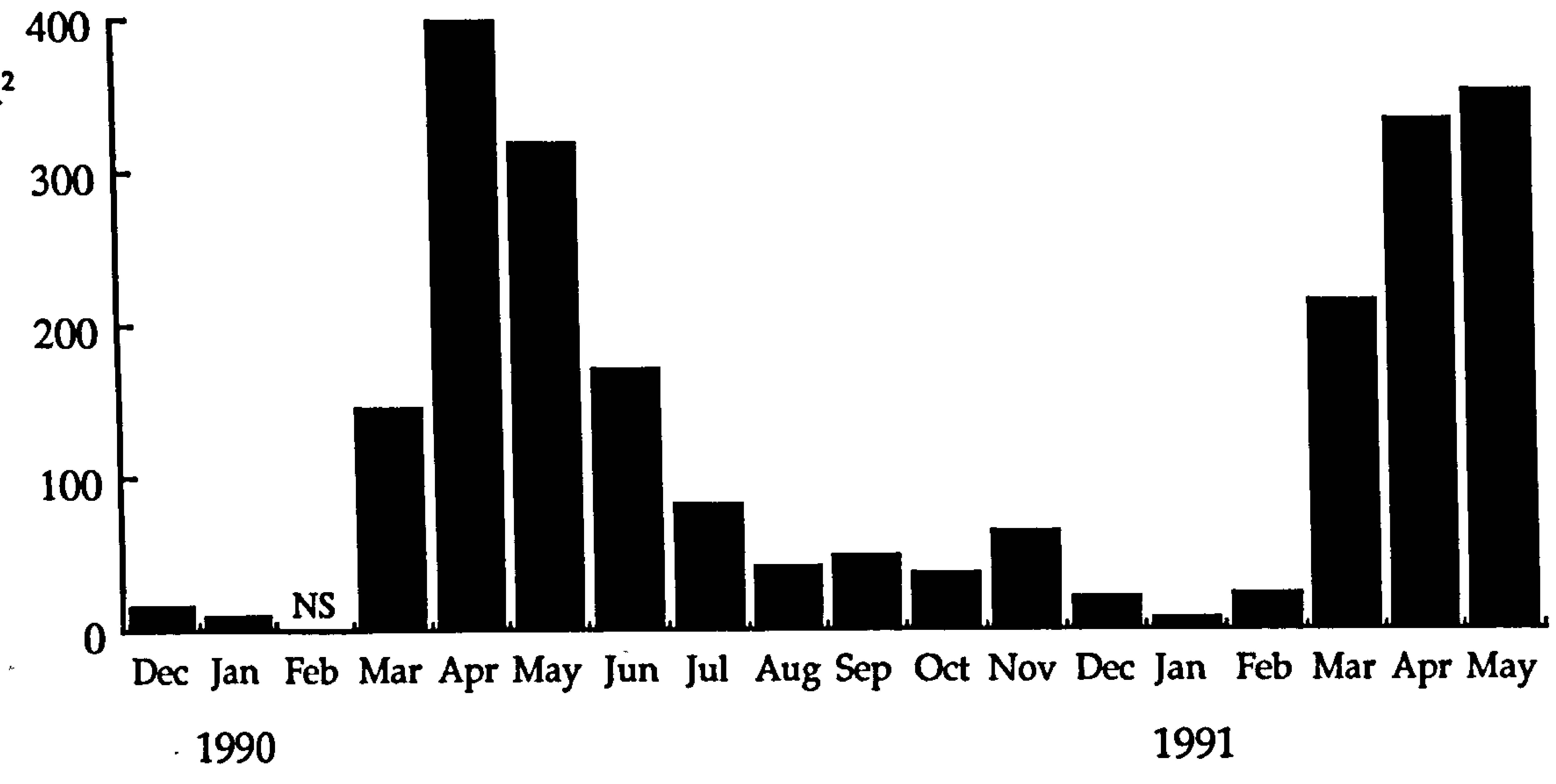


Fig 2.9 Cover of *P. laciniata* (m^2) within the sample area on the north of Port Erin beach measured from maps drawn each month (Fig. 2.1). NS = Not Sampled

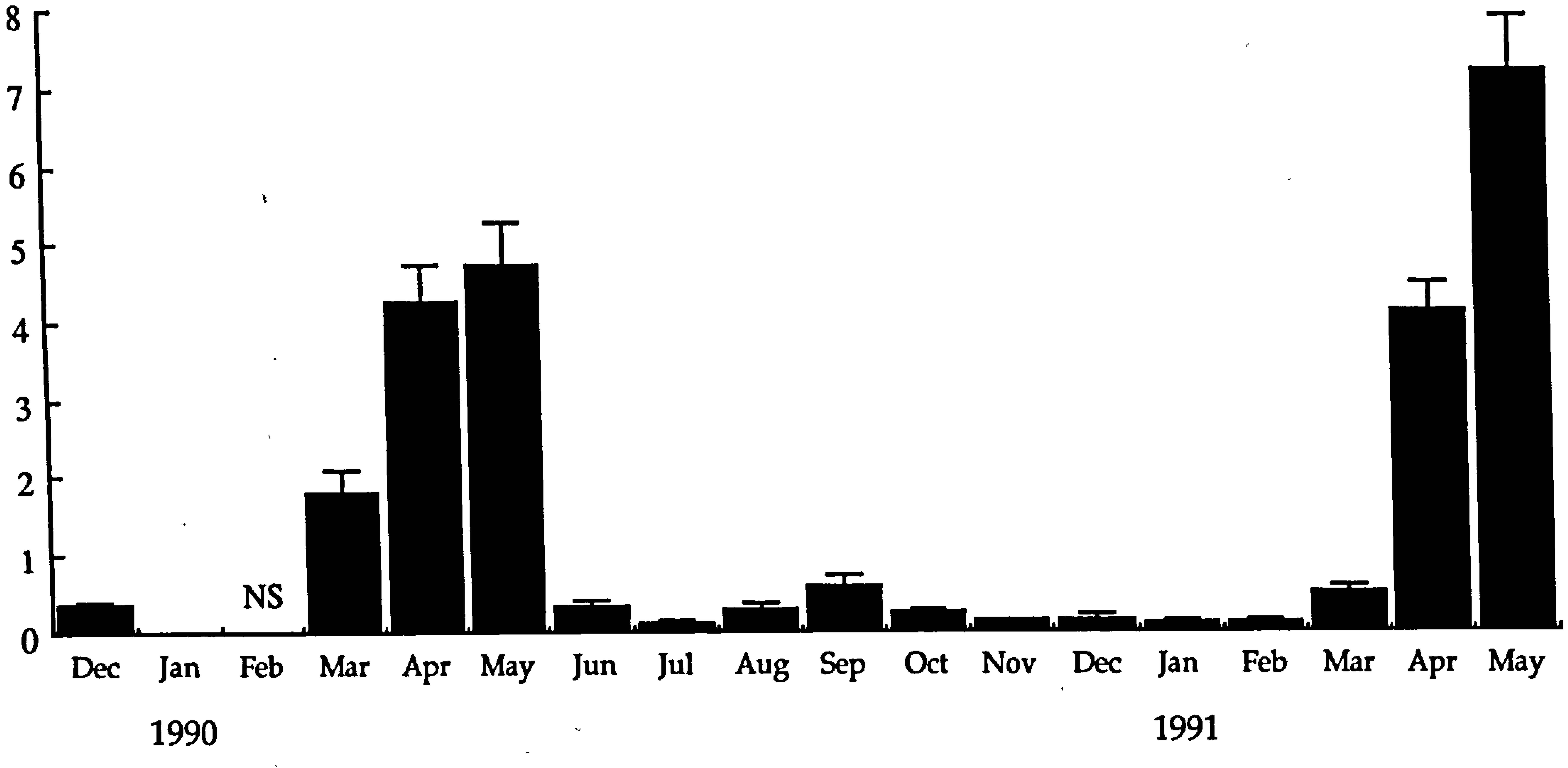


Fig 2.10 Mean percentage cover of *P. laciniata* from quadrats sampled from Port Erin beach (means and standard errors calculated from arcsine transformed raw data)
NS = Not Sampled

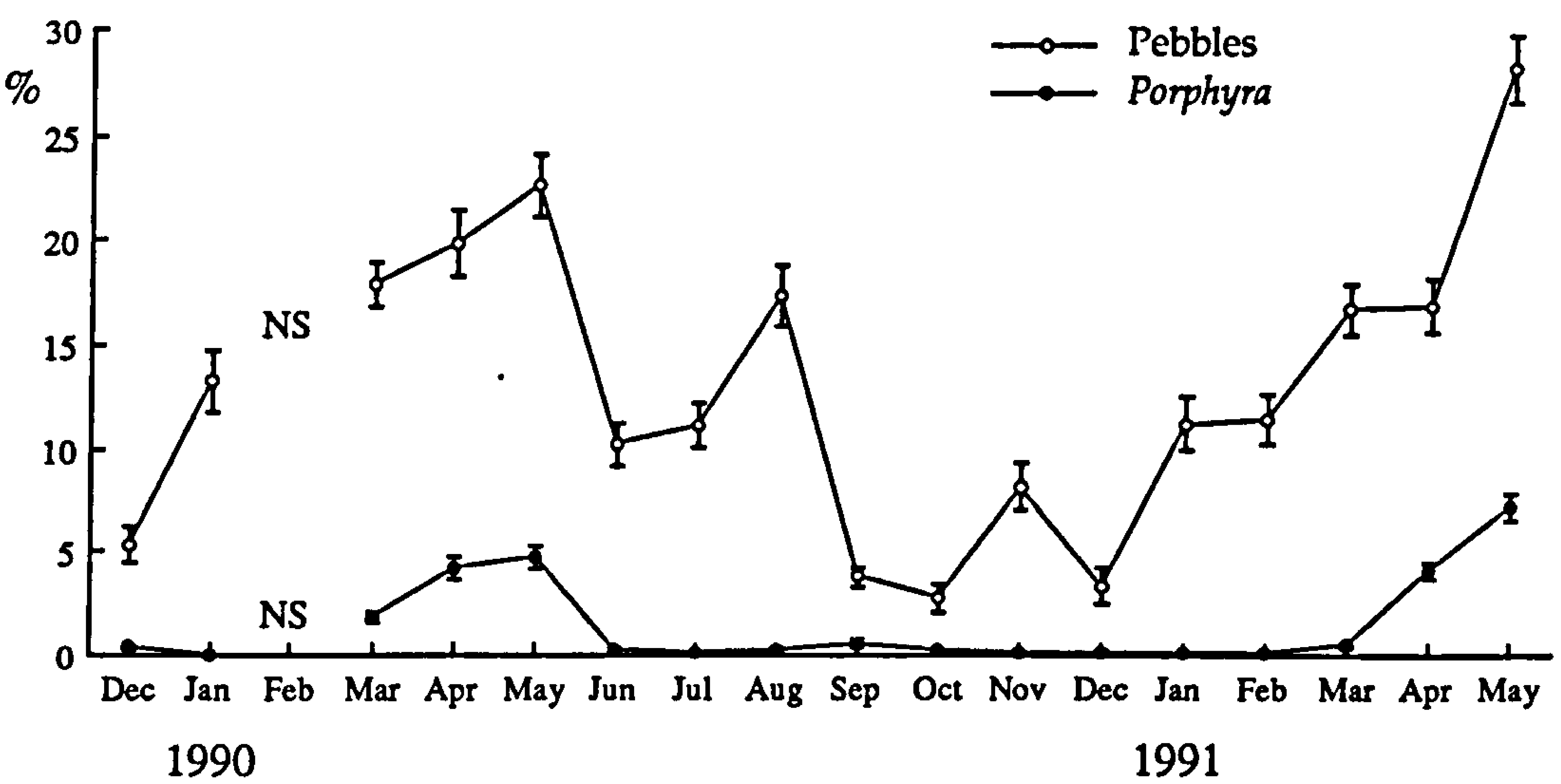


Fig. 2.11a Mean percentage cover of *Porphyra laciniata* and pebbles from quadrat sample data (means and standard errors calculated from asin transformed data) NS = Not sampled

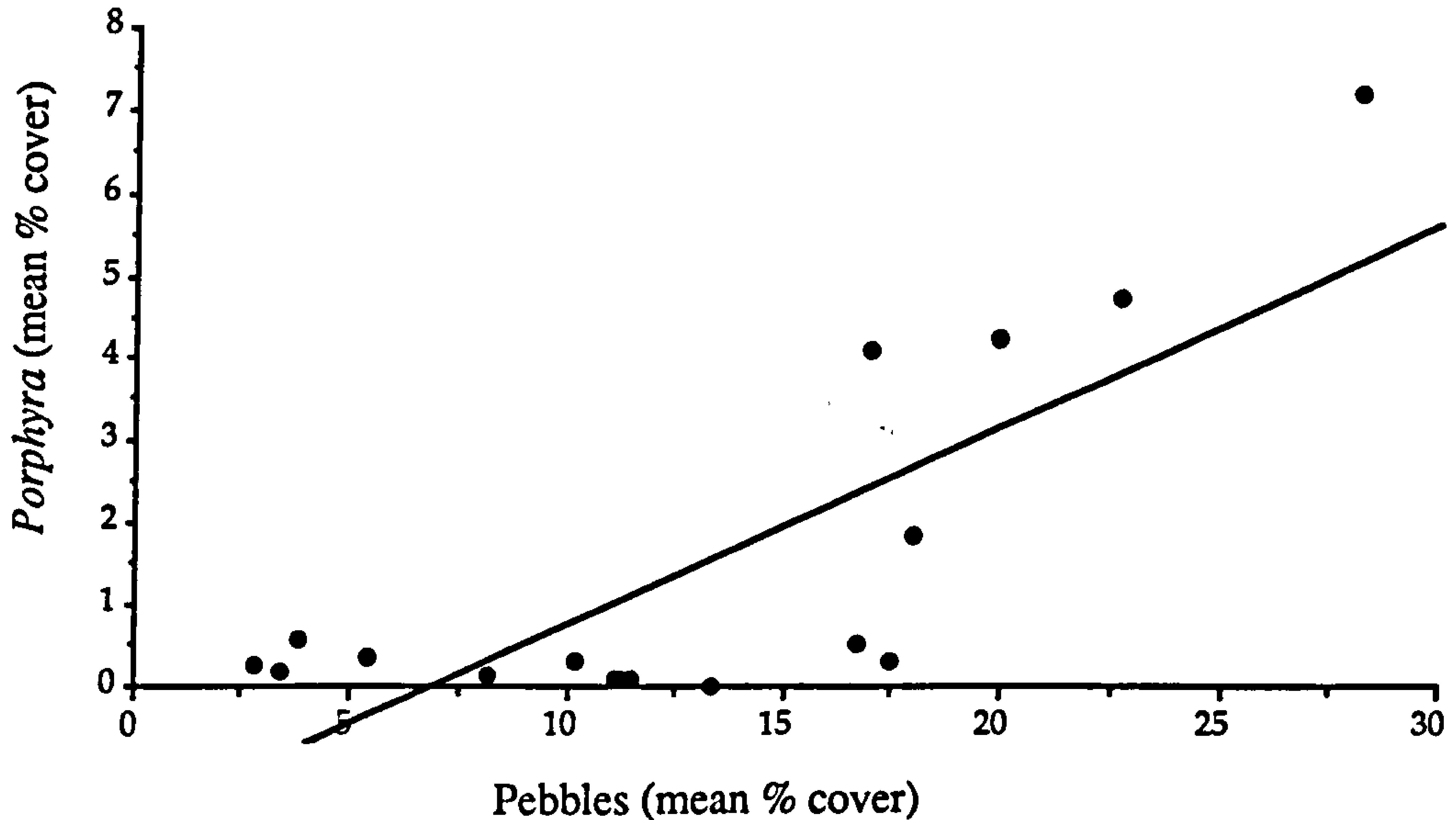


Fig 2.11b Regression for mean percentage cover of *P. laciniata* against mean cover of pebbles

Regression: $y = 0.214x - 1.66$, F test: 25.203, $p << 0.001$

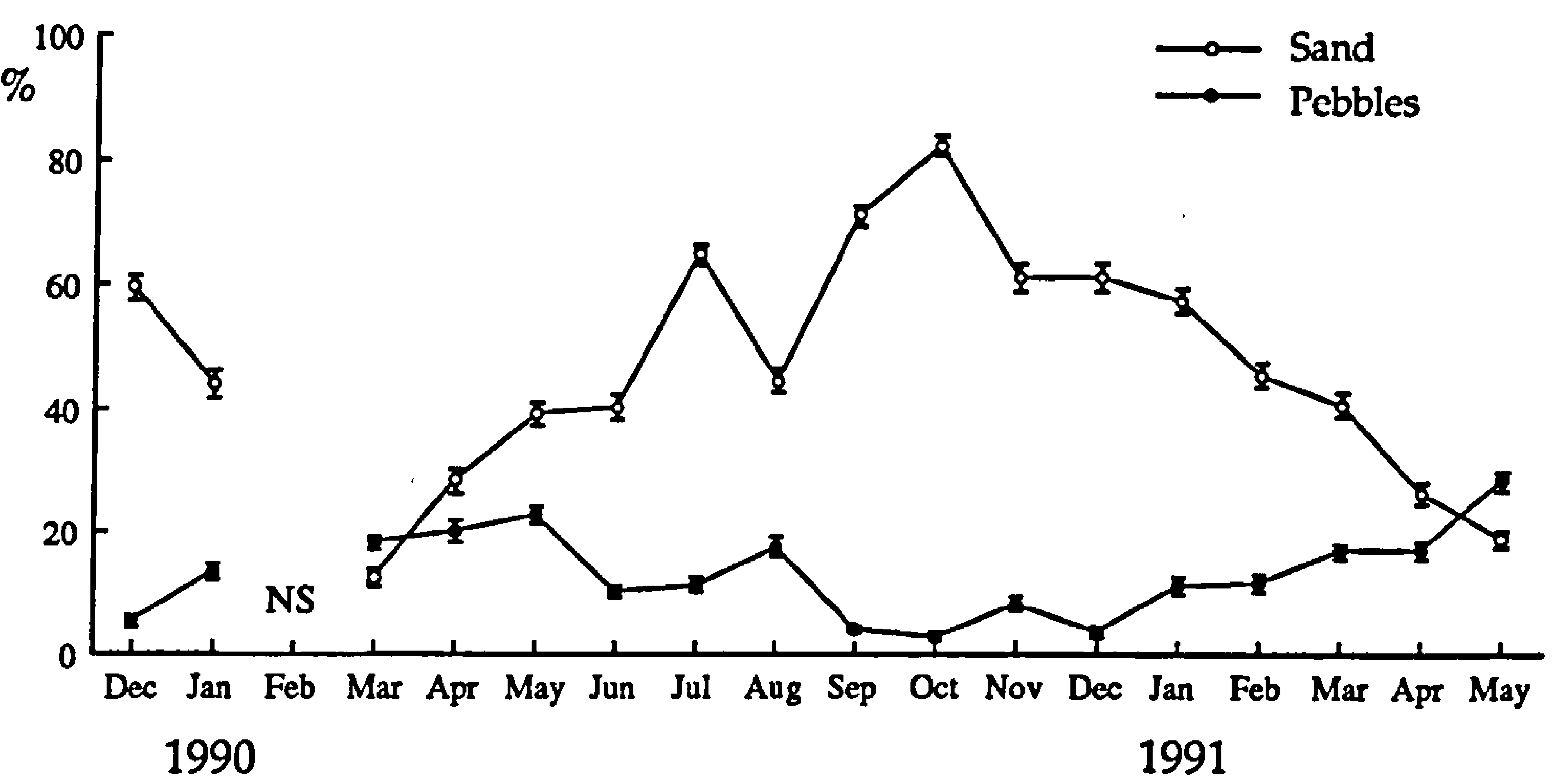


Fig 2.12a Mean percentage cover of pebbles and sand from quadrat sample data (means and standard errors calculated from as in transformed data)
NS = Not sampled

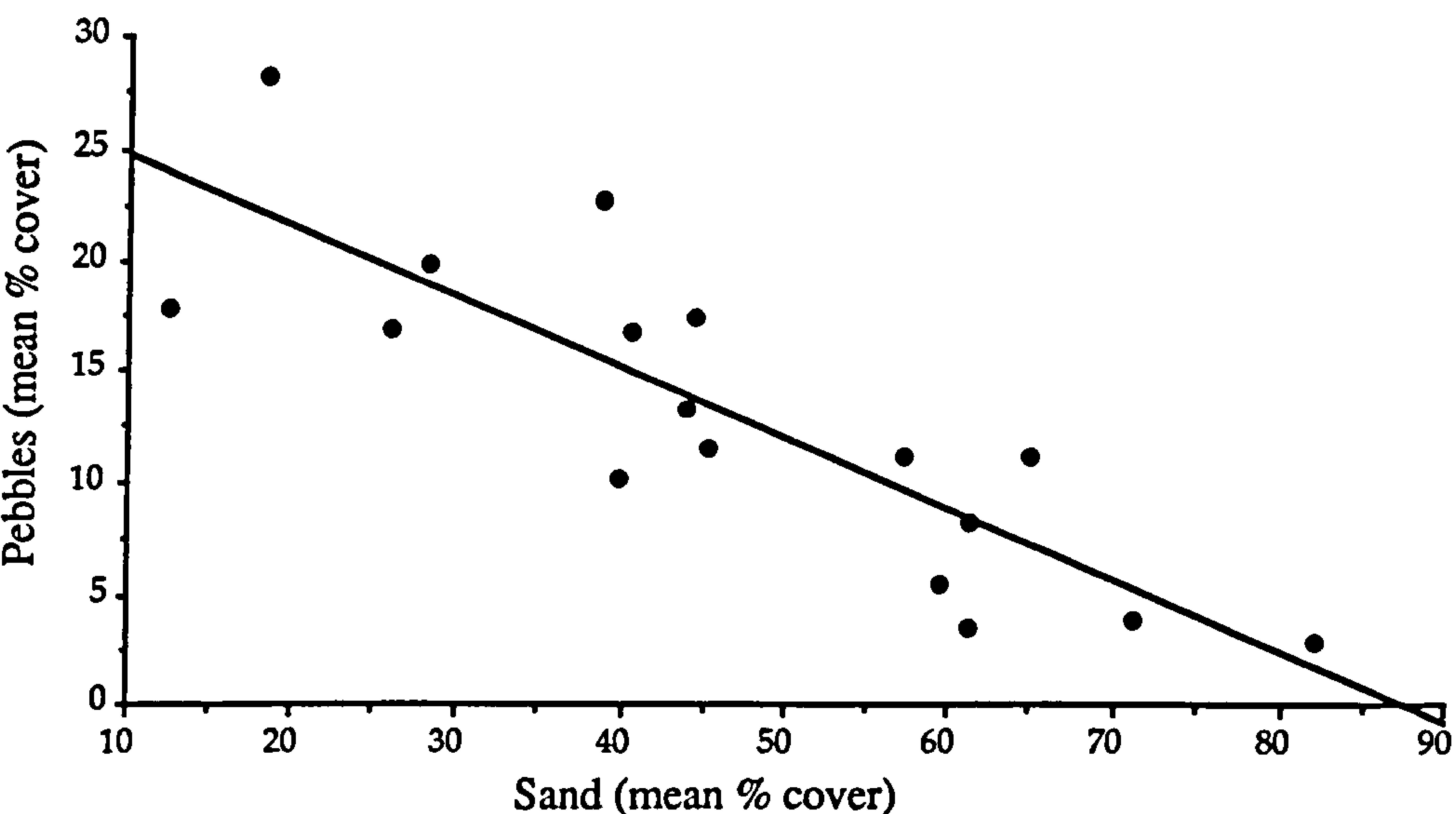


Fig 2.12b Regression for mean percentage cover of pebbles against mean cover of sand

Regression: $y = -0.319x + 27.869$, F test: 37.141, $p << 0.001$

Number

The total number of individuals sampled each month (Fig. 2.13) supports the trends demonstrated by both the map cover and quadrat cover data. The greatest number of individuals was recorded in April 1990 with a decrease in May 1990 to a relatively low level by June 1990. This trend in number repeated itself again in April 1991 but was somewhat smaller than that for the previous April. The mean number of individuals over all the quadrats sampled showed a fairly high variation in the number per quadrat (Fig. 2.13). This is indicative of the patchy nature of *Porphyra* colonisation. The patchiness results in a large proportion of empty quadrats together with quadrats containing high densities, leading to high variation in numbers.

Length

The length data of the *P. laciniata* from the quadrat samples show various results. The average lengths of the ten largest individuals were calculated, to give an indication of the growth of the plants. It can be seen (Fig. 2.14) that a rapid size increase occurred between March and April 1990 leading to a peak size of 60 cm. Size then decreased during May - July 1990 to approximately 17 cm. The mean size of the ten largest plants then stayed reasonably stable until the next sharp rise (to approximately 50 cm) during the following March. The largest plant recorded was 74 cm (in April 1990).

The length frequency histograms (Fig 2.15a-d) show how the population structure changed with the seasons. In December 1989 (Fig. 2.15a) the sample population was made up of mostly small individuals. As the number of individuals increased by March 1990 the majority of the plants were still in the lowest size classes. During April and May 1990 the plants grew, as the peak in frequency shifting to larger sized individuals. From July to November (Fig. 2.15b,c) there was no clear change in length distribution. December 1990 and

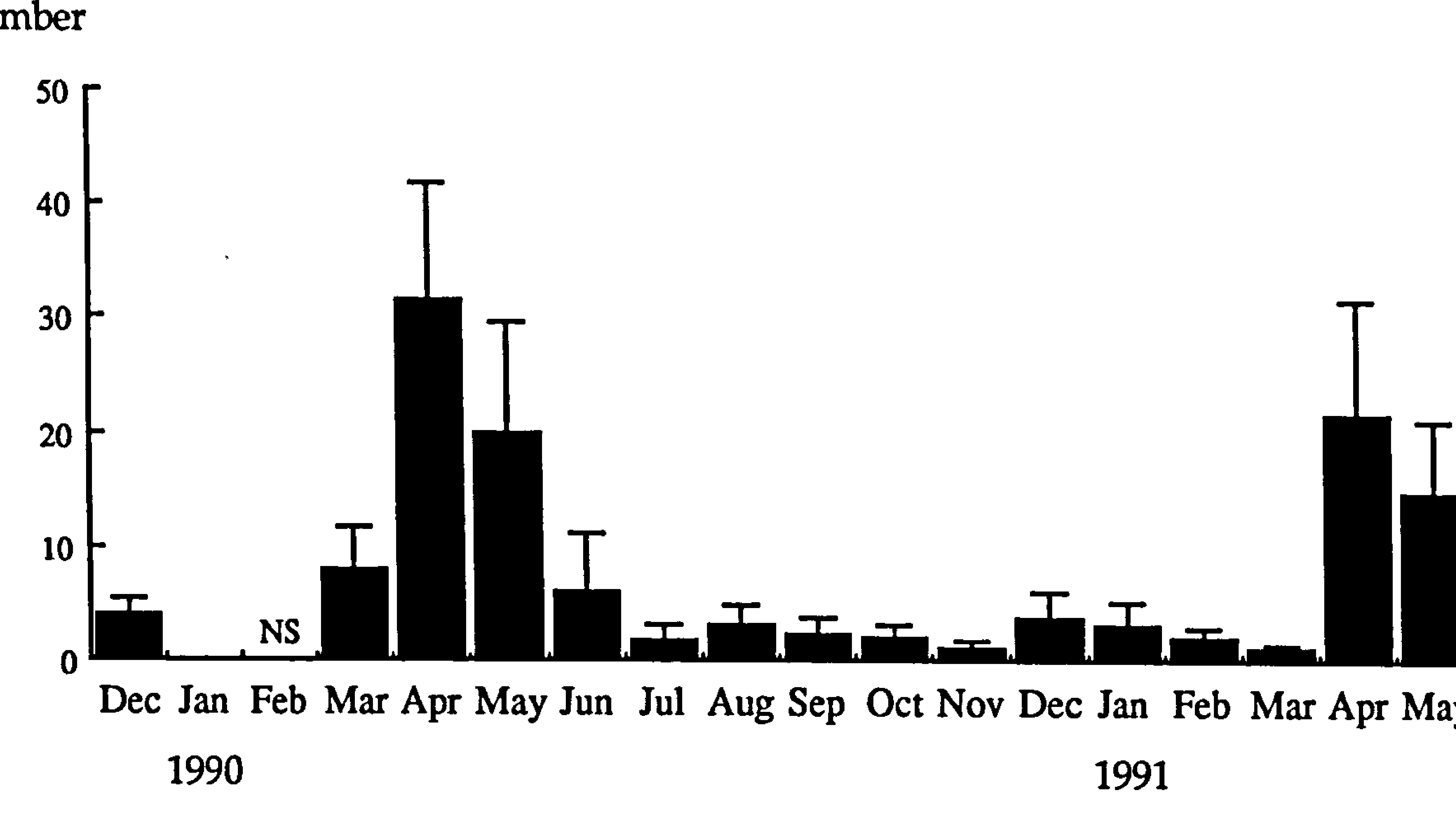


Fig 2.13 Graph showing the mean number of individuals of *P. laciniata* (per quadrat sampled) each month (error bars are single S.E.)

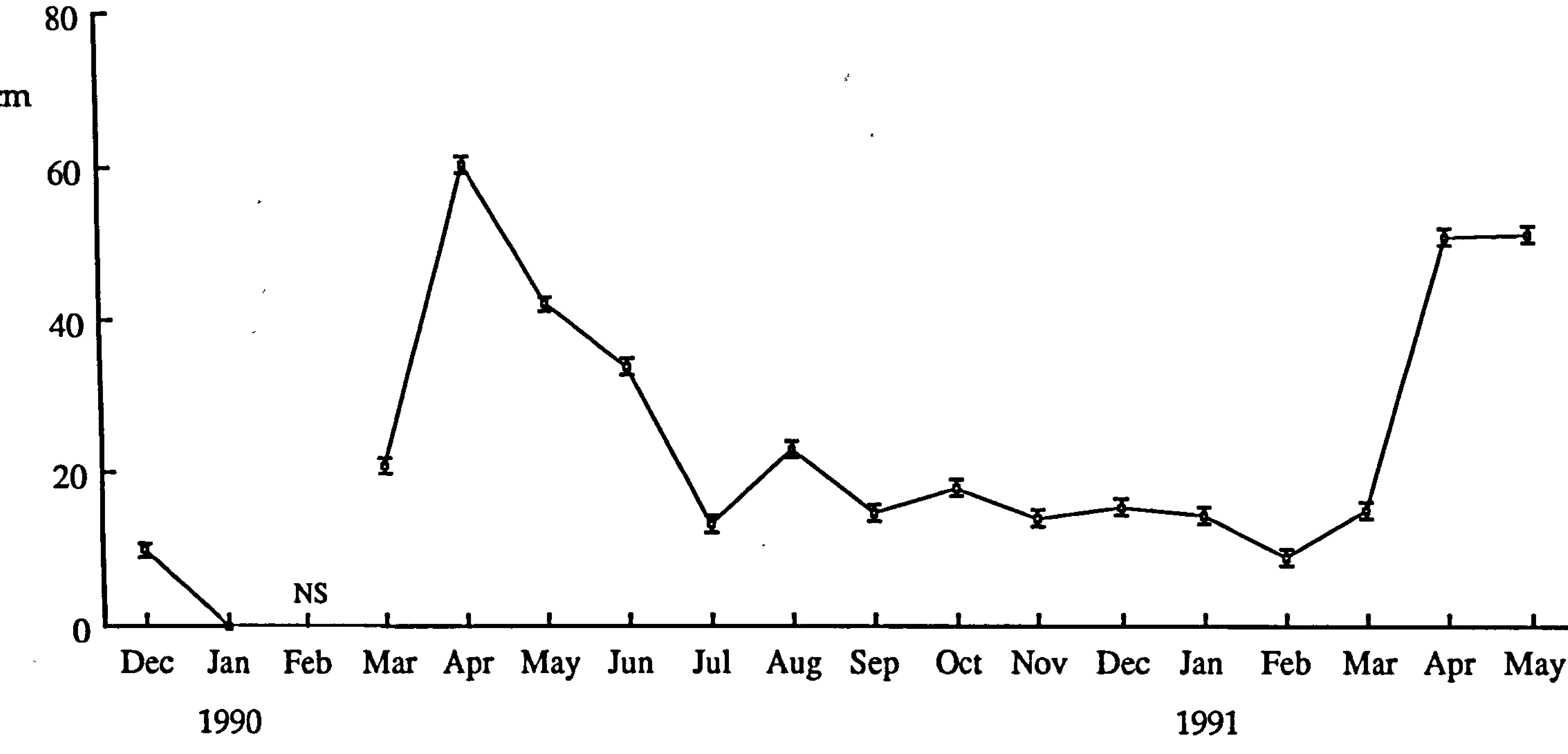


Fig 2.14 Mean length of the 10 largest plants of *P. laciniata* sampled each month during the quadrat sampling (raw data was Logn transformed to calculate means and standard errors)
NS = Not sampled

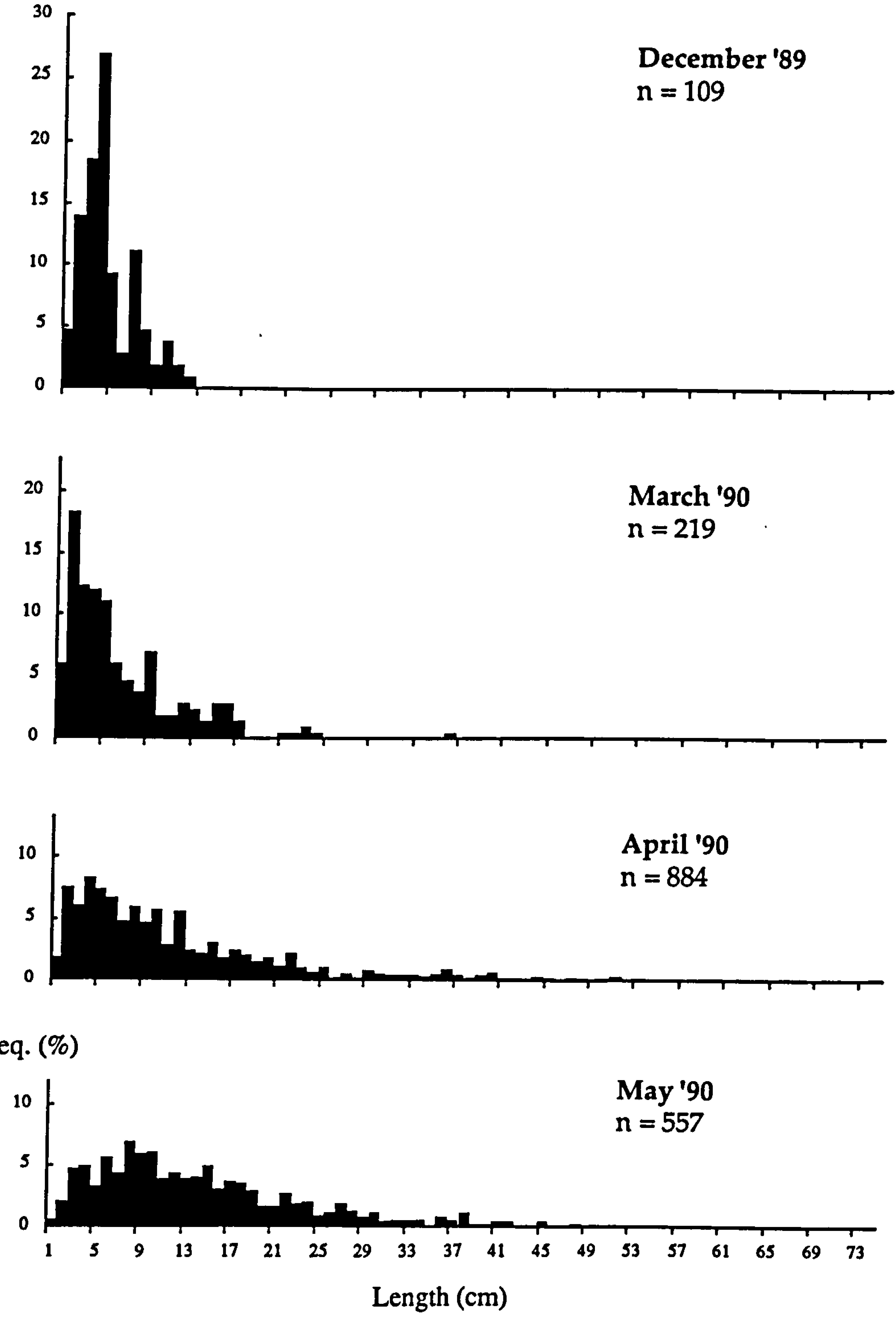


Fig. 2.15a Length frequency histograms for the quadrat samples of *Porphyra laciniata* recorded each month

June '90
n = 166

July '90
n = 51

August '90
n = 90

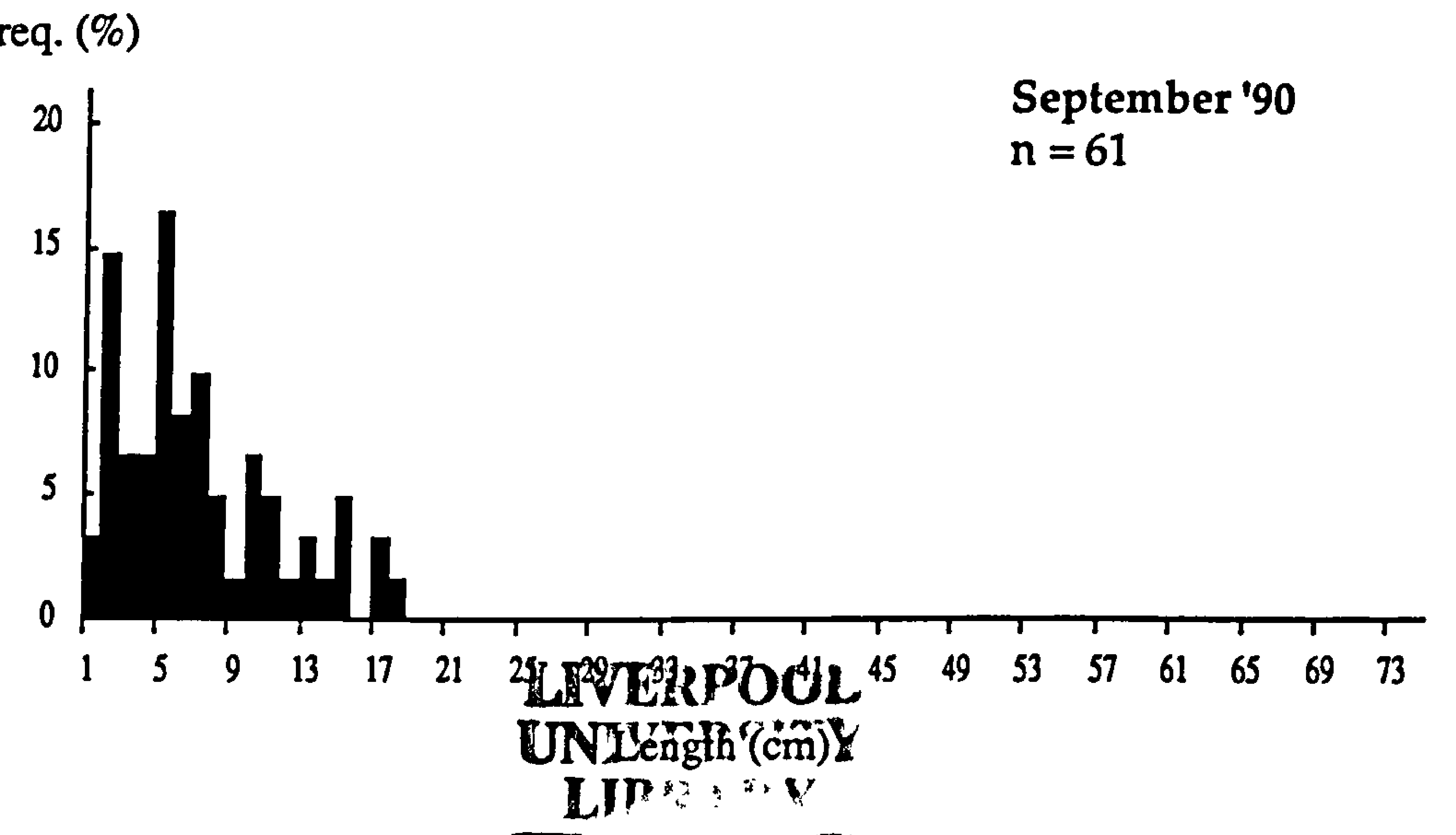


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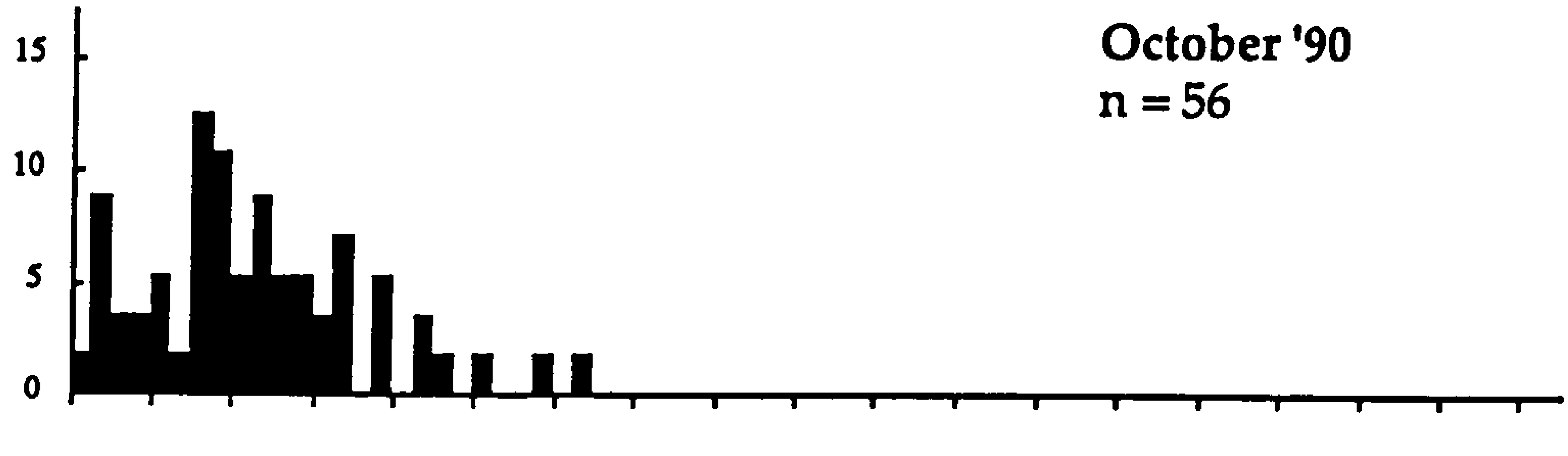
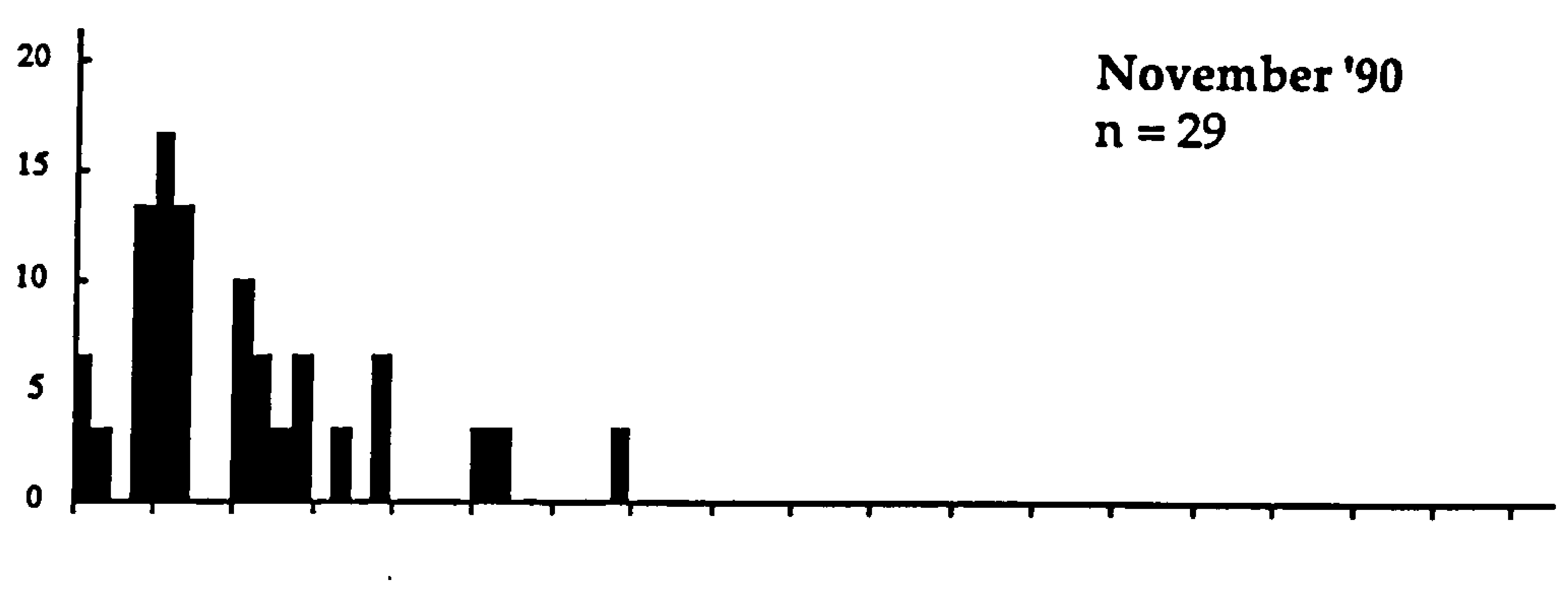
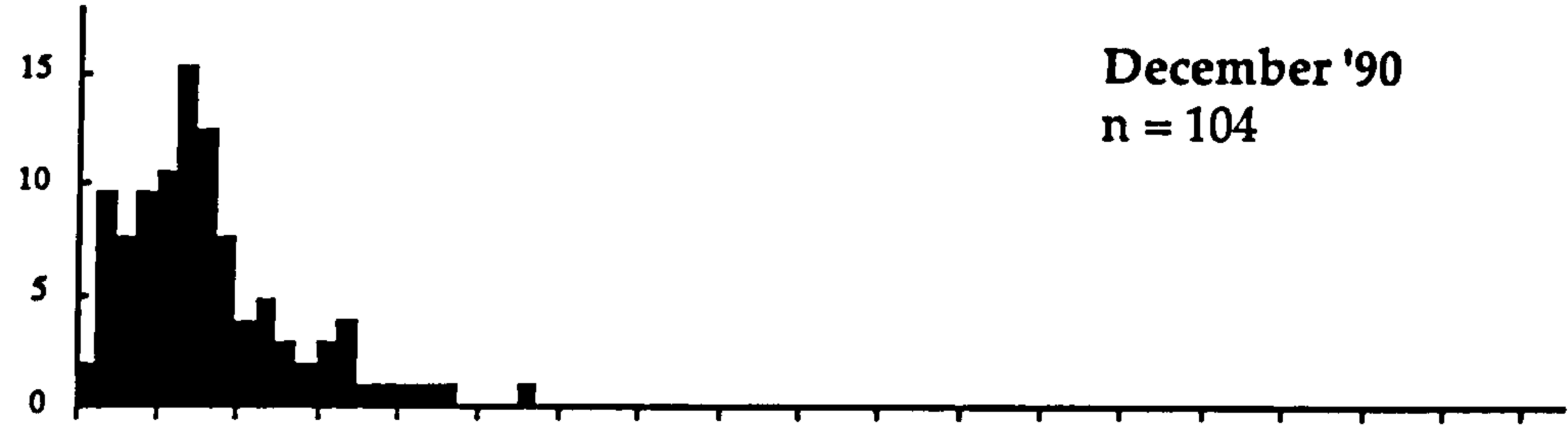
Fig. 2.15b Length frequency histograms for the quadrat samples of *Porphyra laciniata* recorded each month







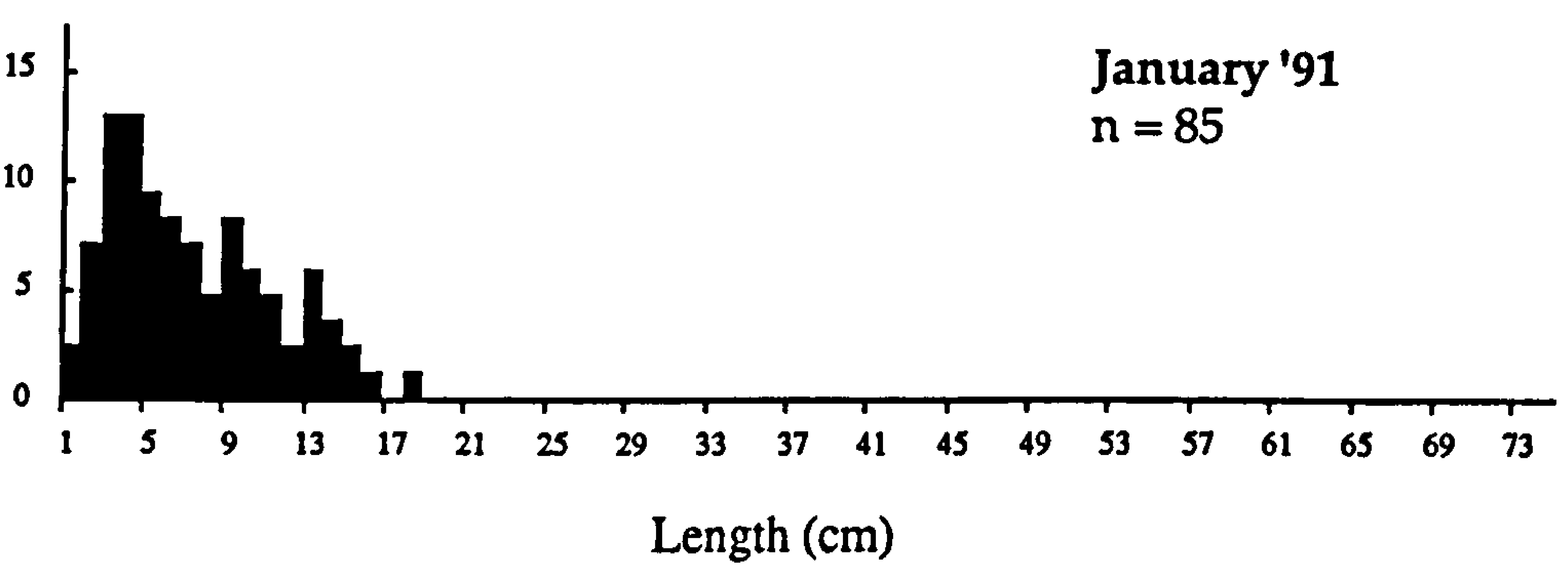


Fig. 2.15c Length frequency histograms for the quadrat samples of *Porphyra laciniata* recorded each month

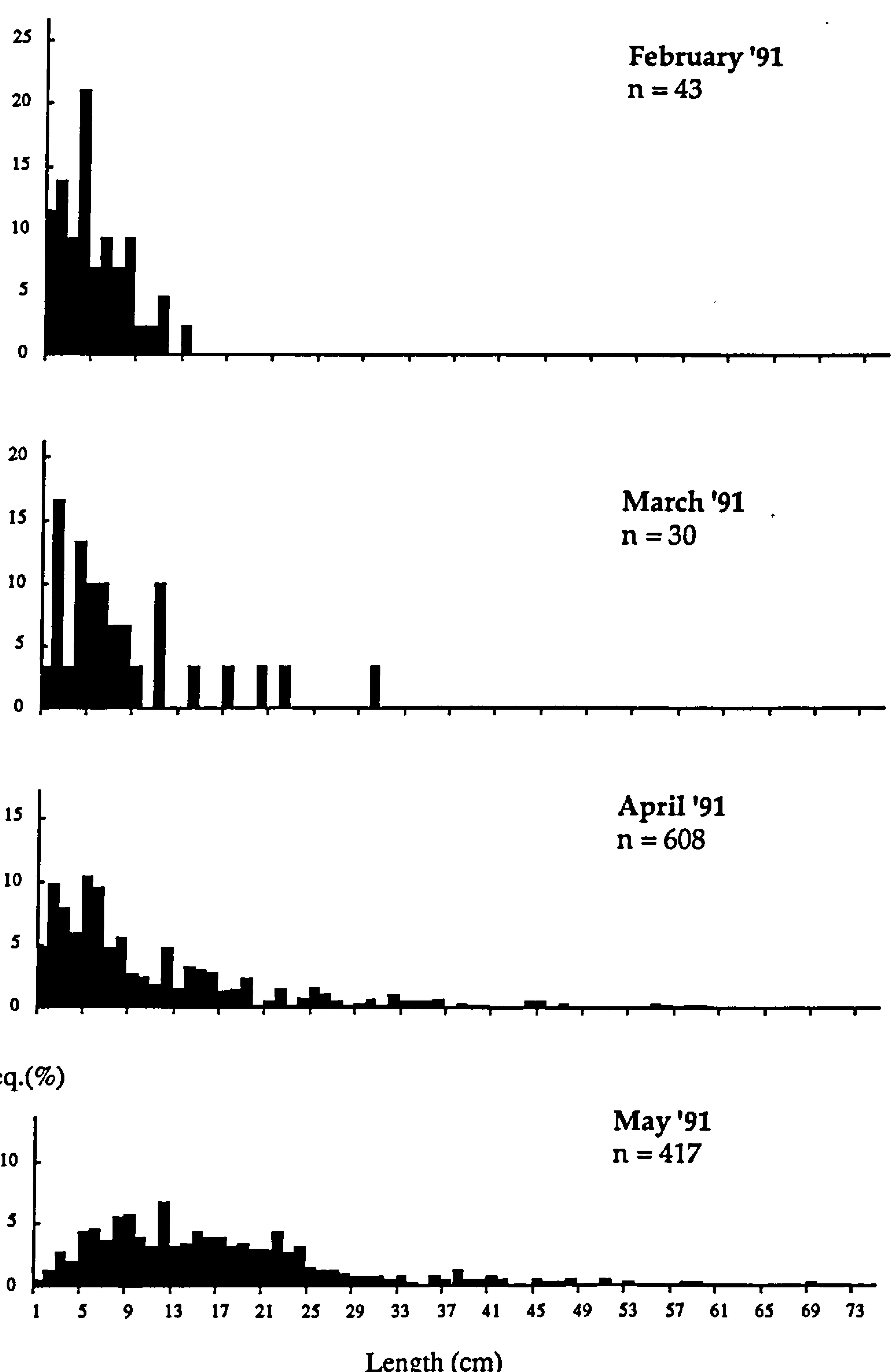


Fig. 2.15d Length frequency histograms for the quadrat samples of *Porphyra laciniata* recorded each month

January 1991 showed a population of relatively small individuals. From March 1991 to May 1991 (Fig. 2.15d) a similar growth trend to that of the previous year was demonstrated.

Weight

The wet and dry weight results (Fig 2.16a,b) demonstrated similar trends for biomass of the *P. laciniata* population as the trends in the previous results for percentage cover and number. In 1990 the peak wet weight occurred in April at 128.4 g m^{-2} with a similar peak in May (Fig 2.16a). A large reduction in wet weight biomass occurred in June falling again in July to a low level of 2.9 gm^{-2}. The wet weight followed a similar trend in 1991 with a peak in biomass occurring in April and May (125.2 & 148.8 g m^{-2}, respectively). The dry weight results (Fig. 2.16b) virtually mirrored the wet weight data with some slight differences during the spring peaks. When the wet weight to dry weight ratio was plotted (Fig. 2.17a) it showed highest values during April in both years at the times of maximum biomass. The regression of wet to dry weight shows a highly significant ($p << 0.001$) relationship as would be expected (Fig 2.17b).

Protein levels

Protein measurements for each of the species showed some trends (Fig. 2.18a,b). *P. laciniata* showed a peak in protein levels around April and May at the time of maximum biomass and lowest mean protein levels during the summer months (July - August 1990).

P. linearis had the highest level of protein of all the species measured (42.63% January, 1991) with a peak protein similarly occurring just before the peak in biomass and abundance on the shore.

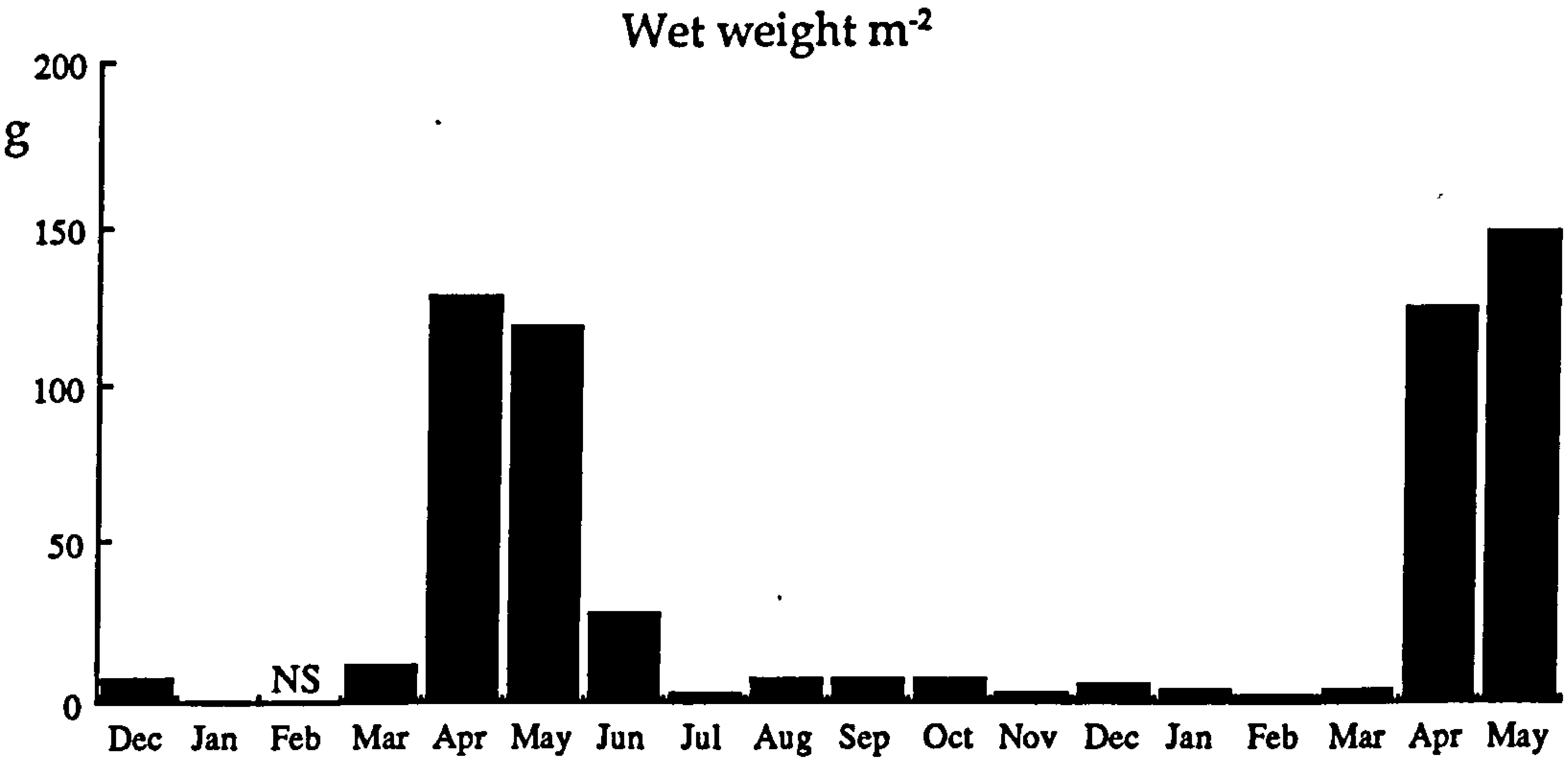


Fig 2.16a Wet weight (g m^{-2}) of *Porphyra laciniata* from quadrat data sampled on the north end of Port Erin beach. NS = not sampled

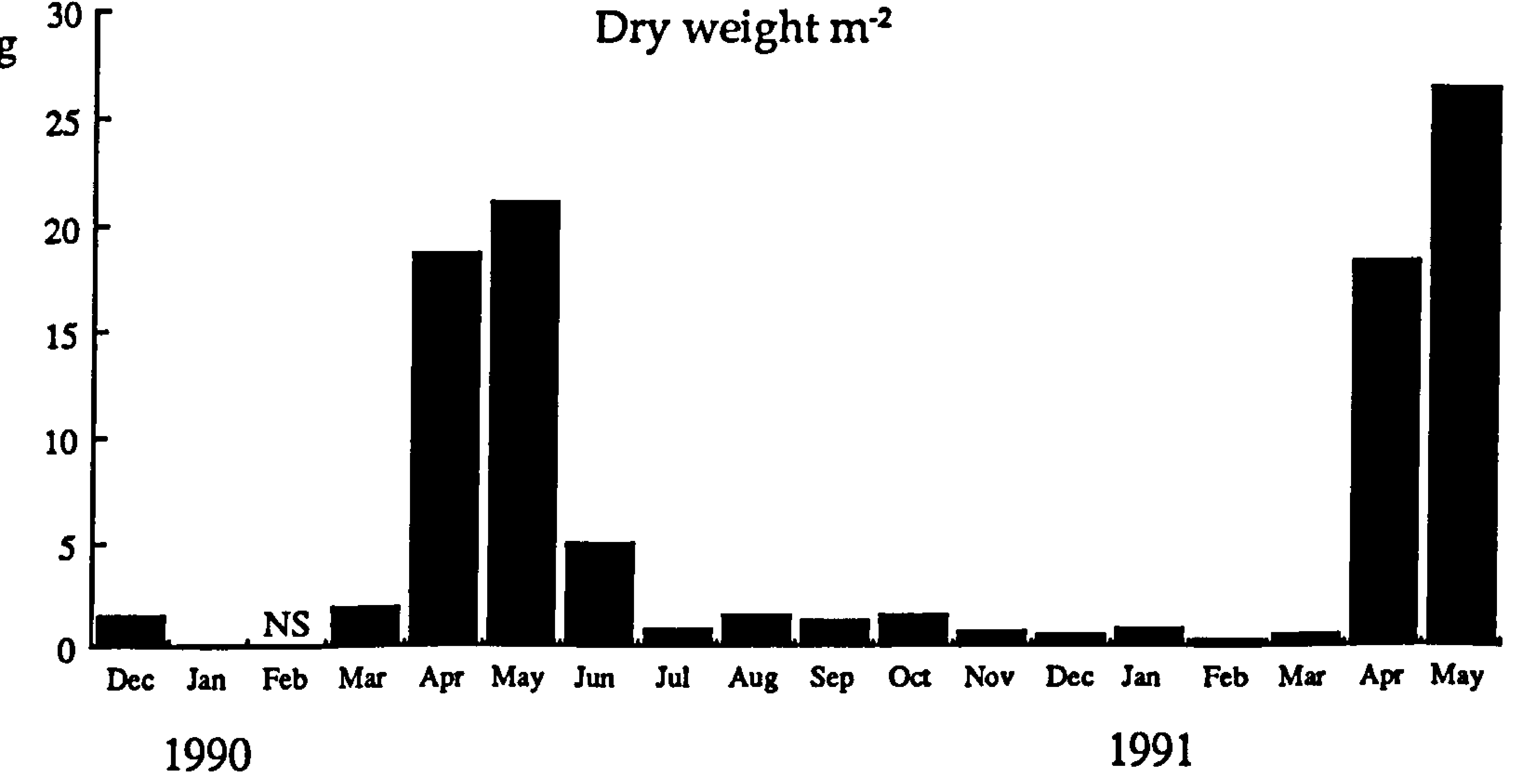


Fig 2.16b Dry weight (g m^{-2}) of *Porphyra laciniata* from quadrat data sampled on the north end of Port Erin beach. NS = not sampled

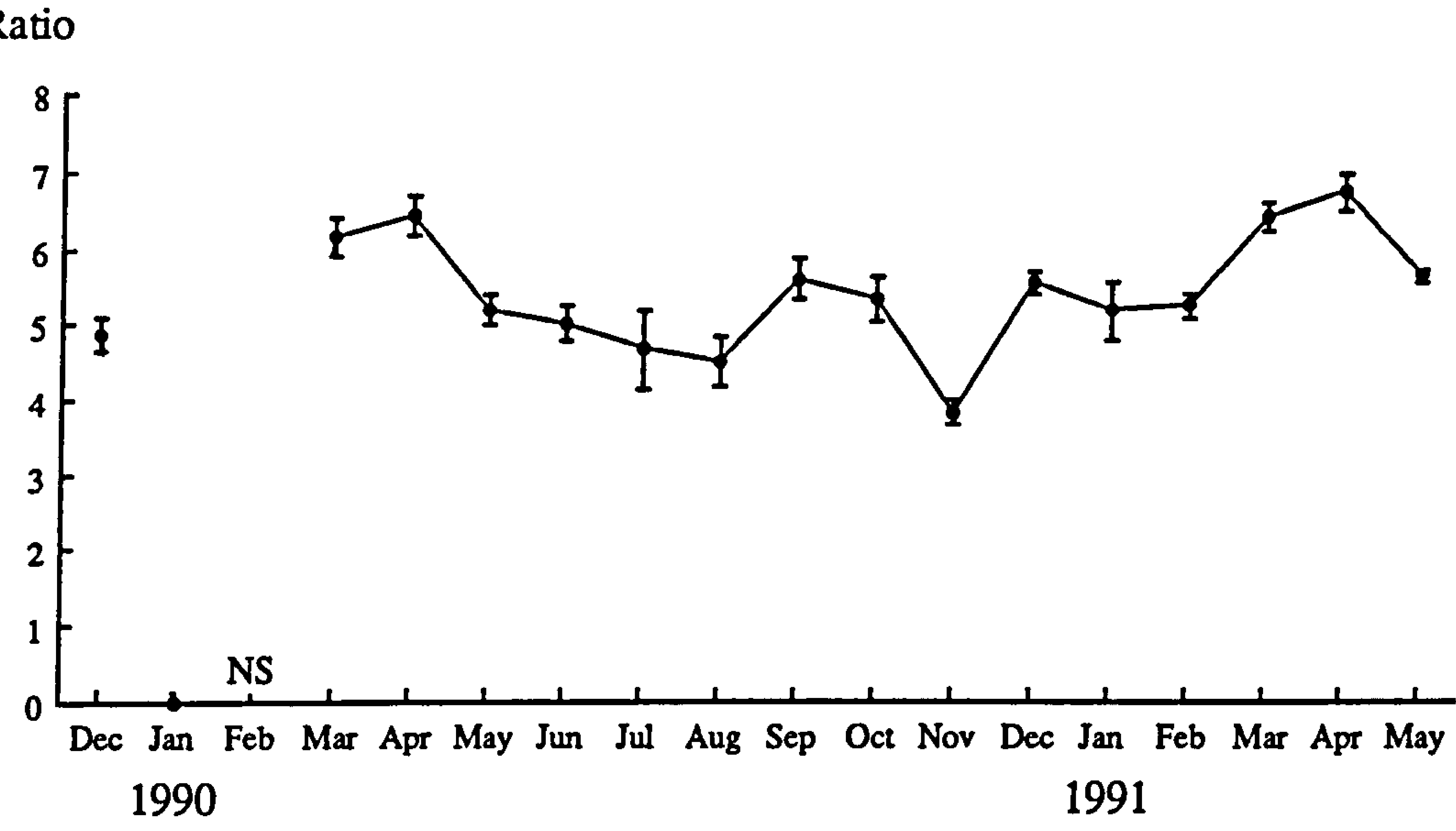


Fig 2.17a Wet weight to dry weight ratio for *P. laciniata* from Port Erin, from quadrat data (error bars are one standard error)
NS = Not sampled

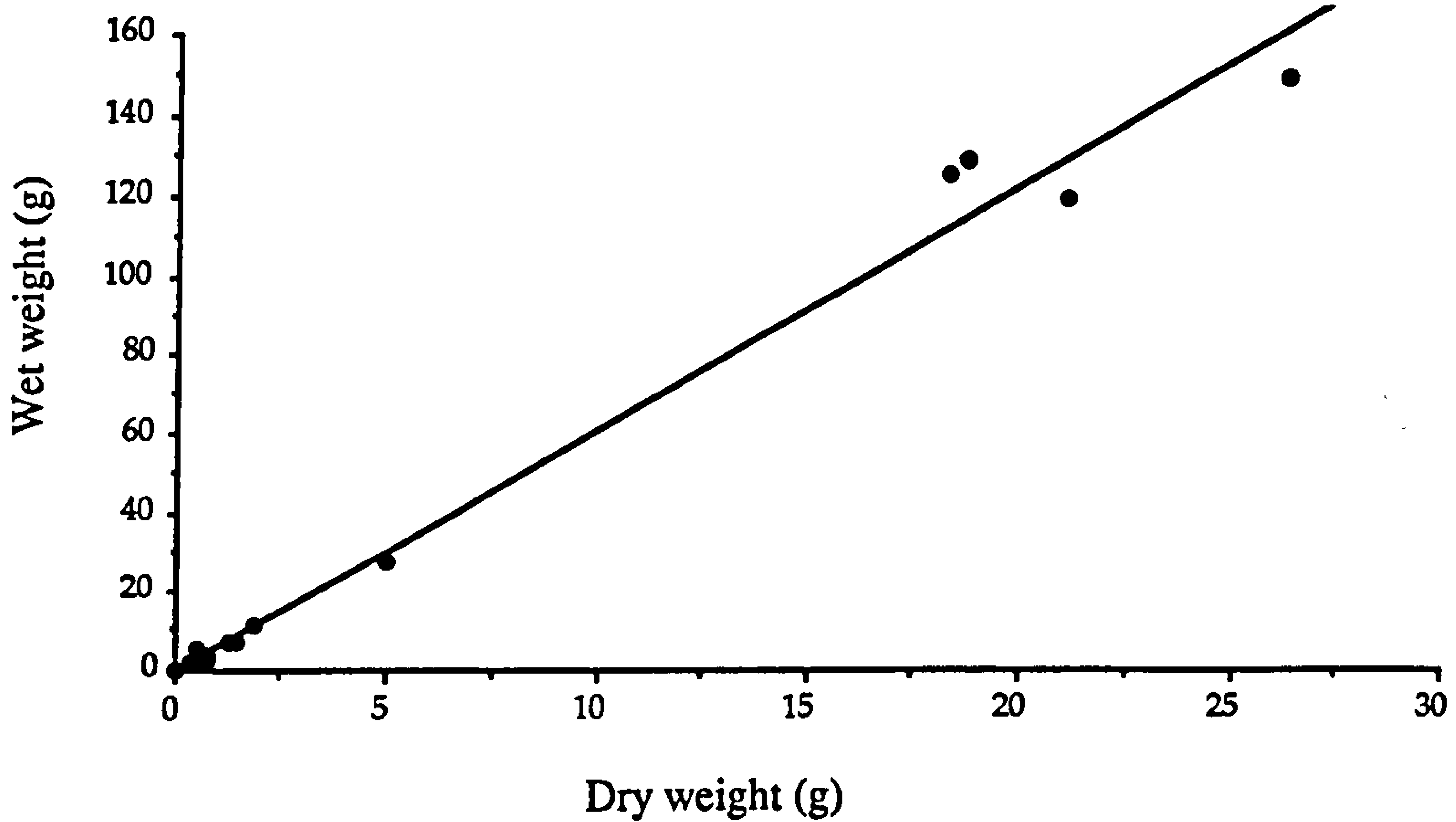
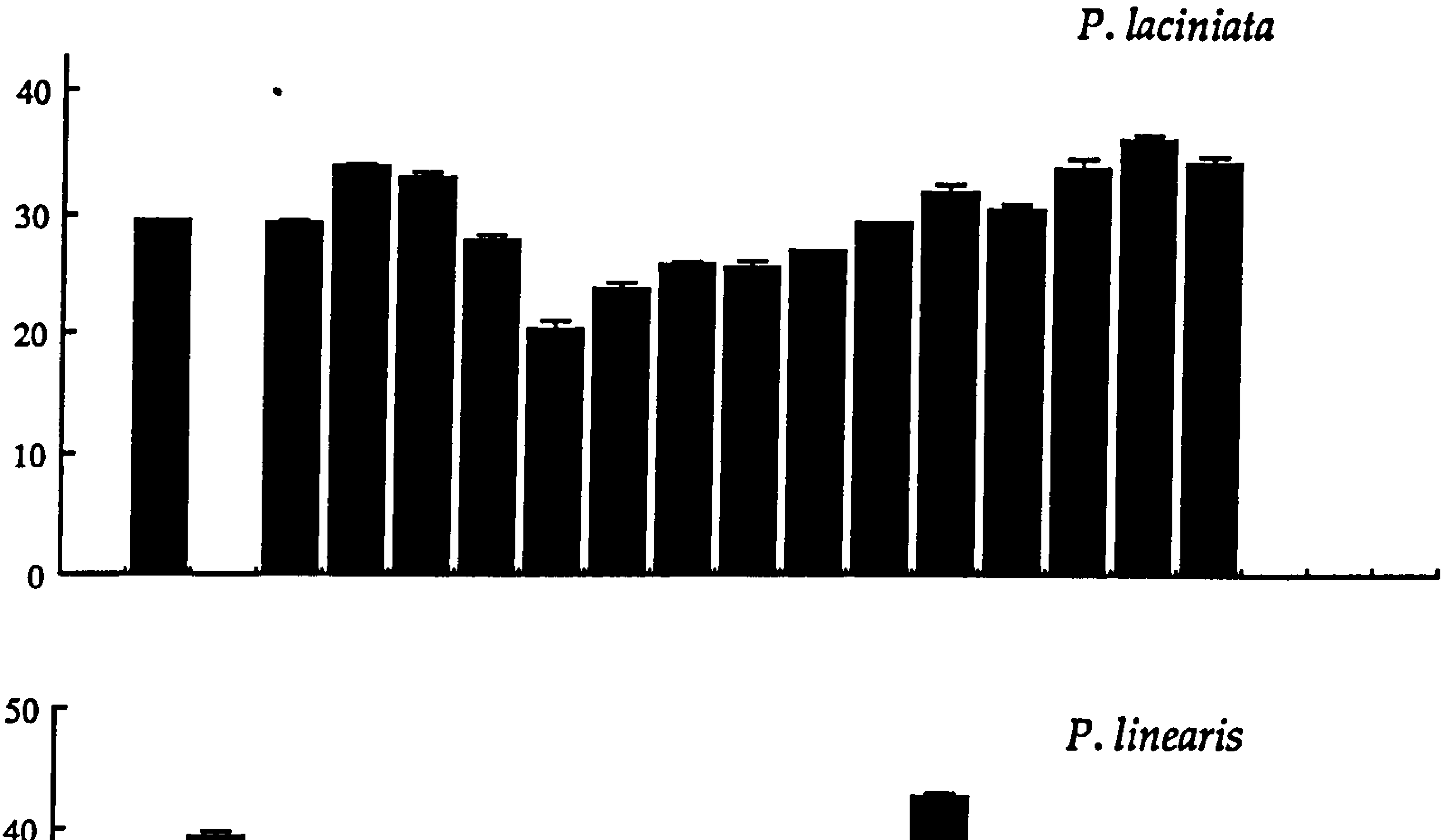


Fig 2.17b Regression for wet weight to dry weight data
(y = 6.114x -0.213, F test 1217.41, p << 0.001)



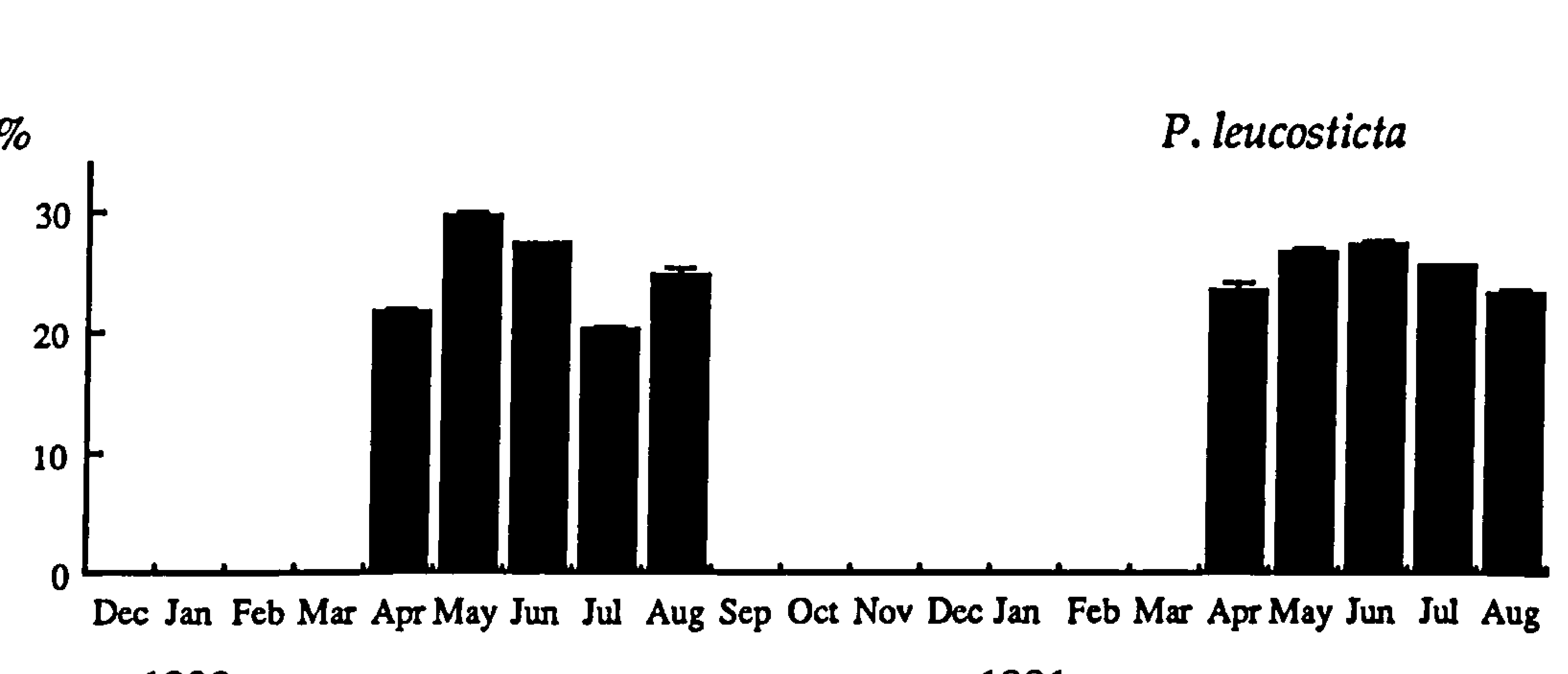
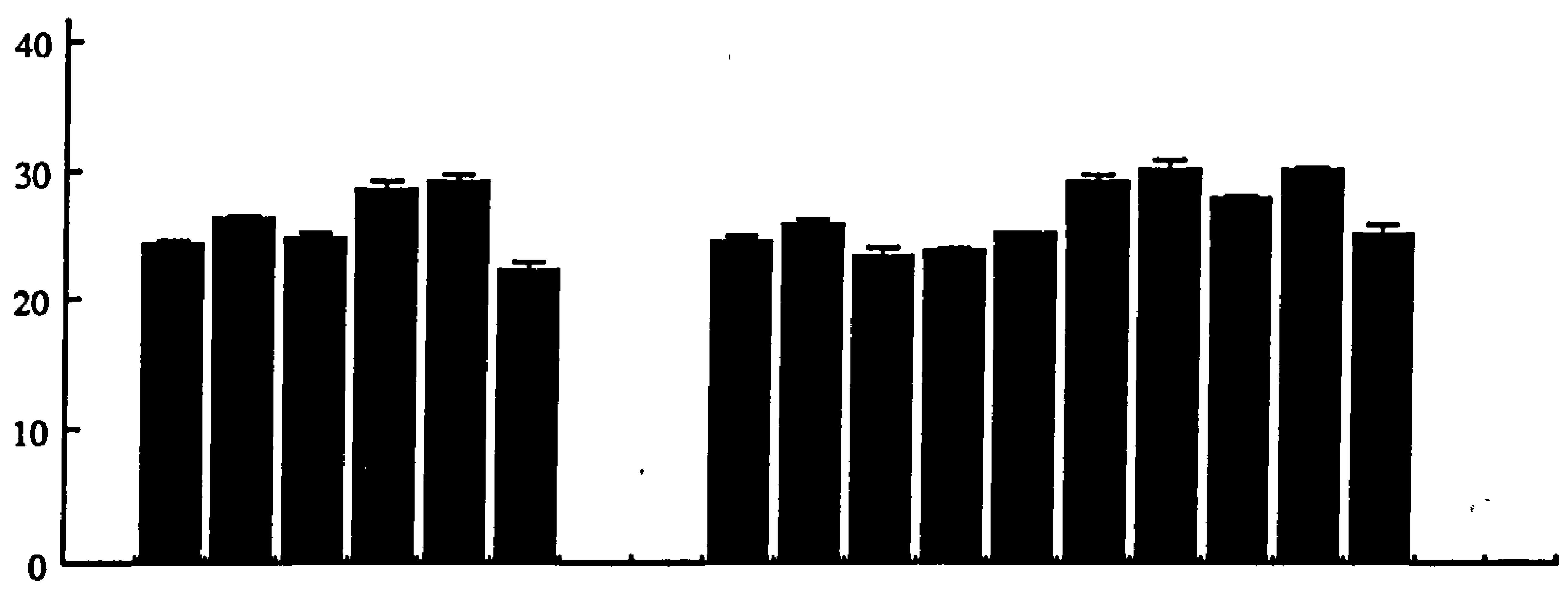


Fig 2.18a Levels of protein (% dry weight) of *Porphyra* measured when species were available (error bars are one standard error of three replicate samples).

P. umbilicalis (high shore)



P. umbilicalis (low shore)

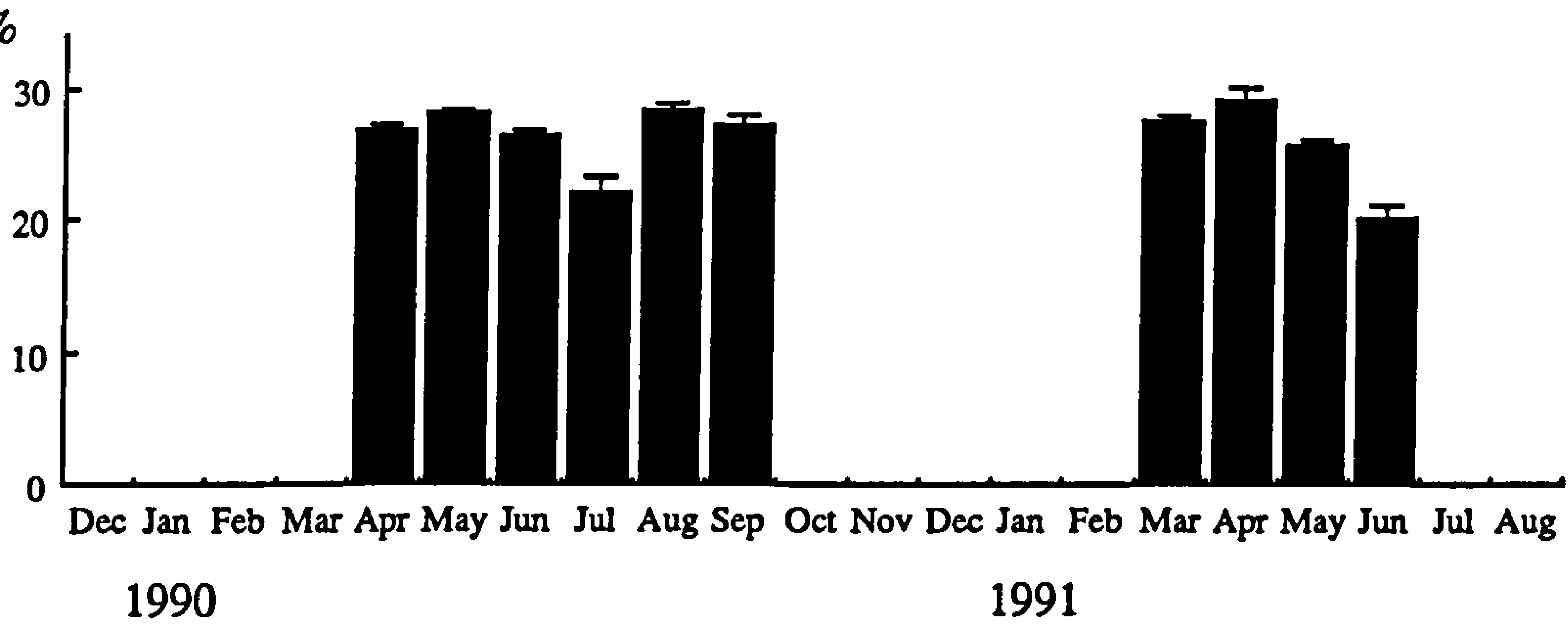


Fig 2.18b Levels of protein (% dry weight) of *Porphyra* measured when species were available (error bars are one standard error of three replicate samples).

P. leucosticta showed the lowest protein level (20.23 %, Fig. 2.18a) of the species. These levels are towards the low end of measurements made by Zavodnik (1987) of 23 - 33 %. A peak level occurred during May 1990 and June 1991 again around the times of optimum biomass.

High shore and low shore *P. umbilicalis* was measured but no discernible differences were seen (Fig. 2.18b). From April (1991) to June (1991) the low shore *P. umbilicalis* showed a reduction in protein levels although this did not occur to the same extent in the previous year.

When annual mean protein levels from all data for each species were calculated (Fig. 2.19). *P. linearis* showed the greatest level (35.95 %) with *P. laciniata* the second highest. The other species tested had similar protein levels around 26 %, *P. leucosticta* having the lowest mean values at 25.07 %.

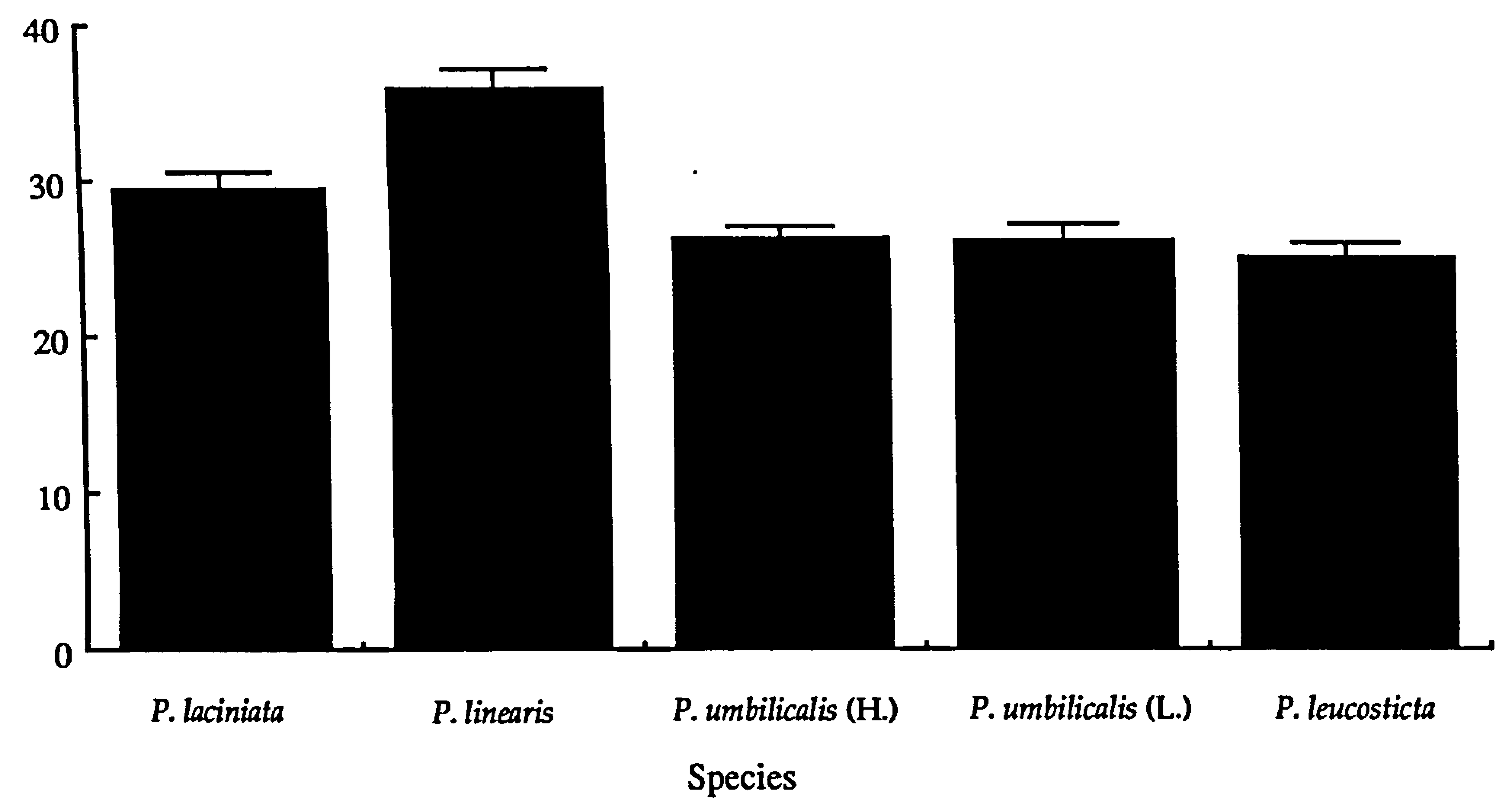


Fig 2.19 Mean protein levels of 4 species of *Porphyra* as % dry weight (H. = high shore, L. = low shore). Means are calculated from all monthly measurements (Fig 2.18) for each species (error bars are one standard error)

DISCUSSION

In this study *P. linearis* was found to grow high on the shore in the supralittoral zone with a well defined winter season as already described by Conway (1964a), Edelstein & McLachlan (1966), Bird (1973) and Munda & Markham (1982). Plants first appeared in October when the sea water temperature was approximately 13 °C. Bird (1973) demonstrated the release of conchospores in cultured conchocelis material at similar temperatures in Nova Scotia which may indicate that the populations of *P. linearis* around the Isle of Man arise as a result of conchospore release. Populations increased and extended higher up the shore after a period of a few weeks, particularly after periods of high wave action or storms. This extension of patches up the shore is presumably the result of decreased desiccation stress on spores as the higher splash zone areas remain wet during the periods of increased wave action. It is also possible that the extension of patches maybe as a result of monospore release from the existing blades. Patches of plants were observed to rejuvenate after large storms during which plants were lost. This was also noted by Munda & Markham (1982) in *P. linearis* in Helgoland.

The evidence produced by mapping patches of *P. linearis* demonstrated how patches occurred in the same positions for two consecutive years with the same phenomenon being observed the previous un-mapped year. This would be hard to explain by settlement of conchospores released from conchocelis from any great distance away, for example in the subtidal, particularly when surrounding rock areas around the observed patches appear to have equally suitable places for growth of the adult thalli. This repeat of patches in the same positions each year raises questions as to what parent populations these thalli originate from and their likely position in the shore environment. A few explanations are possible for this phenomenon. Conchocelis has been shown to have the ability to grow within certain areas in the intertidal [discussed by

Conway & Cole (1977); Boney (1978)] e.g. in intertidal barnacles (Dixon & Richardson, 1969; Mumford, 1975; Martinez, 1990); in limestone (Drew, 1954; Ogata, 1955, 1961) and endophytically in some intertidal algae as suggested by Boney (1978).

In the case of this study populations of conchocelis could be living nearby in the large area of barnacles (Fig. 2.6). Conchocelis filaments were observed growing in the intertidal barnacles during October 1991 at both this site and at the detailed study site in Port Erin but could not be assigned species status. Their presence is however evidence that intertidal conchocelis communities exist in these areas of shore and could consequently produce conchospores necessary for the production of the *P. linearis* populations. Further study of conchocelis communities from intertidal barnacles combined with identification of the species would help to examine this.

After conchospore release localised flow conditions may lead to settlement of these spores and the consequent growth of the thalli in the same place each year although the chance of these flow conditions being so precise from year to year is low. If the conchocelis is very close by (growing in the limestone) the spores from the parent conchocelis may only travel very small distances or germinate *in situ*. This latter hypothesis would appear to be the most likely explanation. If this were the case a study of the genetic makeup of these patches of *P. linearis* using the simple electrophoretic techniques described in Chapter 3 would give strong indications as to the level of isolation of these patches of thalli and thus an indication of whether they originated from different parent (conchocelis) populations. A distance hierarchy could be produced of *P. linearis* patches on an area of shore therefore producing evidence of the degree of population distinction between the patches.

Another possible explanation for the patches growing in the same positions from year to year is that *P. linearis* may survive the summer as dwarf plants

(skeletons) particularly in sheltered cracks within the patch areas. These dwarf plants have been observed in populations studied on the Isle of Man and by Bird (1973) in Canada. Once suitable conditions arise these individuals could re-establish the large dense patches of adult thalli (Dixon, 1973). Similar surviving basal portions of adult thalli showed no signs of life when cultured by Bird (1973) and so it would seem unlikely that although present in some areas these portions of thalli would survive through the summer. If plants did survive this would require the presence of monospores in this species in order for the old remaining thalli portions to spore and produce the large dense populations of the adult thalli. Monospores were found evident in populations in Scotland (Cole & Conway, 1980) but not evident in the *P. linearis* plants studied in Nova Scotia (Bird, 1973).

P. umbilicalis was often found associated with *P. linearis* with the high exposure of the *P. linearis* habitat producing the high shore morphotype of this species.

P. laciniata was relatively easy to distinguish from high shore *P. umbilicalis* morphotypes. However, when *P. umbilicalis* appeared in moderate exposed conditions with *P. laciniata* occurring in the same location the two morphologies became almost indistinguishable. Hence in this 'grey area' great care should be taken when separating the two species. Conway (1964a) noted that P. *laciniata* (as *P. purpurea*) was often found on rocks subject to a high degree of sand cover, and in this study this was particularly the case at the site in Peel (Plate 2.1). This demonstrates the ability of the species to tolerate a high level of abrasion. It is also noticeable that very few, if any, of the intertidal grazing animals e.g., limpets, will tolerate this kind of sand abrasion and so the grazing pressure will be greatly reduced in areas of high sand mobility. These areas of *P. laciniata* were often associated with freshwater runoff from the upper area of the beach. Reduced

salinity e.g., in estuaries, was often used in primitive cultivation techniques in Japan (Miura, 1975) so in this situation the freshwater may have helped to induce spore release from the conchocelis.

The mapping work on *P. laciniata* showed clearly how patches of this species come and go on an area of intertidal shore. The maps also demonstrated how dramatically the substratum can change in the 'transition zone' from a sandy to rocky shore area. The growth of *Porphyra* here is primarily on cobbles and although the cobbles are often covered with sand the *Porphyra* has the ability, as mentioned previously, to withstand this kind of sand abrasion. Some damage and loss of plant material did occur but not to any great degree. It is possible that the appearance of these large patches in spring (April to May) of *P. laciniata* is as a result of sand being removed from the cobbles in the earlier months thus exposing areas for settlement. However this movement of sand will probably be of great importance during the periods of conchospore settlement as it leads to larger areas of cobbles being exposed (February and March). Once settled the developed *P. laciniata* thalli would be less affected by the influx of sand unless it is for a long periods or of great depth of cover over the *Porphyra*. Therefore, when considering the population of this species in this area a particular year may have an increased biomass as a result of more suitable conditions i.e. more cobbles on which spores could settle during late February and early March prior to the major growth season (April to May).

The maps demonstrate a second peak in cover of *P. laciniata* and to some degree this method of mapping was used to bypass some of the problems of setting up a program to sample such a dynamic shore area containing a patchy species. If drawn accurately these types of maps can be used to calculate a quantitative degree of cover within the area (Fig. 2.9).

The timing of a peak in the *P. laciniata* population during the spring was also observed by the quadrat sampling method although large variations were noted

as a result of the patchy nature of the species and of the great mobility of the sand in this area. Plants may appear or disappear between one sample and the next, not just as a result of growth or death but because the plants may be invisible to the sampling technique as a result of sand cover.

A relationship of the cover of *Porphyra* and the presence of cobbles was noted which indicates that the *Porphyra* relies on the cobbles in the area as a substratum on which to grow.

A similar seasonal spring peak in the number of individuals, wet weight and dry weight biomass was demonstrated using the quadrat sampling method. The increase of the population at this time of year indicates a production of conchospores prior to the increase and also suggests that optimum conditions (presence of cobbles) exist at this time of year for the germination and further growth of the thalli of *P. laciniata*.

The plant length data illustrates the changes in growth of individuals within the seasons. Rapid growth of the plants occurred at approximately the same time each year, this being a period when the growth of the individuals (in terms of length) surpasses the loss of material from the plants (due to wave or insolation damage or due simply to the age of an individual). Fewer individuals were recorded in March 1991 than in the previous year but within the sample there was still a high proportion of smaller individuals. The reduction in number in March 1991 may have been a result of the quadrat technique missing patches of *P. laciniata* resulting in a lower than expected population size or alternatively it could be due to slight differences in seasonal conditions. The former reason is the most likely since the maps of cover show the area of cobbles increased dramatically after January, so creating high potential settlement areas for the *Porphyra*. A marked increase in the patches of *Porphyra* was observed in March 1991 when compared to the previous few months. The area covered by *Porphyra* was actually greater in 1991 than 1990 (Fig. 2.9).

The biomass of the population of *P. laciniata* showed a dramatic increase during April and May for both years, illustrated well by both the wet weight and dry weight data. The increase in biomass corresponds to levels of nutrients of approximatley half the winter peak these levels may still be high enough in the seawater at this time to aid increased growth (Thomas & Harrison, 1985). As the light levels increase during the spring the plants will then be able to grow rapidly. The biomass of *Porphyra capensis* was found to be low in winter and higher in the early summer and this was said to correspond (with a lag phase for the conversion of photosynthetic products) to a high spring photosynthetic rate (Levitt & Bolton, 1991). The peak fresh weight biomass for this species (140 to 160 g m^{-2}) is noticeably lower than that calculated for low shore *P. umbilicalis* (3280 g m^{-2}) by Munda & Markham (1982). The low biomass of *P. laciniata* is a result of the sampling technique of this study. In the study by Munda & Markham (1982) quadrat samples were taken of the prominent populations but samples in the present study were from a set area within which a prominent population occurred and so consequently the biomass values per unit area were low. If quadrats had been placed within prominent populations as in the study of Munda & Markham (1982) then biomass values would have been higher and directly comparable. Peak biomass values are calculated for quadrats that had 100 % cover ranged from 1.5 - 1.8 kg m^{-2}, still somewhat lower than the values calculated by Munda & Markham (1982) of 1.8 - 3.3 kg m^{-2} for *P. umbilicalis.*

Protein measurements are a good indication of the ability to produce high quality food products (Johnston, 1972; Fujiwara *et al.*, 1984) from *Porphyra* species and are important when considering the value and quality of a species for commercial cultivation (Miura, 1975). Plants with the highest protein contents are those which produce the highest quality nori products (Noda, 1971). The protein level is an important factor when considering the value of a species in terms of its potential for a cultivated crop (Fujiwara *et al.*, 1984).

Protein measurements of the four indigenous species tested show that there are significant differences between the species. *Porphyra linearis* had the highest protein content of all the species tested with a peak of 42 % in January (1991) and an overall mean of 35 %. This result is similar to that of Munda & Markham (1982) who calculated a peak of 40 % in January for *P. linearis* in Helgoland. The high value of protein in Manx *P. linearis* would indicate that this species would yield a higher quality product than the other species present around the island. More detailed work on flavour substances by McLachlan *et al.* (1972) indicated that *P. linearis* had the best flavour of Nova Scotia species.

P. umbilicalis has high spring protein values (~ 29 %) and lower values of 20 - 22 % in June as compared to *P. umbilicalis* studied by Munda & Markham (1982) which had high values of 28-30 % in spring and declining values in June to a lowest value of 19 % in September. Differences in protein levels between the high and low shore populations of this species were not obvious in this study whereas Munda & Markham (1982) found differences in protein content between the low shore populations with higher protein levels in general than the high shore.

P. laciniata had the second highest protein level with a peak value of 36 % in the spring (April, 1991) and a low value (20.5 %) in July 1990. When dried and tasted by a colleague, an experienced (Korean) nori eater, this species was said to have a good flavour although the texture was too thick. This gives some indication as to the potential of this species for human consumption. Protein levels appear to be related to the observed health of plants. At the time of peak biomass (April and May) plants appeared healthiest and had a corresponding high protein level. Later on in the season, during mid-summer, plants appeared less healthy and many were pale and damaged, possibly as a result of high insolation and sporing; this corresponded to the lowest recorded protein levels.

The highest protein level shown by *P. linearis* occurs during its peak biomass in the winter (January). At this time of year the effect of high insolation and desiccation damage is much reduced. Nutrient levels in the sea are much

higher than in the summer (Fig. 2.1b) therefore are unlikely to be a limiting factor to the growth and consequent protein value of this species. Kudoh (1987) noted that the nitrogen content of *Porphyra* was higher in cultivated species grown in areas with higher seawater nitrate levels. Zavodnik (1987) also found that in general the fluctuations of protein levels in *P. leucosticta* followed the fluctuations of nutrients in the sea. These factors may contribute to the high values of the *P. linearis* plants at this time of year although it should be noted that other species present at this time of the year do not show the same high levels probably as a result of the low growth of other species (*P. laciniata* and *P. umbilicalis*) when compared to the peak in growth of *P. linearis* during the winter period.

It would appear from the results of the protein measurements that *P. linearis* would yield the highest quality product and would consequently be the most viable species for cultivation. It also has the added advantage that it is a winter species and so the present nori cultivation techniques would be suited to this species. Further advantages of its growth in winter such as reduced epiphyte growth and fouling and increased nutrient levels in the seawater would also add to the greater suitability of this Manx *Porphyra* species for cultivation.

CHAPTER 3

SOME ASPECTS OF THE TAXONOMY OF *PORPHYRA* SPECIES USING THE TECHNIQUE OF STARCH GEL ELECTROPHORESIS

INTRODUCTION

Species of *Porphyra* have traditionally been identified and distinguished using morphological features. These features include: number of cell layers of the thallus; number of chloroplasts per cell; arrangement of reproductive structures on the thallus; thallus shape, size, colour, thickness and microscopic spore arrangements (Conway *et al.*, 1976; Garbary *et al.*, 1981). Other techniques have also assisted with the classification of species. Chromosome counts have provided some taxonomic evidence (Mumford & Cole, 1977; Krishnamurthy, 1984; Tseng & Sun, 1989), their number and morphology helping to distinguish species. The season of occurrence, and type of habitat, have also provided additional information for the distinction of species (Kornmann & Sahling, 1991).

The more traditional methods for species distinction mentioned above have been most commonly used. Studies of the taxonomy of algal species using genetic techniques have been made less frequently (Holton, 1973; Cheney & Babbel, 1978; Price *et al.*, 1987; Miura, 1988). The evidence for genetic differentiation between species has rarely been obtained by direct methods (Chapman, 1974; Rueness & Rueness, 1975) and often genetic differentiation has been inferred from morphological characteristics, with little understanding of whether the basis for variation is environmental or genetic. It has been suggested (Dixon, 1963, 1966, 1970) that the failure to recognise morphological plasticity within species has been a confounding factor of many taxonomic problems.

As morphological features on which species are often distinguished appear to vary intra- as well as inter-specifically, the technique of starch gel electrophoresis was tested in the present study to help with the differentiation of local species of *Porphyra*. Generally horizontal starch gel electrophoresis provides data unbiased by environmentally induced morphological variation.

Cases of environmentally induced variation do occur but are rare. Consequently it was hoped that this technique would provide additional useful information for more reliable species differentiation.

Recent work on *Porphyra* (Mumford, 1973; Miura, 1984, 1985, 1988; Kornmann & Sahling, 1991) and work involving the use of electrophoretic techniques (Holton, 1973; Cheney & Babbel, 1978; Fujio *et al.*, 1985; Miura, 1985; Price *et al.*, 1987; Lindstrom & South, 1989; Lindstrom & Cole, 1990) have lead to the solution of some taxonomic problems presented by algal species.

Starch gel electrophoresis is a technique widely used in studies of the systematics and population biology of higher plants (Gottlieb, 1981; Crawford, 1983). The advantage of this technique is that the results, in the form of protein banding patterns, quantify the direct gene products themselves rather than the secondary morphological phenotypic characteristics. The method consequently provides a measure of genetic differentiation independent of morphological differentiation. This technique is also known to be particularly useful at and below the congeneric species level and is consequently ideal for the present study. Thorpe (1979) noted that a large number of studies on a wide range of species had shown generally close agreement between conventional taxonomic schemes based on morphological characteristics and those based on interspecific enzyme variation. He also warned of the dangers of accepting precise figures from electrophoretic studies and the consequence of using these figures alone to sweep aside existing taxonomic ideas. The use of electrophoretic techniques for species separation should thus be combined with other taxonomic techniques such as morphological characteristics.

In comparison to the number of studies on animals and land plants, electrophoresis has still only been used relatively little on algae. Recent electrophoretic work (cited above), however, demonstrates how these techniques can be applied to algal taxonomy. Miura (1978), Fujio *et al.* (1985), Lindstrom & Cole (1990) and Miura *et al.* (1979) studied the genetic variation and

differentiation in various populations of *Porphyra*, Lindstrom & South (1989) studied species of *Palmaria* and Cheney & Babbel (1978) surveyed enzymes (14 Loci) in *Eucheuma*. Work by Fujio *et al.* (1987) demonstrates the applied use of electrophoresis as a tool for genetic differentiation involving comparisons of wild and cultivated populations. Fujio *et al.* (1985) demonstrated high levels of genetic differentiation and genetic variability in natural populations of the haploid laver *Porphyra yezoensis*.

Many of the studies involving marine algae are on cultured or cultivated species with only relatively few studies on wild populations (e.g. Malinowski, 1974; Cheney & Babbel, 1978; Yamazaki, 1984; Fujio *et al.*, 1985; Fujio *et al.*, 1987; Lindstrom & South, 1989; Lindstrom & Cole, 1990).

In this study traditional taxonomic methods were used along with electrophoretic techniques. Electrophoresis was used:

i) to demonstrate the level of genetic differentiation between five *Porphyra* species occurring on the Isle of Man;

ii) to test the validity of intraspecific morphotypes.

METHODS

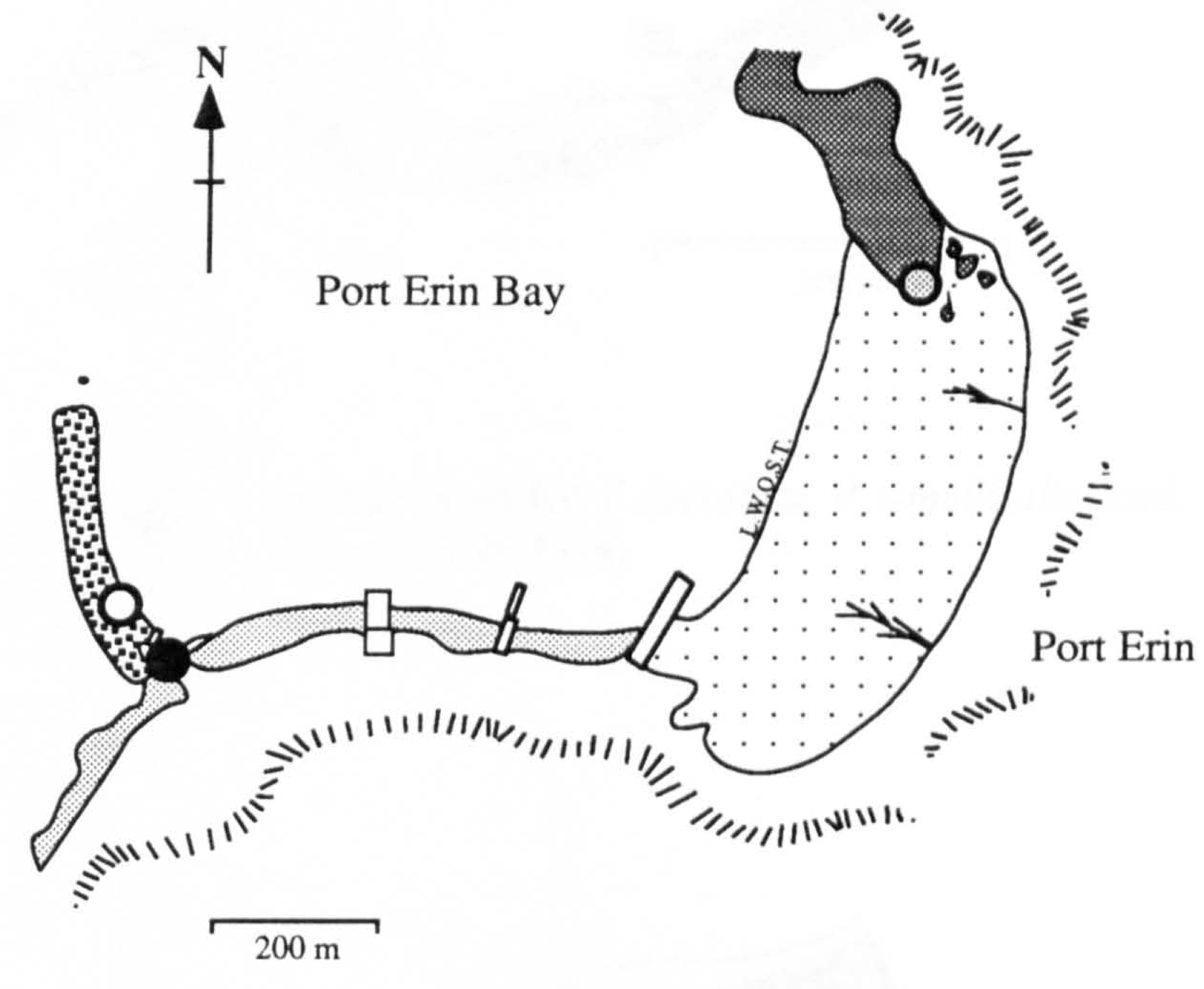
Sample selection

Four species of *Porphyra*: *P. linearis*, *P. laciniata*, *P. umbilicalis* and *P. leucosticta*, distinguished by the characteristics presented by Conway (1964a, b, c), were selected from sites around the Isle of Man. Where feasible plants of different species were selected from sites as close together as possible, so that the populations of each species could be considered to be sympatric.

P. linearis and *P. umbilicalis* were collected in February 1990 from a site on the exposed western side of Port Erin breakwater (54°05'12"; 4°46'25", Fig. 3.1). *P. leucosticta* was obtained from the same site in June 1990. *P. laciniata* plants were collected in March 1990 from the exposed north end of Port Erin beach (54°05'20" ; 4°45'30", Fig. 3.1); *P. laciniata*, *P. umbilicalis* and *P. linearis* from the main beach at Peel (54°13'26"; 4°41'18", Fig. 3.2) and from the north east end of Douglas Bay (54°09'57"; 4°27'12", Fig. 3.3); *P. laciniata* and *P. umbilicalis* from the middle of Port Soderick Bay adjacent to the sewer outfall (54°07'30"; 4°31'36", Fig. 3.4) and *P. umbilicalis* and *P. linearis* from Port St Mary Ledges (54°03'48"; 4°45'00", Fig. 3.5). A small sample of *P. laciniata* was also analysed from Ramsey (54°19'50" ; 4°22'58", Fig. 3.6).

Sample storage

Samples were sorted and cleaned in fresh sea water and dried using paper towels. In most cases samples of the thalli were electrophoresed while they were still fresh. When this was not possible they were wrapped in paper towels, sealed in plastic bags and stored frozen at -18°C for future electrophoretic examination. Initially a number of preservation methods for the storage of the thallus material were tested: freezing at -18°C, freeze drying and keeping material live in sea



● Site where *P. linearis* occurs during the winter

○ Site of collections of *P. leucosticta* on the old collapsed breakwater

◉ Site of collections of *P. laciniata*

Fig. 3.1 Map of Port Erin bay showing sample sites

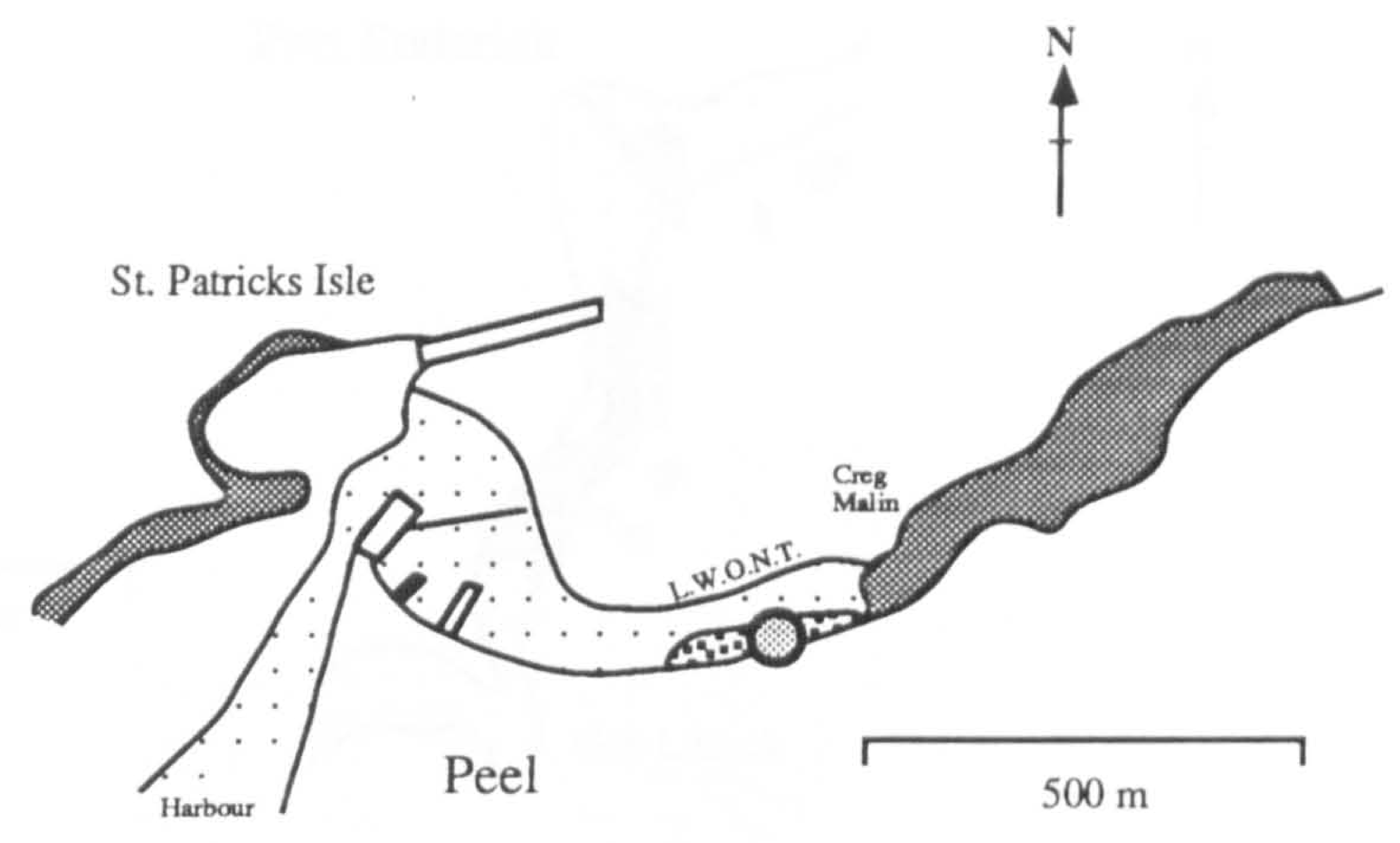


Fig. 3.2 Sample area for *P. laciniata, P. umbilicalis* and *P. linearis* in Peel.

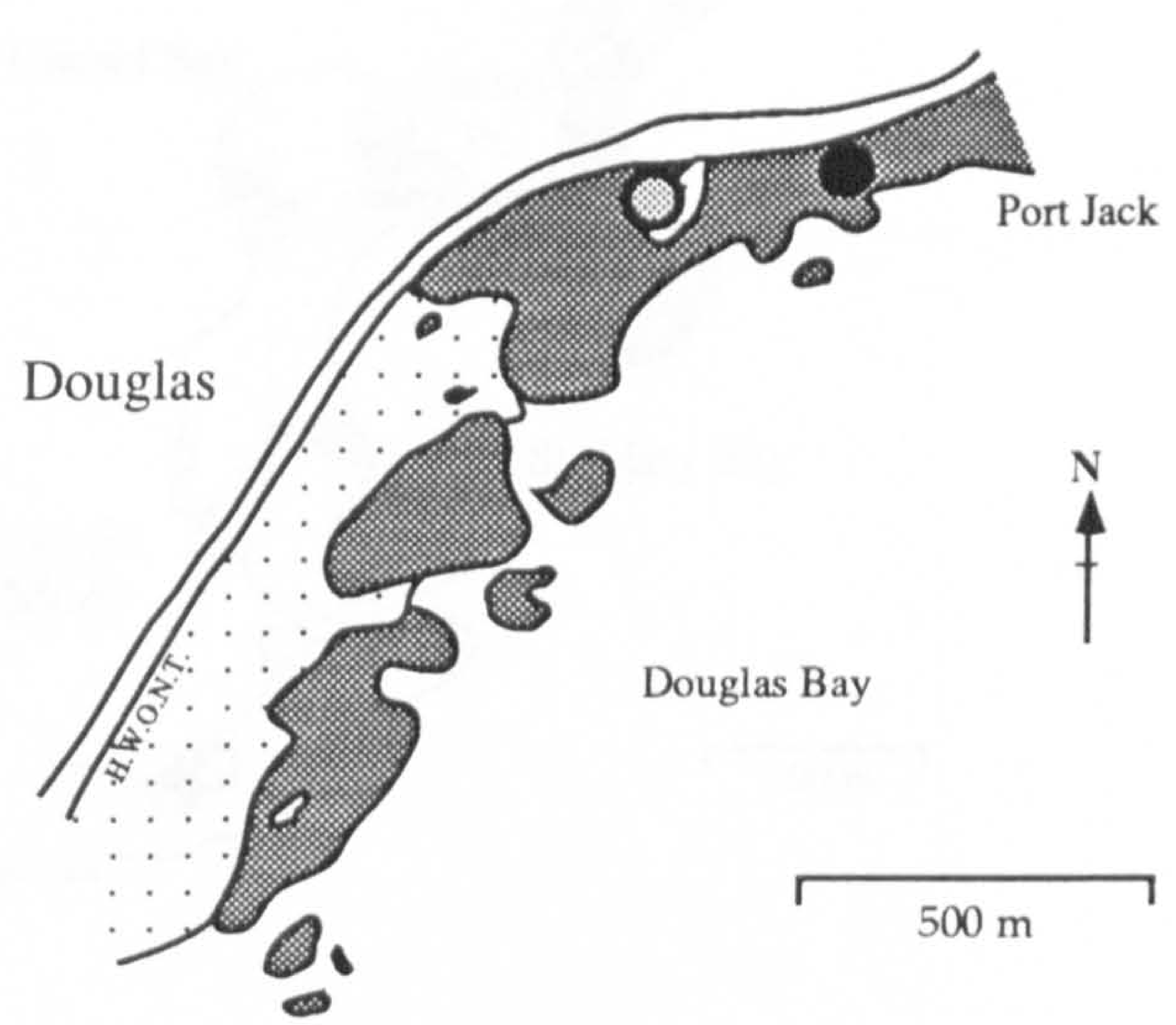


Fig. 3.3 Sample site of *P. laciniata* in Douglas Bay

Sample site of *P. linearis*

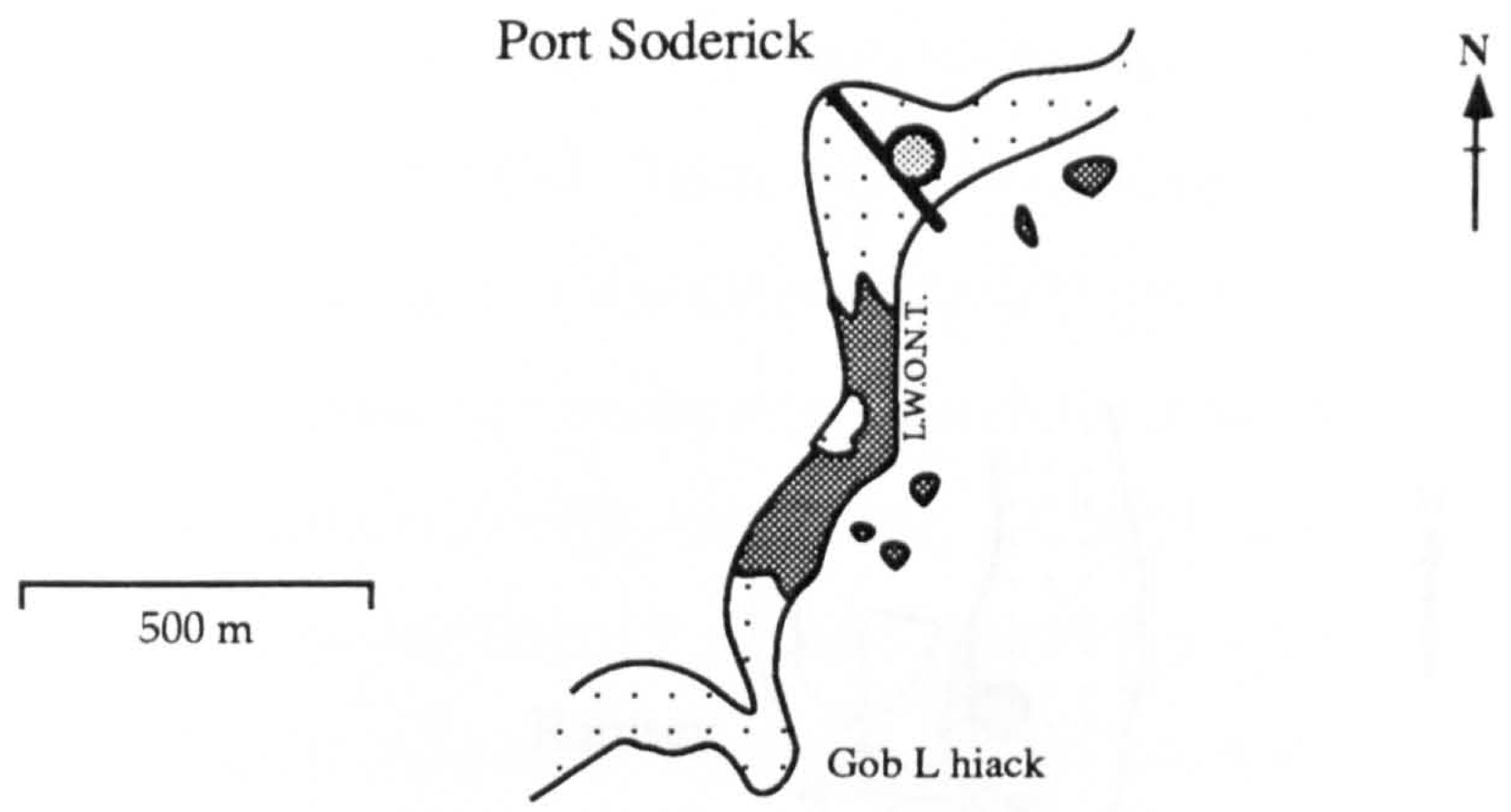


Fig. 3.4 Sample site of *P. laciniata* and *P. umbilicalis* in Port Soderick.

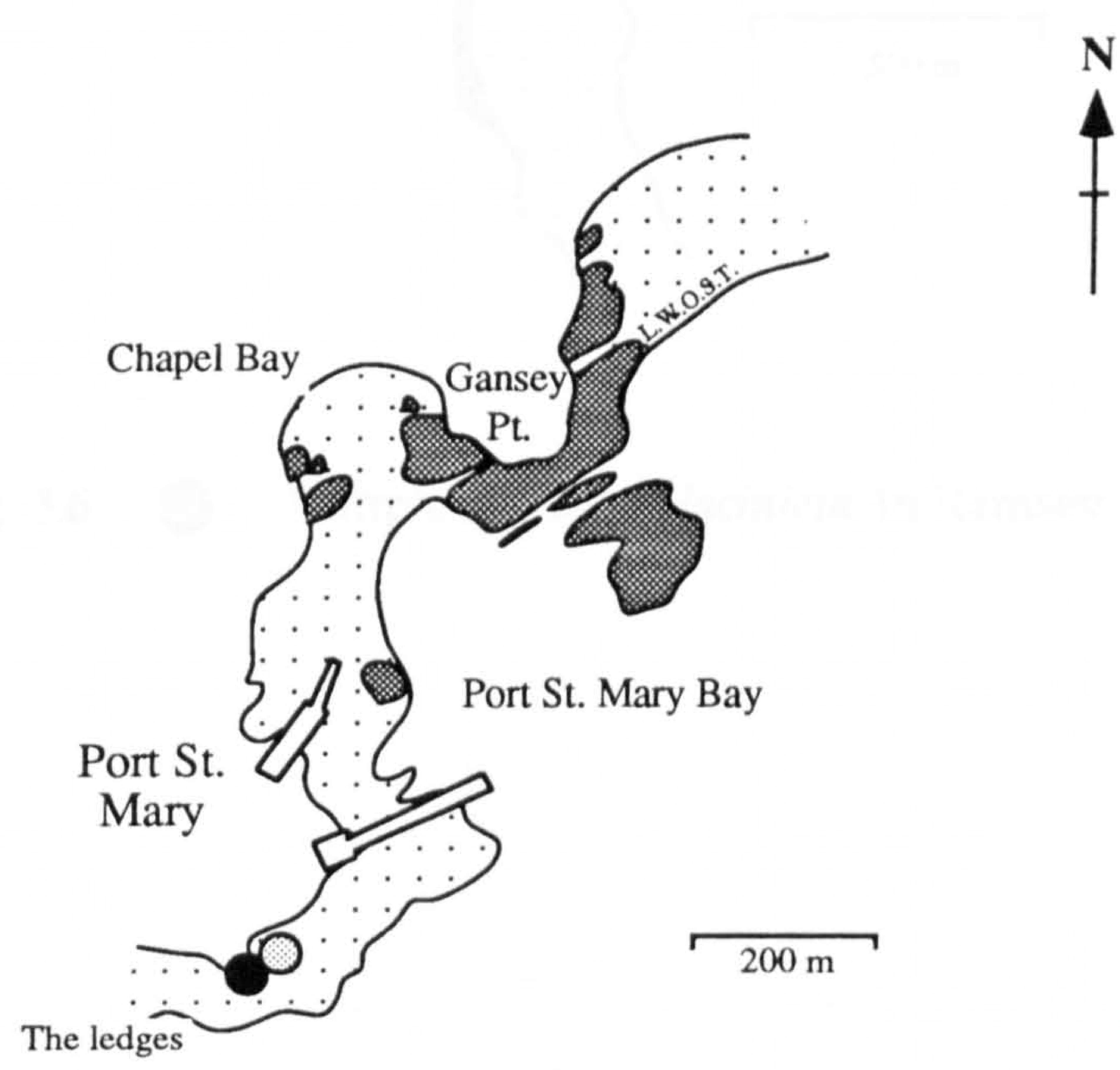


Fig. 3.5 Sample site of *P. linearis* in Port St. Mary

Sample site of *P. umbilicalis*

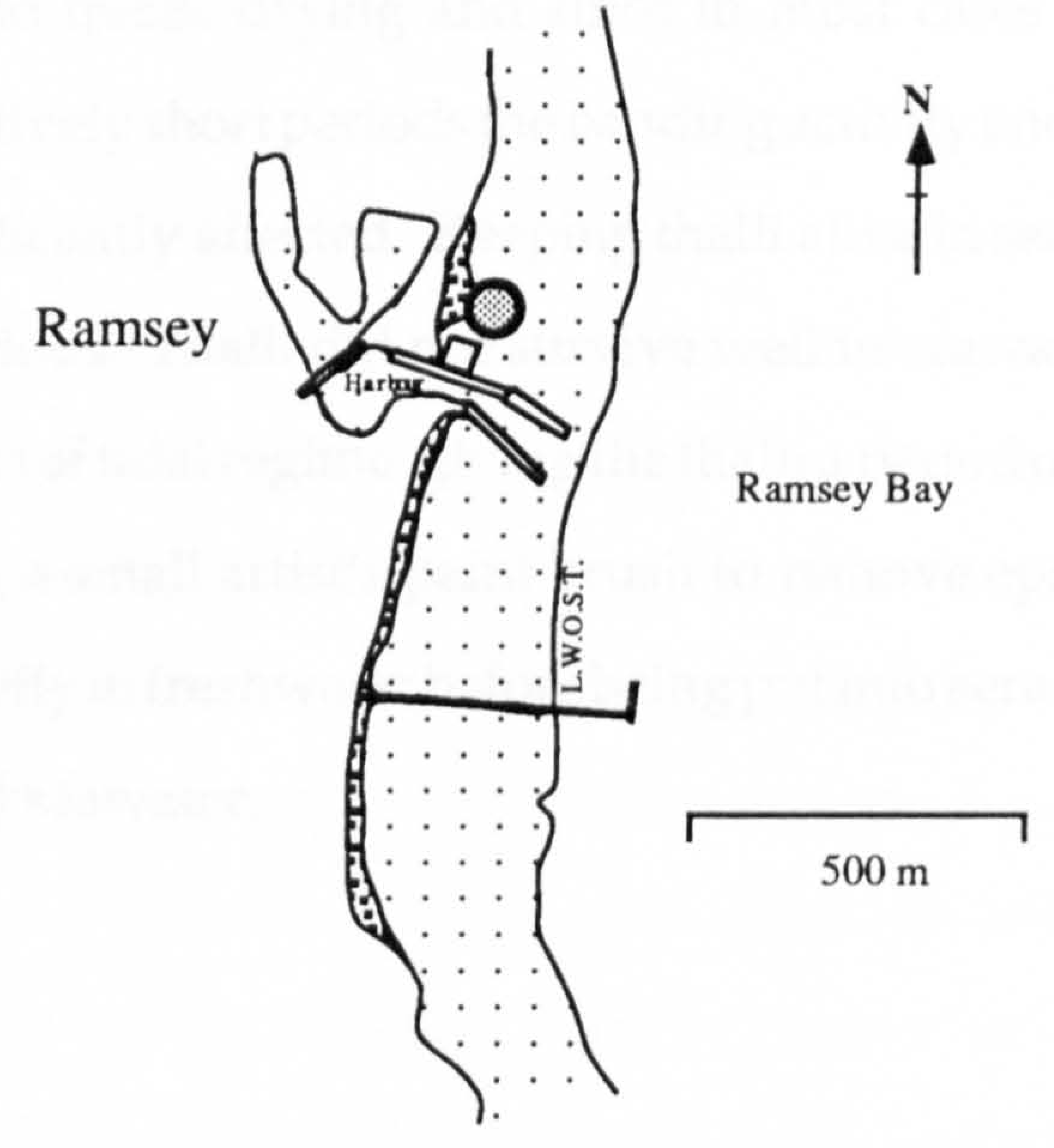


Fig. 3.6 Sample site for *P. laciniata* in Ramsey

water. For freezing the samples were stored as described above. For freeze drying the *Porphyra* thalli were washed in fresh water and then a 4 cm^2 segment was selected and freeze dried. The method of freezing at -18 °C was the technique generally used in this study if thallus material had to be stored as the method was simple when compared to freeze drying and since in most cases thalli were electrophoresed after relatively short periods the banding activity and resolution did not appear to be significantly affected. Keeping thalli alive in sea water was used mainly for short periods. Thalli did not survive well in seawater for long periods without some form of tidal regime, giving the thalli a period of emersion. Thalli were cleaned using a small artist's paint brush to remove epiphytes etc. and were then washed briefly in freshwater before being put into aerated buckets of freshly filtered (0.2 µm) seawater.

Electrophoresis

Electrophoretic techniques were modified from those of Miura *et al.* (1978a, b), Fujio *et al.* (1985), Lindstrom & South (1989), Lindstrom & Cole (1990) and Lindstrom (pers. comm). For general basic electrophoresis techniques see Leary & Booke (1990).

Gel preparation

The electrophoretic support medium was starch gel. Starch gels were prepared from 10 % w/v starch (Sigma S4501), 2.5 % Electrostarch and 3 % sucrose. Tris-EDTA-borate (TEB: 0.18 M Tris-HCl, 0.004 M Na_2EDTA, 0.1 M boric acid, pH 8.6) was used for the electrode buffer, and quarter strength TEB was used in gel preparation in a 1,000 ml Pyrex side arm flask. This mixture was stirred vigorously over a bunsen flame until the material had changed from a viscous opaque state to a transparent fluid and had boiled. This molten gel was

degassed using the side arm of the flask connected to a tap aspirator and then poured into a perspex former. Gel moulds were 180 mm long x 150 mm wide x 10 mm deep.

Gels were prepared the evening before use and were stored for at least 1 hour in the refrigerator before sample loading.

Sample preparation and buffers

Samples were taken from the freezer and separated into individuals. In some cases freezer damage had occurred. The evidence for this was a different colouration of the thallus (usually a dark to clear purple colouration), so care was taken to select for areas of the thalli in the best condition (selected as the areas with natural colouration and appearance of cells), similar areas (i.e. middle) of the thalli were used for a particular run. Samples were stored for upto 12 months but generally used within 3 months. Similar sized vegetative sections of the plants were used (approx. 1 cm^2), the size being dictated by the size of the perspex wells in which the plant material was crushed. The selected thalli sections were crushed, using a modified screwdriver, in a fixed amount (100μl) of extraction buffer, (50 mM MOPS, 5 % w/v PVP40, 40 mM disodium EDTA, 5 mM 2-mercaptoethanol, 20 mM sodium metabisulphite and 200 mM sodium ascorbate, adjusted to pH 7.5). The amount of crushing each thallus section received was standardised (approximately 20 repetitions of the crushing process). The crushing process was carried out on a cooling block, in a fume cupboard. If samples were not used immediately they were stored in a refrigerator until required.

Filter paper wicks (3 x 12 mm) were used to extract the supernatant from the crushed material in the wells. One wick was used per well unless replicate gels were used in which case two wicks were placed in each well.

A 2.5 cm wide strip was cut from one side along the long axis of the gel. The wicks were placed 1 mm apart along the end of the main gel section starting

2.5 cm in from the left side of the gel. The wicks were usually placed in groups of 5 or 10 approximately 1 mm apart with a larger gap (2 mm) between the groups, thus simplifying the scoring of the gels when developed. When all the wicks were in place the end gel segment was replaced, followed by the mould with extra perspex wedges placed at the end to compress the gel against the wicks. This ensured that no gaps were present around the wicks which might have affected the movement of the proteins.

Loaded gels were covered with a polythene sheet and then a block of ice in a plastic tray to prevent overheating of the gel during the running process. The gels were placed in a Shandon Model 600 electrophoresis chamber and the whole unit was put in a refrigerator at 4°C and run horizontally at approximately 200 V and 30 mA for 6 - 8 h. Voltages were maximised to reduce the running time (migration being directly proportional to volt hours) whilst attempting to keep the power below 7 Watts in order to reduce the generation of heat within the gel. Heat affects the enzyme activity and may distort the isozyme patterns. The gels were checked after 2 - 3 hours and if the ice in the plastic tray had melted appreciably it was replaced. Permanently having water in the trays of ice ensured that the temperature was also not too low.

Each gel was cut into 8 - 10, 1 mm thick slices. The slices were stained for enzymes using the following recipes :

Glucose-6-phosphate (G6PDH) : 50 ml 0.1 mol Tris-HCl pH 7.5, 0.5 ml 1 M $MgCl_2$, 40 or 80 mg glucose-6-phosphate, 7.5 mg NADP, 10 mg 3-[4,5-Dimethyliazol-2-yl]-2,5-diphenyltetrazolium bromide = MTT, 2 mg Phenazine methosulphate = PMS;

Shikimate dehydrogenase (SkDH) : 100 ml 0.1 M Tris-HCl, pH 7.1, 100 mg shikimic acid, 15 mg NADP, 20 mg MTT, 4 mg PMS.

Phosphogluconate dehydrogenase (PGD) : 50 ml 0.1 M Tris-HCl pH 8.0, 0.5 ml $MgCl_2$, 20 mg 6-phosphogluconic acid, 7.5 mg NADP, 10 mg MTT, 4 mg PMS;

Glutamate oxaloacetate transaminase (GOT, also known as AAT) : 100 ml 0.1 M Tris-HCl pH 8.5, 100 mg alpha-ketoglutarate, 200 mg aspartic acid, 10 mg pyridoxal-5-phosphate, 75 mg fast BB salt;

Mannose phosphate isomerase (MPI) : 50 ml Tris-HCl pH 8.0, 20 mg mannose-6-phosphate, 5 mg NADP, 25 µl G6PDH, 50 µl PGI, 5 mg MTT, 10 mg $MgCl_2$, 1 mg PMS;

Malate dehydrogenase (MDH) : 50 ml 0.2 M Tris-HCl pH 8.0, 50 ml 0.2 M DL-malic acid adjusted to pH 7.4-7.7 with 1 N NaOH, 30 mg NAD, 20 mg MTT, 4 mg PMS (Check pH);

Diaphorase (DIA) : 30 ml Tris-HCl, 10 mg NADH, 1 mg 2,6 dichlorophenol-indophenol (DCPIP), 7 mg MTT;

Leucine amino peptidase (LAP) : 100 ml Tris-malate pH 6.0, 50 mg L-leucyl-B-napthylamide in 1 ml acetone, 50 mg Fast Black K salt;

Glucosephosphate isomerase (PGI) : 50 ml 0.1 M Tris-HCl pH 7.5, 0.5 ml $MgCl_2$, 20 mg fructose-6-phosphate, ~20 units G6PDH, 7.5 mg NADP, 10 mg MTT, 4 mg PMS;

Aldolase (ALD) : 50 ml 0.1 M Tris-HCl pH 8.0, 125 mg fructose-1,6-diphosphate, 75 mg Na_2HASO_4, 15 mg NAD, 15 mg MTT, 2 mg PMS, ~200 units glyceraldehyde-3-phosphate dehydrogenase;

Phosphoglucomutase (PGM) : 50 ml 0.1 M Tris-HCl pH 7.5, 0.5 ml $MgCl_2$, 20 mg glucose-1-phosphate, 5 mg NADP, 10 mg MTT, 2 mg PMS, ~40 units G6PDH;

Bromoperoxidase (BrPer) 50 ml 0.01 M KPO_4 pH 6.0, 0.25 g pyragallol, 45 mg KBr, 0.5 ml H_2O_2;

Glucose dehydrogenase (GDH); 50 ml 0.05 M phosphate buffer, pH 7.5, 9 g D-glucose (1.0 M final conc. in reaction mixture), 20 mg NAD, 5 mg MTT, 5 mg PMS;

Catalase (CAT) : 10 ml 0.1 M phosphate pH 7.0, 5 ml 3 % H_2O_2, 7 ml 0.06 M $Na_2S_2O_3$, 78 ml distilled water, followed by 100 ml 0.045 M KI;

Adenosine deaminase (ADA); 50 ml phosphate buffer pH 7.5, 15 mg Adenosine (1.1 mM final conc. in reaction mixture), 25 μl (25 units) Nucleoside phosphorylase, 25 μl (4 units/ml) Xanthine oxidase, 5 mg MTT, 5 mg PMS;

Adenylate kinase (AK), 50 ml Tris-HCl pH 8.0, 10 mg ADP, 1.0 ml $MgCl_2$, 40 mg glucose, 2 ml H_2O, 5 mg NADP, 25 μl (280 units) Hexokinase, 25 μl (140 units) Glucose-6-phosphate dehydrogenase, 2.5 mg MTT, 2.5 mg PMS;

Lactate dehydrogenase (LDH) : 50 ml 0.1 M Tris HCl, pH 7.5, 0.5 ml 1 M $MgCl_2$, 5 ml lactate stock solution (49 ml 1.0 M Na_2CO_3, 10.6 M 85% DL-lactic acid), 7.5 mg NADP, 10 mg MTT, 2 mg PMS

Malic enzyme (ME) : L-malic acid (15 mM-final concentration in the reaction mixture) - 100 mg dissolved in 20 ml 0.1 M Tris-HCl , pH 7.0. Then adjusted to pH 7.0 with Na OH, 0.2 M $MgCl_2$, 5 mg NADP, 5 mg MTT, 0.5 mg PMS;

D-Aspartate oxidase (DASOX); D-Aspartic acid : 200 mg dissolved in 50 ml 0.5 M Tris-HCl, pH 8.0, pH then adjusted to 8.0 (30 mM final concentration in the reaction mixture), 8 mg FAD, 5 mg Peroxidase (100 units/mg), 1 ml (25 mg/ml) 3-Amino-9 ethyl carbazole

Aconitase (ACON); Cis-aconitic acid 75 mg dissolved in 20 ml 0.4 M Tris-HCl pH 8.0, adjusted to pH 8.0 (8.3 mM final conc. in reaction mixture), 5 ml $MgCl_2$, 5 mg NADP, 0.1 ml (20 units) Isocitrate dehydrogenase, 5 mg MTT, 2 mg PMS;

Superoxide dismutase (SOD) shows up as white bands on gels stained with 7 mg MTT, 1 mg PMS and 50 ml Tris-HCl and on many of the gels stained at pH 7.5 or 8.0.

The stain ingredients were mixed (dry ingredients in advance and wet ingredients just prior to use, to minimise the deterioration of stains, many of which are light labile) in 100 ml Pyrex beakers and were then poured over the gel slices in perspex trays. Gels were stained at room temperature and were covered with a piece of card to cut out the light. They were viewed at regular, 5 minute intervals until no further staining was apparent and 'scored', noting the presence and number of stain bands, their distance moved and density of staining (activity and resolution) once development had taken place. All gels were left for 12-14 hours after staining and the scoring was then checked.

Data Analysis

Genotypes and allele frequencies were examined using the FORTRAN computer program BIOSYS-1 (Release 1.7) (Swofford & Selander, 1981). The values of genetic identity (*I*) (probability of allelic identity or genetic similarity)

and genetic distance (*D*) (Nei, 1972) were calculated. The scale of *I* ranges from 1 (no difference) to 0 (no alleles in common) whilst *D* ranges from 0 (no difference) to infinity (no alleles in common). Once calculated these values were utilised to produce dendrograms using the unweighted pair-group arithmetic mean (UPGMA) cluster analysis algorithm (Sneath & Sokal, 1973) for diagrammatic presentation of the data.

Chromosome Staining

Fresh thallus material was used for chromosome staining. Thalli with spermatia present (usually as a pale margin around the thallus) were collected from the shore. Once back in the laboratory the thalli were cleaned using an artist's paint brush, to remove any epiphytes. Thalli with particularly well developed spermatia were then selected (preferably with the spermatia beginning to be released) and a small piece of spermatia material removed. This material was then placed onto a microscope slide and excess seawater was wicked off with a paper towel. One drop of aceto-orceain stain was then added taking care to push away any air bubbles. The stain was agitated to move it across the cells. The slide was then heated very carefully for 3-5 seconds by waving over a gentle bunsen flame, taking care not to boil the stain, and a coverslip added. The cells were then squashed using mild pressure on the coverslip with the thumb. The stained cells were then examined using initially, 400X magnification followed by 1,000X magnification (oil immersion) once areas of stained chromosomes had been identified. Chromosome numbers were recorded for each of the species. This involved focusing up and down around the image of the chromosome many times to ensure that whole chromosomes were counted. Once chromosome staining had been achieved areas showing well orientated chromosomes were photographed using colour slide film for permanent records.

Morphology

P. umbilicalis has been recognised as having three major morphotypes within the species (Conway, 1964a, b; Edelstein & McLachlan, 1966). Using the techniques described above examples of plants of each of the three described morphotypes were electrophoresed to ascertain whether they were in fact all members of the same species. The possible existence of significant differences in allele distributions between morphotypes of *P. umbilicalis* was investigated using the two tailed Fisher Exact test (Fisher, 1958).

The Fisher Exact test was carried out using the SAS/STAT (Release 6.03) statistical package (SAS Institute Inc, 1988) on the University of Liverpool IBM 3083 mainframe computer. This test calculates exact significant probabilities and avoids the difficulties encountered using the chi-squared distribution for small samples (Haldane, 1954).

In the case of the unknown species *P.* sp. mixed within samples of *P. laciniata* the morphology was recorded and any unusual or unique features compared to the other species studied were noted. The unknown species appeared to be somewhat thinner than *P. laciniata,* the species with which it was most easily confused, and so measurements of the thickness of 30 plants (previously electrophoresed to ensure validity) were carried out using a micrometer (0.01 - 10 mm). Plants were cleaned using an artists paint brush to remove epiphytes and then placed in fresh filtered (0.2 μm) seawater for half an hour prior to measurement.

RESULTS

Storage

Since freezing was the simplest storage method and proved to be suitable for this particular study it was adopted as the main storage method. Ideally storage of the material by freezing at -80 °C would be an improved freezing method than storing at -18 °C however, when this facility is not available, this study has shown that results can be obtained with samples frozen at -18 °C for upto 6 - 12 months. The use of fresh material gave the best results and so should be used where possible. Frozen specimens proved useful as standards when analysing a new species present at a different time of year.

Enzyme activity

Of the 25 enzymes for which gels were stained, 15 resolved producing 12 which resolved frequently enough to be of use for data analysis (Table 3.1). Allele frequencies at the 12 successfully resolving loci are presented in Table 3.2.

The 12 enzymes most commonly used stained and resolved as follows:

Superoxide dismutase (SOD)

SOD resolved well in most cases with both Tris-borate and Tris-citrate buffers. In many cases activity and resolution was best when staining a gel for a different enzyme. Good results were still obtained when using the SOD recipe itself. SOD gave good distinguishing results for *P. laciniata* which was easily distinguished from all other species (Table 3.2). All the five species tested showed a single band or allele, two alleles were resolved in total.

Enzyme	Activity	Resolution	Species
SOD	+++	++	1,2,3,4,5
ALD	+++	+++	1,2,3,4,5
PGI	++++	++++	1,2,3,4,5
BRPER	++	++	1,2,3,4,5
LDH	+++	+++	1,2,3,4,5
MDH	+++	++	1,2,3,4,5
PGM	+++	+++	1,2,3,4,5
G6PDH	++++	++++	1,2,3,4,5
PGD	++++	+++	1,2,3,4,5
MPI	+++	+++	1,2,3,4,5
DIA	++++	+++	1,2,3,4,5
SKDH	++	+++	3,4
CAT	++	+	1,4
GOT	++	+	1,2,3,4
ME	+	o	1,2
DASOX	o	o	1,2
LAP	o	o	1,2
ADA	+	o	2
GDH	o	o	1,2,3,4,
AK	+	++	1
ACON	+	+	1
XOD	+	++	1,2,4
HBDH	o	o	1,2,
FUM	+	o	1

Key:

o	=	No activity/resolution	1 = *Porphyra linearis*
+	=	Poor	2 = *P. umbilicalis*
++	=	Reasonble	3 = *P. leucosticta*
+++	=	Good	4 = *P. laciniata*
++++	=	Excellent	5 = *P.* sp.

Table 3.1 The level of activity and resolution of all enzymes tested in *Porphyra* species.

Locus	Allele	*P. umb.*	*P. lacin.*	*P. lin.*	*P. leuc.*	*P.* sp.
Sod	A	1.000	0.000	1.000	1.000	1.000
	B	0.000	1.000	0.000	0.000	0.000
	n	61	115	31	31	50
Ald	A	1.000	1.000	1.000	1.000	1.000
	n	31	36	36	31	5
Pgi	A	0.000	0.000	0.000	0.000	1.000
	B	0.985	0.685	1.000	0.000	0.000
	C	0.015	0.067	0.000	0.043	0.000
	D	0.000	0.248	0.000	0.957	0.000
	n	66	165	33	46	66
BrPer	A	0.000	0.000	0.000	1.000	0.100
	B	0.600	0.032	0.000	0.000	0.000
	C	0.100	0.000	0.800	0.000	0.900
	D	0.300	0.968	0.200	0.000	0.000
	n	11	31	5	20	10
Ldh	A	0.000	0.188	0.000	0.000	0.600
	B	1.000	0.813	1.000	1.000	0.400
	n	25	16	33	5	5
Mdh	A	0.300	0.000	0.200	0.000	1.000
	B	0.700	1.000	0.300	0.286	0.000
	C	0.000	0.000	0.500	0.714	0.000
	n	10	5	10	7	5
Pgm	A	0.000	0.000	0.250	0.000	0.000
	B	1.000	1.000	0.250	0.800	1.000
	C	0.000	0.000	0.500	0.200	0.000
	n	6	14	5	15	10
G6pdh	A	0.000	0.006	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.027
	C	0.000	0.000	0.000	0.000	0.973
	D	0.696	0.013	0.028	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000
	F	0.304	0.981	0.972	0.000	0.000
	G	0.000	0.000	0.000	1.000	0.000
	n	46	157	36	33	74

Table 3.2a Allele frequency table for the 12 best staining loci.

Locus	Allele	*P. umb*	*P. lacin*	*P. lin*	*P. leuc*	*P.* sp.
Pgd	A	0.000	0.019	0.000	0.000	1.000
	B	1.000	0.981	0.182	0.000	0.000
	C	0.000	0.000	0.030	0.000	0.000
	D	0.000	0.000	0.788	1.000	0.000
	n	51	53	33	40	26
Mpi	A	0.000	0.000	0.000	0.000	0.156
	B	0.000	0.991	0.000	0.026	0.844
	C	1.000	0.009	0.833	0.000	0.000
	D	0.000	0.000	0.167	0.000	0.000
	E	0.000	0.000	0.000	0.974	0.000
	n	36	107	6	39	32
Dia-1	A	0.018	0.000	0.000	0.000	0.000
	B	0.982	0.019	0.192	1.000	0.000
	C	0.000	0.093	0.808	0.000	0.000
	D	0.000	0.860	0.000	0.000	0.160
	E	0.000	0.009	0.000	0.000	0.000
	F	0.000	0.019	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.840
	n	56	107	26	39	50
Dia-2	A	0.000	0.000	0.000	1.000	0.000
	B	1.000	0.611	1.000	0.000	1.000
	C	0.000	0.389	0.000	0.000	0.000
	n	36	18	26	6	8

(n = number of individuals)

P. lin = *Porphyra linearis*
P. umb. = *P. umbilicalis*
P. leuc. = *P. leucosticta*
P. lacin = *P. laciniata*
P. sp. = *P.* sp.

Table 3.2b Allele frequency table (cont.)

Aldolase (ALD)

ALD was monomorphic for all species tested and so was of no use as a distinguishing. Activity and resolution were good in all cases.

Glucosephosphate isomerase (PGI)

PGI was one of the best resolving enzymes. Activity and resolution were excellent in all cases. It resolved well in both Tris-borate and Tris-citrate buffers and with or without the extraction buffer. It proved to be the most resilient enzyme in terms of storage, whether frozen, freeze dried, or kept in sea water for long periods. Good resolution and activity was still possible after storage in the freezer at -18°C for 8-10 months.

Bromoperoxidase (BRPER)

BRPER had reasonable activity and resolution and was useful for distinguishing species. It resolved much better with the Tris-Borate and extraction buffer regime. Some of the species tested were polymorphic, particularly *P. umbilicalis, P. laciniata, P. linearis* and the unknown species *P.* sp. Best results were obtained with fresh rather than frozen material.

Lactate dehydrogenase (LDH)

LDH gave good activity and resolution and worked well in most cases with the Tris-citrate or Tris-Borate buffers. *P.* sp. appeared to show the most obvious difference from the other species at this locus.

Malate dehydrogenase (MDH)

MDH gave good activity and reasonable resolution. As with LDH this enzyme worked best with the extraction buffer and Tris-Borate regime. It distinguished well the species *P.* sp. and *P. laciniata*, two easily confused species, and showed useful differences between the other three species.

Phosphoglucomutase (PGM)

PGM gave good activity and resolution for all species. Working with both Tris-Citrate and Tris-Borate buffers although much better with the latter, particularly with the extraction buffer. PGM showed three alleles for *P. linearis* and two for *P. leucosticta* and one allele for the remaining three species.

Glucose-6-phosphate dehydrogenase (G6PDH)

G6PDH was one of the best enzymes used, working in all tests. It gave the best results with the Tris-Borate and extraction buffer regime with 7 alleles resolved. It survived storage well and good activity and resolution was still possible after 4 - 6 months of frozen storage. However, it did not survive storage as well as PGI when stored for this length of time. After 4-6 months the quality, in terms of banding activity and resolution, of the results obtained were reduced.

Phosphogluconate dehydrogenase (PGD)

PGD gave excellent activity and good resolution in most cases and was a reliable enzyme in each of the gel runs carried out. It did not appear to be badly affected by the storage technique although did not store as well as G6PDH or PGI.

Mannose phosphate isomerase (MPI)

MPI worked well in most cases particularly with the extraction buffer and Tris-Borate regime. It gave good activity and resolution particularly with fresh material. After 3-4 months of storage resolution and activity was greatly reduced. Most of the five species had two alleles, one common and one rare, with *P. umbilicalis* having only the one common allele.

Diaphorase (DIA)

DIA gave excellent results in nearly all of the runs carried out. It took a little longer than others to resolve but gave clear results and a large number of alleles

in total. Seven alleles were scored in total with most species having two, one common and another rarer allele, *P. umbilicalis, P. linearis, and P.* sp. *P. laciniata* showed four alleles and *P. leucosticta* one allele. This enzyme is useful for distinguishing these five species from each other, particularly when comparing *P. laciniata* and the morphologically similar unknown species *P.* sp.

There was a second locus which appeared as a second band on the gels stained for diaphorase and resolved well when it appeared, although it did not appear in all cases.

Of the remaining enzymes tested with these five species (Table 3.2) only SKDH, CAT, GOT worked reasonably well although often they did not work at all. Storage appeared to have a noticeable affect on these enzymes with the best results being obtained with fresh material. The remaining enzymes tested ME, DASOX, LAP, ADA, GDH, AK, ACON, XOD, HBDH, and FUM (Table 3.1) gave little and in many cases no banding patterns at all.

Some additional results were obtained for a small sample of *P. laciniata* from Ramsey and North Wales. When compared with the other species these samples came out as very similar to examples of *P. laciniata* from Port Erin and Peel.

The levels of genetic identity (*I*) and distance (*D*) (Table 3.3) ranged from 0.296 and 1.216 (respectively), between *P. leucosticta* and *P. laciniata* the least similar pair of species to 0.719 and 0.330 between *P. linearis* and *P. umbilicalis* the most similar pair of species.

The dendrogram of Nei's (1972) genetic identity (Fig. 3.7) shows graphically the results in Table 3.3. It can be seen that the species *P. linearis* and *P. umbilicalis* are the most genetically similar. This group is then most similar to *P. laciniata* with *P.* sp. and *P. leucosticta* being the most dissimilar from the other groups.

Species	1	2	3	4	5
1. *P. linearis*	****	0.719	0.459	0.515	0.454
2. *P. umbilicalis*	0.330	****	0.460	0.617	0.449
3. *P. leucosticta*	0.778	0.777	****	0.296	0.303
4. *P. laciniata*	0.665	0.484	1.216	****	0.383
5. *P. sp.*	0.790	0.801	1.192	0.960	****

Above diagonal : Nei (1972) genetic identity (*I*)

Below diagonal : Nei (1972) genetic distance (*D*)

Table 3.3 Matrix of Nei's (1972) Genetic Identity (*I*) and Distance (*D*) between all species pairs.

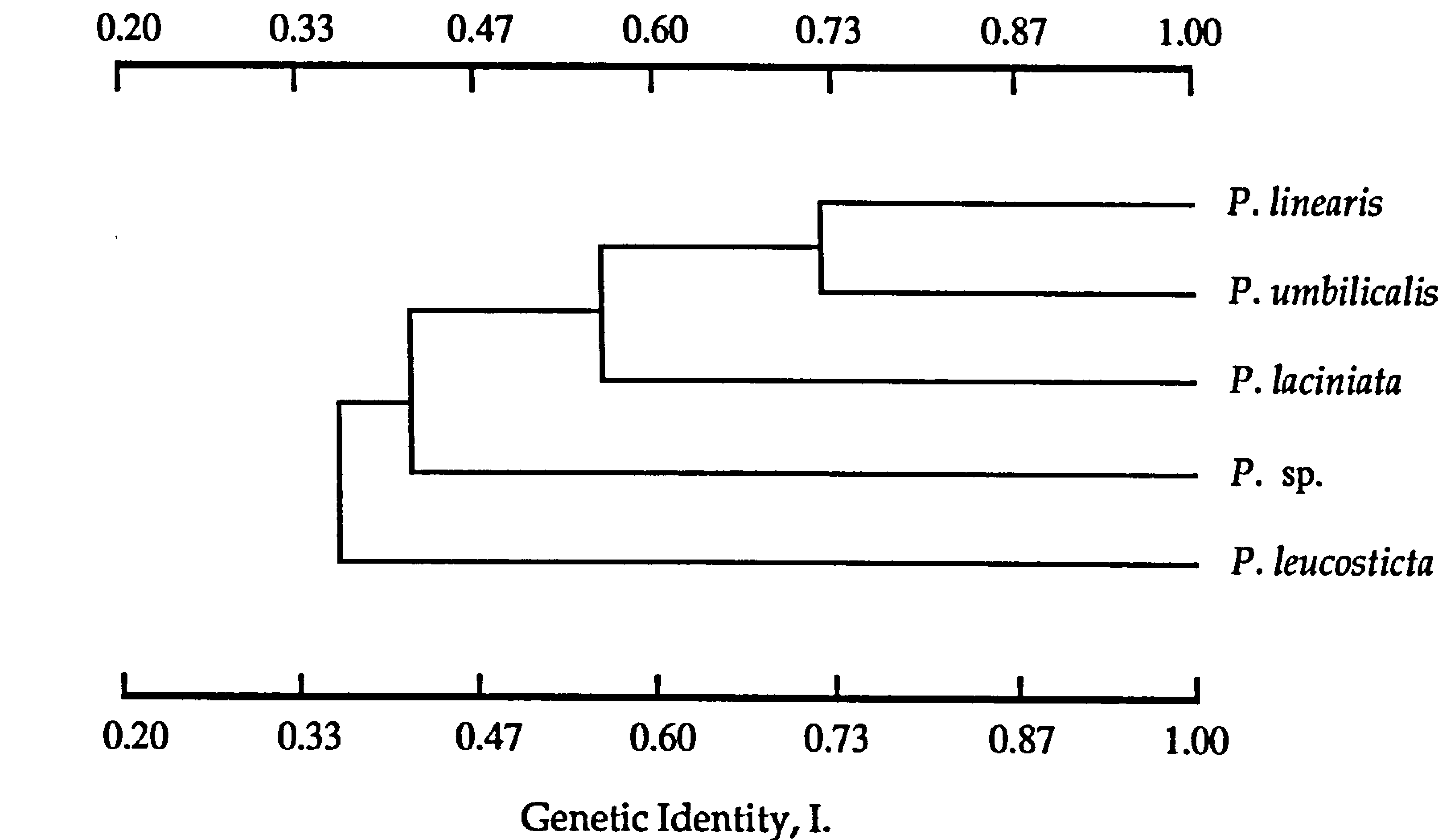


Fig. 3.7 UPEGMA Dendrogram of Nei's (1972) Genetic Identity (I) between all species analysed

The expected levels of heterozygosity which give an indication of levels of polymorphism for the species tested are presented in Table 3.4. These figures are of potential use for looking into and relating to theories such as variability, times of colonosation or nature of dispersal.

Chromosomes

The results of chromosome counts are given in Table 3.5, along with results from previous studies or reviews (for comparison) and photographs on Plates 3.1 - 3.3. The number of chromosomes in the haploid thallus phase of *Porphyra* species is small and so noticeable differences between species are reduced. *P. leucosticta*, *P. linearis* and *P. umbilicalis* have the same number of chromosomes (4). *P. laciniata* has the greatest number of chromosomes (5) and *P. miniata* the smallest (3).

Morphology

Examples of the three morphotypes of *P. umbilicalis* were electrophoresed to ascertain whether they were the same species.

Distribution of alleles between morphotypes was investigated using the Fishers Exact test, which revealed no significant differences at the 5 % level. It can therefore be concluded that the three morphotypes tested share a common gene pool and as such are representative of a single biological species.

An unknown species (*P.* sp.)

Using electrophoresis the species so far named as *P.* sp. was discovered within populations of *P. laciniata* at Port Soderick, Douglas and later in samples from Peel. From observations of individuals it was apparent that the blades of

Locus	*P. umb*	*P. lacin*	*P. lin*	*P. leuc*	*P. sp.*
ALD	0	0	0	0	0
PGI	0.03	0.465	0	0.082	0
BRPER	0.540	0.063	0.320	0	0.180
LDH	0	0.304	0	0	0.480
MDH	0.420	0	0.620	0.408	0
PGM	0	0	0.625	0.320	0
G6PDH	0.423	0.037	0.054	0	0.053
PGD	0	0.037	0.345	0	0
MPI	0	0.018	0.278	0.051	0.310
DIA-1	0.035	0.251	0.310	0	0.269
DIA-2	0	0.475	0	0	0
SOD	0	0	0	0	0
Mean of H_L	0.121	0.138	0.238	0.072	0.104

Expected heterozygosity was calculated as $H_L = 1- X_i^2$ where X_i is the frequency of the ith allele at a locus

Mean of H_L is the sum of H_L over all loci (including monomorphic loci where $H_L = 0$) divided by the total number of loci examined

Table 3.4 Expected heterozygosity calculated from allele frequency

	P. leuc	*P. lacin*	*P. umb*	*P. lin*	*P. min*
Present Study	4	5	4	4	-
Kain (1991)	4	5	4	-	3
Kito (1967)	-	-	-	4	-
Kapraun (1991)	4/3	-	-	-	-
Coll (1977)	4	-	-	-	-
Krishna (1959)	-	5	-	-	-
Kapraun (1987)	4	4	4	-	-
Yabu (1963)	-	5	-	4	-
Mumford (1977)	-	-	-	-	3

Table 3.5 Haploid chromosome numbers of *Porphyra* species found on the Isle of Man compared with previous studies and reviews.

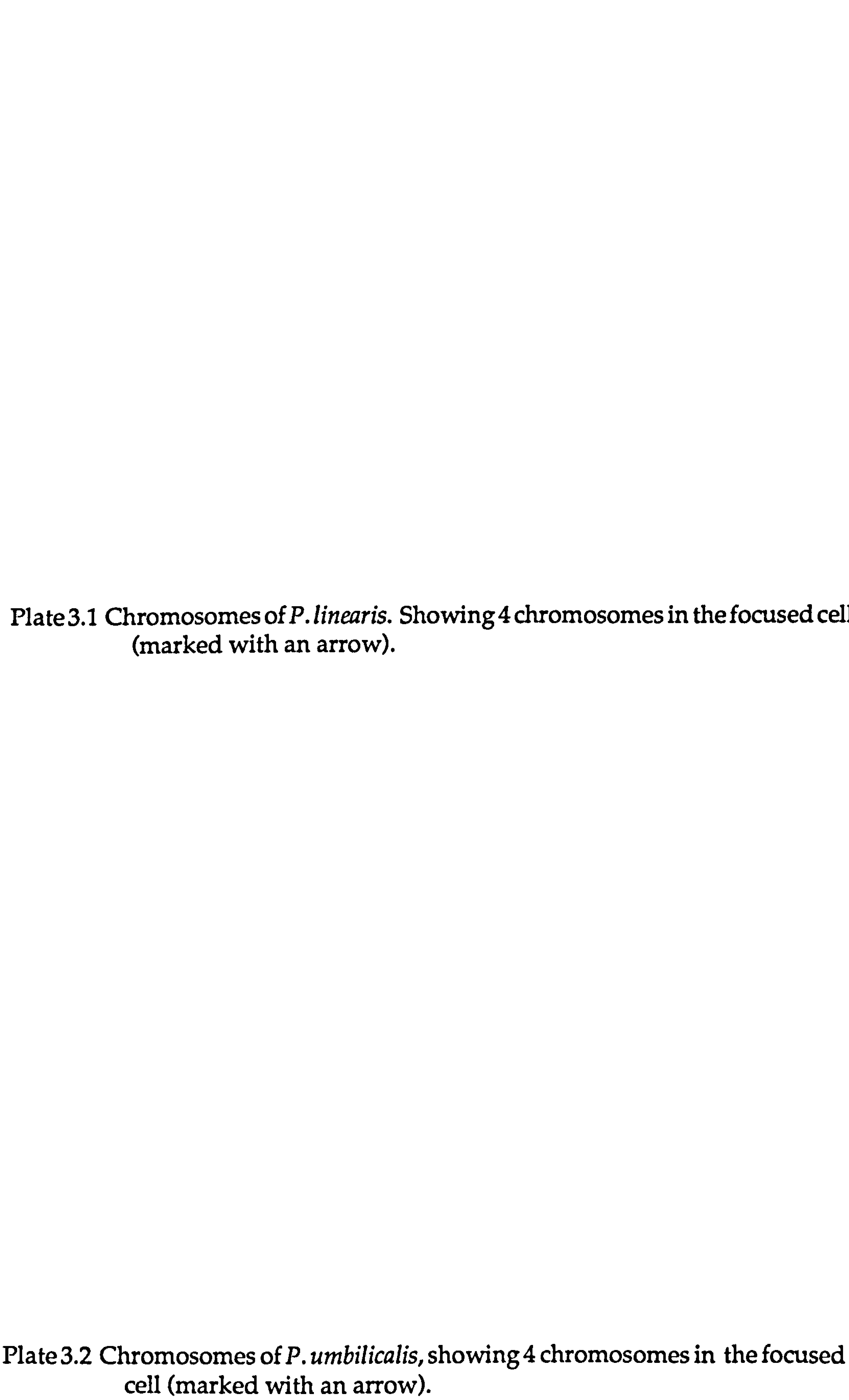


Plate 3.1 Chromosomes of *P. linearis*. Showing 4 chromosomes in the focused cell (marked with an arrow).

Plate 3.2 Chromosomes of *P. umbilicalis*, showing 4 chromosomes in the focused cell (marked with an arrow).

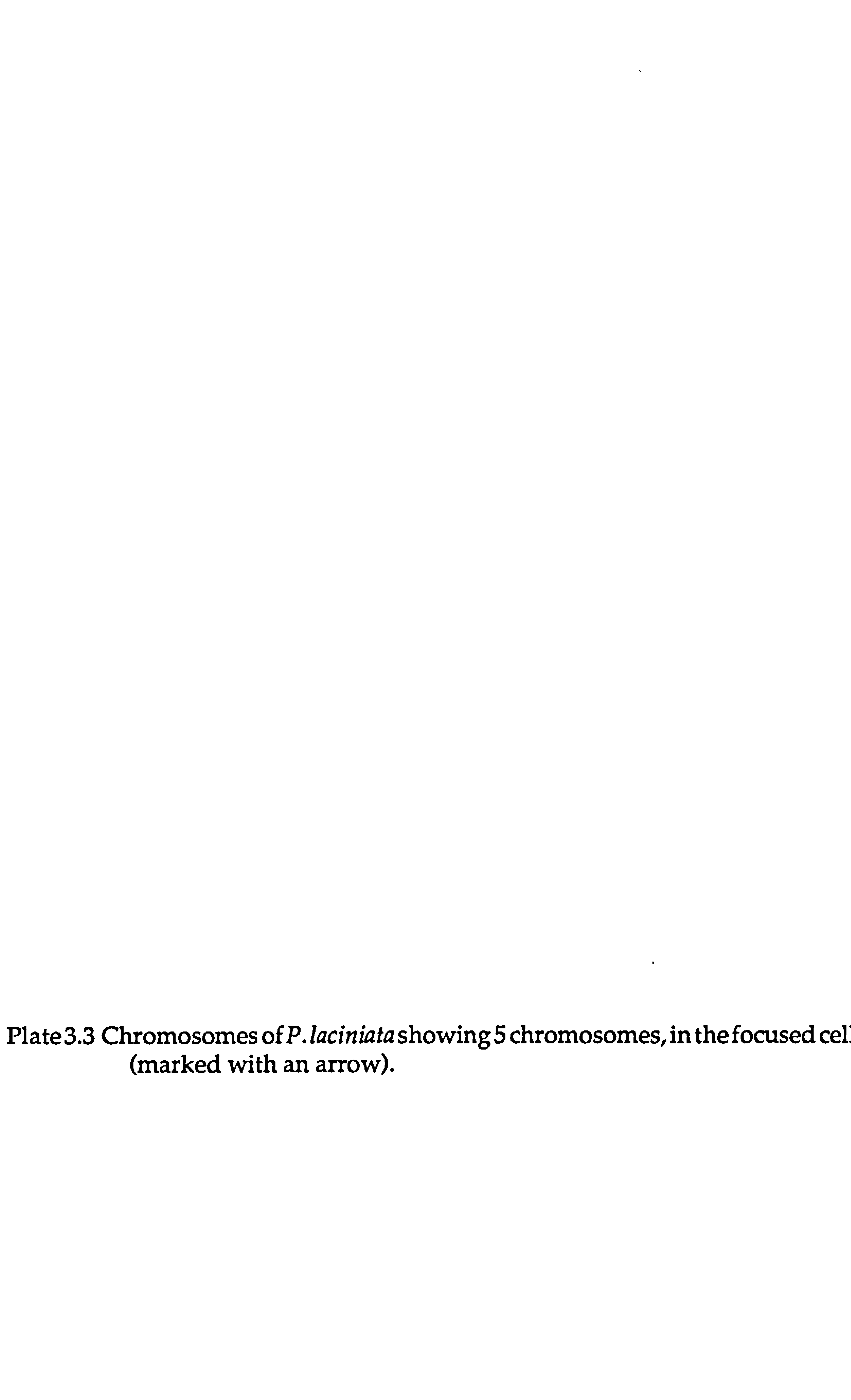


Plate 3.3 Chromosomes of *P. laciniata* showing 5 chromosomes, in the focused cell (marked with an arrow).

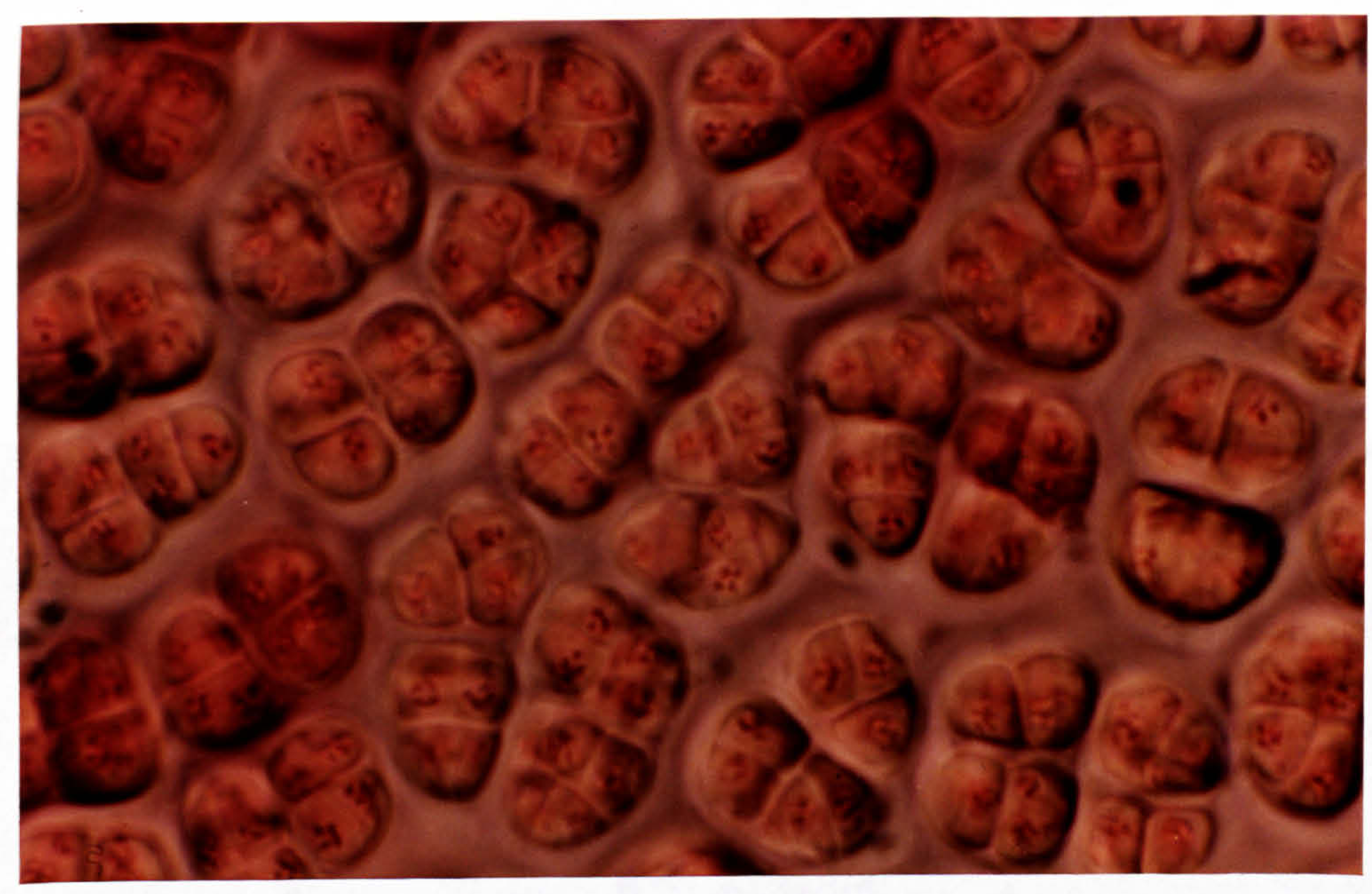


Plate 3.1 Chromosomes of *P. linearis*. Showing 4 chromosomes in the focused cell (marked with an arrow).

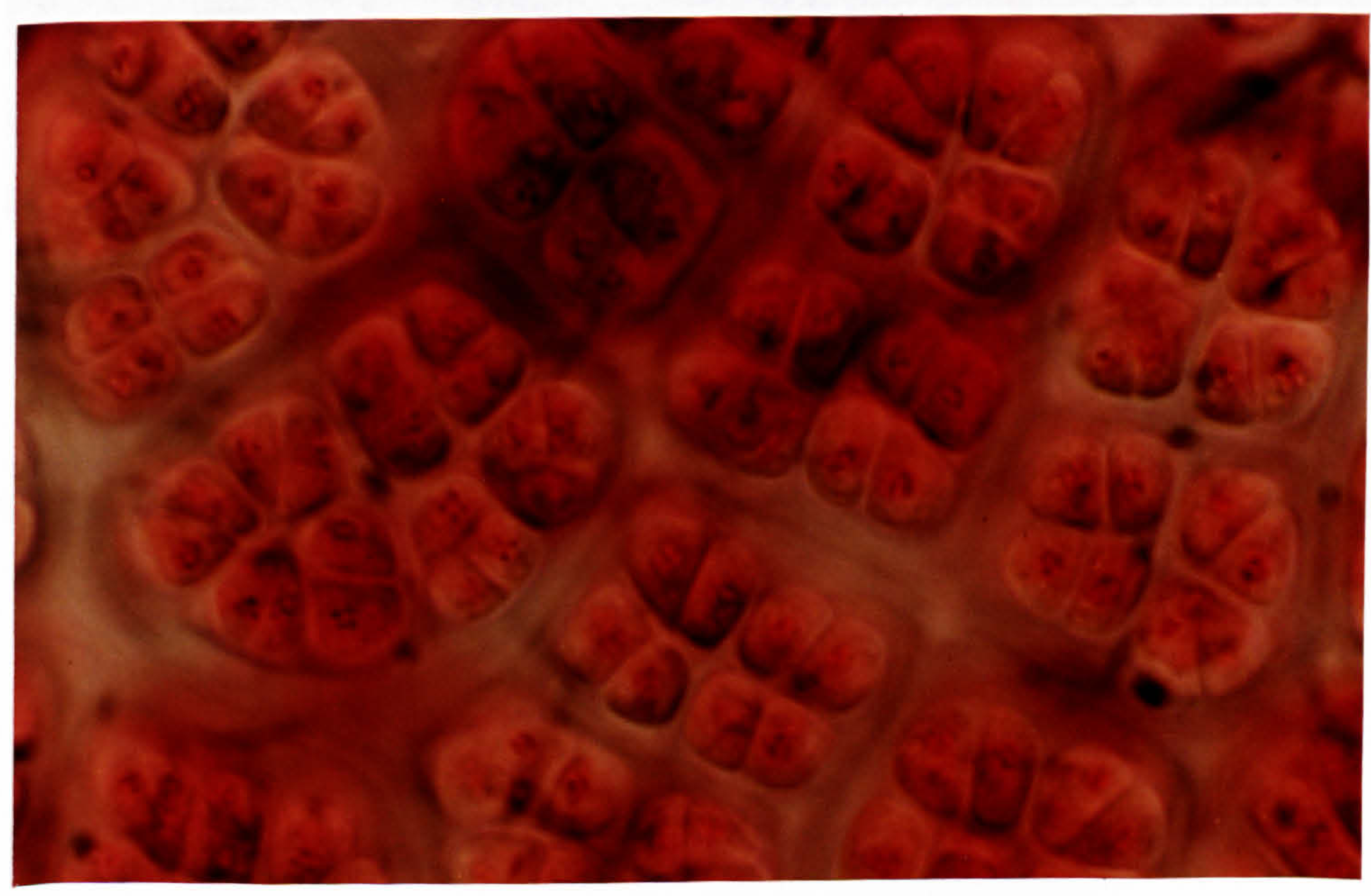


Plate 3.2 Chromosomes of *P. umbilicalis*, showing 4 chromosomes in the focused cell (marked with an arrow).

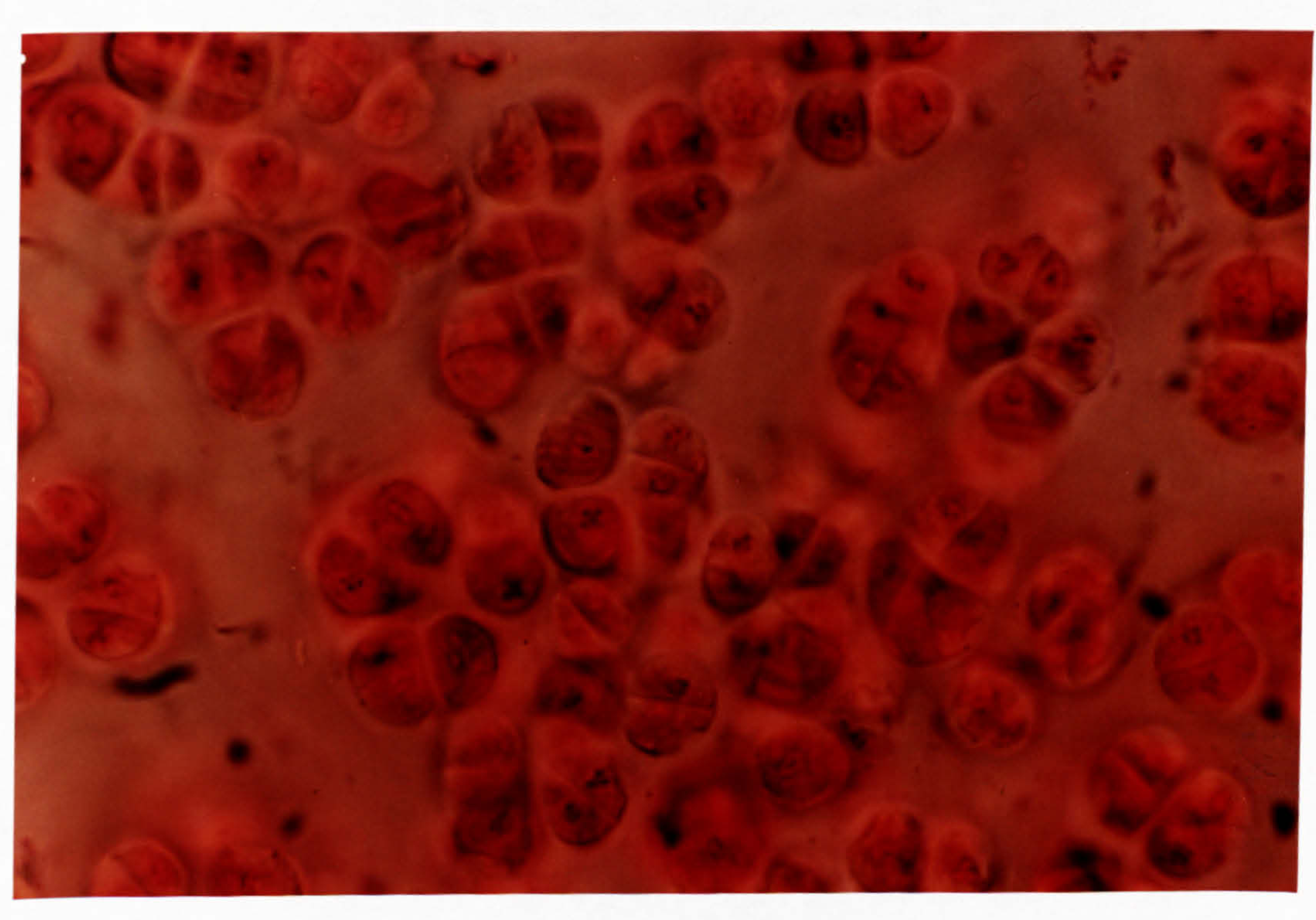


Plate 3.3 Chromosomes of *P. laciniata* showing 5 chromosomes, in the focused cell (marked with an arrow).

P. sp. were thinner than the blades of *P. laciniata*. Consequently the thickness of blades of the two species was measured after portions of the blades had been electrophoresed to ensure species identity. From Fig. 3.8 it can be seen that the two species do in fact have a different blade thickness. There is variation of thickness measurements within each species but these do not overlap with one another. *P. laciniata* had the thicker blades with the majority being approximately 51 - 56 μm thick and *P.* sp. thinner blades approximately 35 - 40 μm.

As a result of an interest in the application of electrophoresis to other algal species a sample of *Laminaria hyperborea* (Gunn.) Fosl. when tested resolved for Pgi and G6pdh when run on a test gel. With further development and research with this and other genera of seaweeds more enzymes would be found, increasing the potential of this kind of study.

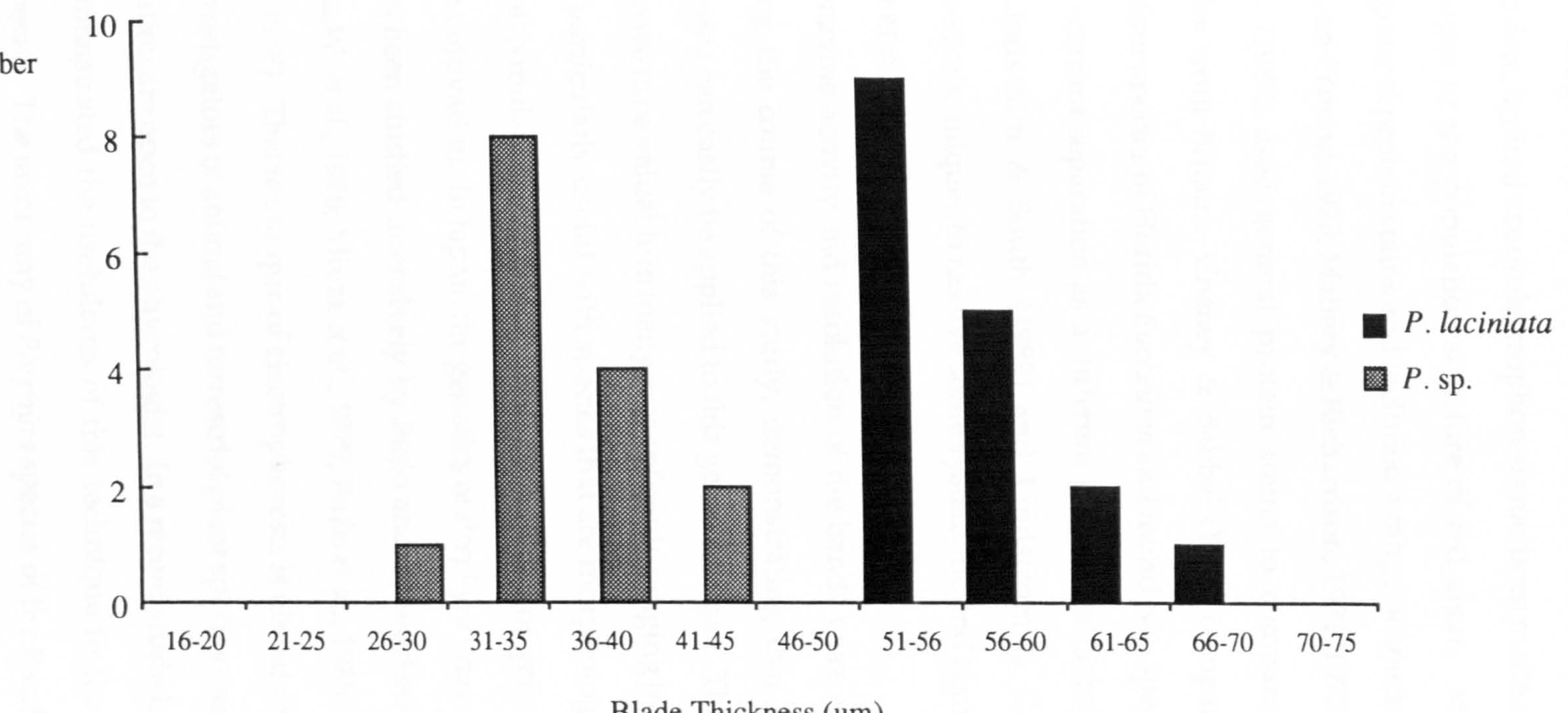


Fig. 3.8 Thickness measurements of *P. laciniata* and the unknown species *P.* sp. from individuals confirmed by electrophoresis.

DISCUSSION

A knowledge of systematics and evolution is fundamental to all areas of biology. To date, limited use of electrophoresis has been made in either studying the systematics or the population structure of red algae. Most early papers employed general protein stains and included a range of species not necessarily closely related (Young, 1970; Mallery & Richardson, 1971, 1972). More recently, Price *et al.* (1987) used general protein stains to compare five species of *Callithamnion* from Britain. Cheney & Babbel (1978) compared five enzyme systems in four species of Florida *Eucheuma* and found two species to be distinct enough to warrant separation as a different genus [see Gabrielson & Cheney (1987)]. Lindstrom & South (1989) and Lindstrom & Cole (1990) used electrophoretic techniques to resolve some systematic and taxonomic problems in *Porphyra* and *Palmaria.*

The enzyme activity and resolution of the bands were excellent in many cases during the course of this study, demonstrating that the technique of electrophoresis can easily be applied to this genus of algae. This technique may therefore prove to be valuable in many areas of study ranging from the taxonomy of species (particularly useful with species that are morphologically similar) to the study of population genetics and the applied use of genetic selection and breeding in cultivation. In Japan, the genetics of *Porphyra yezoensis* (a cultivated species) has been studied intensively by Fujio and co-workers (Miura,A. *et al.*, 1978; Miura,W. *et al.*, 1978; Miura *et al.*, 1979; Fujio *et al.*, 1985; Fujio *et al.*, 1987; Fujio *et al.*, 1988). This technique of electrophoresis is already widely exploited by other investigators of animal and terrestrial plant species and so similar paths of investigation are open to the phycologist. In a recent study Lindstrom & Cole (1992a) demonstrated the usefulness of this technique in the classification of *Porphyra* species. The taxonomy of *Porphyra* species of the Pacific coast of North

America has been studied several times (Krishnamurthy, 1972; Conway *et al.*, 1976; Garbary *et al.*, 1981) and yet as Lindstrom noted 'it still provides taxonomic surprises'.

In the present study in which 12 loci regularly resolved, polymorphism was detected in 5 loci for *P. umbilicalis*, 8 for *P. laciniata*, 7 for *P. linearis*, 4 for *P. leucosticta*, and at 5 for *P.* sp. (Table 3.2). The available electrophoretic data for *P. yezoensis* has shown polymorphism at 6 out of 8 loci tested in wild populations (Miura *et al.*, 1979).

P. linearis in Scotland was shown to produce asexual monospores (Cole & Conway, 1980). If this is the case with the species studied in the Isle of Man then this may affect the consequent number of genetic individuals within the population. If, however, a species is not reproducing the thallus phase asexually then the number of genetic individuals will be equivalent to the number of individuals within the population. It is possible that a relatively small number of individuals produce the majority of descendants in each locality because they reproduce mostly by selfing and asexual reproduction. Low genetic variability has been observed within the asexually reproducing marine diatom *Thalassiosira pseudonna* Clev. taken from wide geographic areas (Murphy & Gillard, 1976). Allard *et al.* (1975) could not find a clear relationship between the mating system and the amount of genetic variability in plants, that is, strongly self fertilising species maintained levels of genetic variation equal to those of outbreeding species. It is known that large genetic variation is present in many natural populations, and these data have been summarised by Babbel & Selander (1974).

Genetic differentiation between isolated species or populations may occur as a result of factors such as mutation, selection and random genetic drift. In general the more closely related species are, the greater is the similarity in their electrophoretic banding patterns. Genetic identity (*I*) indicated that the most closely related species were *Porphyra linearis* and *Porphyra umbilicalis* with a

genetic identity of 0.719 and genetic distance of 0.330. These were also the most sympatric species. The most distantly related species from this study were *Porphyra leucosticta* and *Porphyra laciniata* with an identity of 0.296 and distance of 1.216. If one accepts the published values for the molecular clock, ranging from 5 - 19 million years per unit of distance (D) (Thorpe, 1982, 1989), then the estimated time of divergence for the most closely related species would be 1.7 - 6.3 million years ago and the most distantly related species, between 6.1 - 23.1 million years ago. If the levels of Identity (I), which can be used to distinguish levels of taxonomic separation, are to be accepted then the differences of genetic identity (*I*) (Table 3.3) are quite large when one considers that the region of the value of genetic identity (*I*) which delimits the separation of individuals as separate species is ~ < 0.85 and the value for individuals belonging to a separate genus is ~ 0.2 - 0.3 (Thorpe, 1983). Therefore the two most different species *P. laciniata* and *P. leucosticta* (*I* = 0.296) could be considered as almost belonging to a separate genus. The two most distantly related species, *P. leucosticta* and *P. laciniata* were found at greater geographic distances and so were less sympatric being found on different shore heights.

The results of this electrophoretic study demonstrates that starch gel electrophoresis is an extremely useful tool for identifying species of *Porphyra*. The unique patterns provided by staining a variety of proteins produce identifying profiles that can supplement other methods used in making species distinctions.

The determination of whether a morphological variant is genetically based and thus deserving of taxonomic recognition, or whether it is environmentally induced generally requires the use of experimental taxonomic or biosystematic techniques (Cheney & Babbel, 1978). The three morphotypes of *P. umbilicalis* showed no difference when analysed using electrophoresis therefore it can be concluded that the phenotypic differences observed in these morphotypes do not present a taxonomical problem to the classification of the three forms as the single

species *P. umbilicalis.* This demonstrates how this technique can be used to substantiate the validity of distinguishing morphological variants as the same species or as separate species which may otherwise cause taxonomical difficulties.

Ishikawa (1921) was the first person to study the cytology of *Porphyra.* Since then a great deal of work has been carried out on the number of chromosomes of *Porphyra* species (Krishnamurthy, 1959; Kito, 1966a, b; Kito *et al.*, 1967; Yabu, 1969; Kito *et al.*, 1971; Yabu, 1972; Kito, 1974; Coll & de Oliveria-Filho, 1977; Kito, 1978; Krishnamurthy, 1984; Kapraun & Freshwater, 1987; Sun *et al.*, 1987; Cole, 1990; Kapraun *et al.*, 1991) because of the development of relatively simple techniques and also the ease of staining for chromosomes in the spermatia of the adult blade [see Krishnamurthy (1984) for a review of 43 species].

In many cases the cytological methods have been used to study the position of meiosis in the life history of *Porphyra* species and have been used in relatively few cases to study taxonomic problems. More recently however, cytogenic techniques have been applied to systematic problems, providing additional criteria to distinguish closely related species (Mumford, 1975; Mumford & Cole, 1977). In the study by Kapraun & Freshwater (1987) the chromosome count was used as an aid to a taxanomical study and on the basis of karyotype analysis they suggested that five species were closely related. Most cytological efforts have been concentrated in the North Pacific where *Porphyra* is of significant economic importance. There have been few investigations on the cytology of *Porphyra* in the Atlantic, and these have centred on cold temperate species (Daneguard, 1927; Magne, 1952; Krishnamurthy, 1959; Kito *et al.*, 1971; Kapraun & Luster, 1980; Krishnamurthy, 1984).

The results of the chromosomes counted in the four species analysed (Table 3.5, Plates 3.1 - 3.3) agree with those in the literature. Between the species of *P. umbilicalis*, *P. linearis* and *P. leucosticta* there is no difference in chromosome number (4) and so a simple chromosome count could not be used to distinguish

these species from each other. Four chromosomes were noted by Coll & de Oliveria-Filho (1977) and Kapraun & Freshwater (1987) for *P. leucosticta*. *P. laciniata* however, has a chromosome count of 5 which does distinguish it from the other 3 species studied. Chromosome counting can be an aid too taxonomic differentiation but due to the low number of chromosomes in this genus and often little if any differences in numbers between many of the species further techniques for differentiation of these species are required.

A combination of the more traditional morphological, ecological identification and karyological work combined with the use of a genetic technique such as electrophoresis, unbiased by phenotypic differences, allows greater reliability when making distinctions between species. The results demonstrated that electrophoretic techniques were useful for distinguishing the four Manx species and also with doubts cast *a priori* by morphological characteristics [blade thickness (Fig. 3.2) and colour] revealed and supported the presence of a 'new' sympatric, apparently cryptic species. It is in this situation that allozyme electrophoresis is at its most powerful as a tool to the systematist or taxonomist (Richardson *et al.*, 1986).

Initially in this study descriptions of this unknown species which appeared mixed in populations of *P. laciniata* could not be found. It was thought that this species was either an immigrant or a previously undescribed species. However, during the course of this study the research by Kornmann & Sahling (1991) appears to shed light on this taxonomic problem. Kornmann noted that in the past the literature has been confused as to the identity and nomenclature of *P. laciniata* and *P. purpurea*. He himself apologised for the error in his paper (Kornmann, 1961b) of the supposed identity of *P. purpurea* (Roth) C.Ag as *P. umbilicalis* var. *laciniata* (Lightf.) J. Ag. caused by their similar external morphology. Kornmann (1991) also concluded that the material on which Drew (1954) based her classic study was identical with *P. laciniata*. He also noted, however, that the correct use of this name was still uncertain. In light of

Kornmann's own descriptions and observations and the description of *P. laciniata* as identical to that of Drew (1954) material then this species name is accepted in this study.

The additional species so far named as *P.* sp. found to be masquerading with the *P. laciniata* samples in this study still, however, presents a problem. Kornmann (1991) stated that Krishnamurthy (1972) noted that *P. purpurea* was in fact previously described by Roth himself as *P. purpureo-violacea* and chose to use this latter name in his study. In his description of *P. laciniata* Kornmann (1991) stated that the confusion of this species with *P. purpureo-violacea* was still possible. A possible identity of the unknown species of the study *P.* sp. is that of the species *P. purpureo-violacea* described by Kornmann (1991).

This suggestion is based on the following characters being similar in the two entities:

1. Overall morphology very similar to that of *P. laciniata*
2. Thallus considerably thinner than that of *P. laciniata*
3. Thallus divided by a clear line into male and female half.

Unfortunately other data for comparison of spore configurations and chromosome numbers are not available for the material in the present study. It seems likely, however, that the species referred to as *P.* sp. in this study is in fact the same as the species *P. purpureo-violacea* described by Kornmann (1991). This is by no means conclusive and further work on the taxonomy of this species with more information on spore configurations, cytology and ecological distinctions is necessary. However, Kornmann (pers. comm.) seems to agree with the interpretation.

If the unknown species *P.* sp. is in reality a new species then the fact that it appears to be morphologically similar to *P. laciniata* is probably the reason why it has been overlooked in previous studies of *Porphyra* in the British Isles

(Conway, 1964a, b). If it is a new species then it may have been introduced by shipping as with other marine immigrants or through the transportation of the conchocelis in imported shellfish for culture or consumption. Further ecological and genetic work of this species around the coast of the British Isles would give some indication as to whether this species is fairly common, but overlooked as a separate species, or a record of an isolated introduction of a foreign species as was the case with *P. yezoensis* in Helgoland (Kornmann, 1986). Comparisons with other European species including likely immigrant species is required to be certain of the classification of this 'new' species.

In light of the present study, the recommendation by Lindstrom & Cole (1992a) that the use of at least one non-morphological characteristic (i.e. electrophoresis or cytology) for the correct identification of specimens of such morphologically similar species would appear to be extremely valuable advice.

CHAPTER 4

THE CULTURE OF THE CONCHOCELIS PHASE

OF *P. LINEARIS* AND *P. LACINIATA*

INTRODUCTION

The leafy phase of the genus *Porphyra* grows rapidly and in many cases shows a marked seasonal occurrence often covering significant areas of the intertidal (Conway, 1964a; Conway *et al.*, 1976). On the Isle of Man *P. linearis* has marked winter seasonality and *P. leucosticta* shows marked summer seasonal occurrence (Chapter 2). *P. laciniata* thalli are present for much of the year but show a marked increase in biomass and abundance in spring. The thalli of many highly seasonal species often occur for a relatively short period of the year and so study of the alternate conchocelis phase is of importance in order to understand aspects of the biology and ecology of species of the genus over the complete seasonal cycle.

Most *Porphyra* species have been shown to alternate from a thallus phase to a small and presumably perennial filamentous conchocelis phase. Consequently it would be expected that the growth and alternation from one phase to the other would occur as a response to reliable environmental stimuli that would ensure initiation of the thallus phase at a time of year when conditions are optimal for growth.

A knowledge of the conditions and environmental stimuli required for production and growth of conchocelis, conchosporangium development and conchospore release is necessary for a greater understanding of the seasonal occurrence of the thalli of *Porphyra* species. This information also aids further studies into the potential of a species for cultivation.

A great deal is yet to be learned about the biology of the natural conchocelis phase and as a result of the numerous difficulties encountered when studying such an organism in the field, laboratory culture experiments can be very useful. Laboratory research can be used to gain a greater depth of understanding of environmental stimuli necessary for the alternation from one phase of the life history to the other. Photoperiod and temperature have been shown to be

important regulators of conchospore maturation and release in five *Porphyra* species studied. Changes in these conditions are also those most likely to be found at the time preceding the appearance of the leafy thallus in the field (Waaland *et al.*, 1990).

Photoperiodism is best defined as 'the control of some aspect of a life cycle by the timing of light and darkness' [Hillman (1979) from Dring (1984)].

The number of photoperiodic responses demonstrated for species of algae has increased [see Dring (1984, 1988) for reviews on photoperiodic studies] since the first clear proof was demonstrated independently by Dring (1967) and Rentschler (1967) for the species *P. tenera*. However, many species that occur seasonally have yet to be studied from the point of view of photoperiodic control within the conchocelis phase.

Conchosporangium production in some *Porphyra* species requires a specific environmental stimulus, e.g. a short day photoperiod in *P. tenera* (Dring, 1967; Rentschler, 1967). However, *P. miniata, P. linearis, P. angusta* and *P. torta* require no such stimulus (Chen *et al.*, 1970; Bird *et al.*, 1972b; Chiang & Wang, 1980; Waaland *et al.*, 1987). The winter species *P. tenera* was shown to only release conchospores from September to early November (Kurogi & Hirano, 1956), a time when the photoperiod is changing through a critical daylength. Dring (1967) demonstrated a photoperiodic response (short day) for the initiation of conchosporangia production in this winter species. Chiang & Wang (1980) showed that an increase in temperature was necessary for the production of conchosporangia in *P. angusta* and that photoperiod had no effect. Work on the species *P. abbottae, P. nereocystis, P. perforata* and *P. pseudolanceolata* showed that photoperiod, light and temperature were all involved in the production and maturation of conchospores (Waaland *et al.*, 1990).

Thus the production of conchosporangia appears to occur as a result of environmental stimuli in some cases but not in others. It would also seem that the production of conchosporangium in some cases occurs as a result of

maturation of the growing conchocelis. The production of conchospores and the subsequent control of their release is also under the control of a number of stimuli which varies for different species. Research has shown that a number of variables can be involved in the growth of conchocelis, formation of conchosporangia, conchospore initiation and conchospore release and that the sets of conditions that control these stages can vary greatly from species to species (Dring, 1967; Bird *et al.*, 1972a; Avila *et al.*, 1986; Freshwater & Kapraun, 1986; Waaland *et al.*, 1987; Waaland *et al.*, 1990). Similarly conchocelis cultured in shell substrata would also appear to behave differently.

In light of these potential differences it is important to study the conditions necessary for growth, maturity and spore release for as many species in a given study area as possible in order to understand aspects of the biology and ecology of consequent phases in the life history of species of the genus *Porphyra*.

METHODS

The culture of conchocelis stocks for use in further experiments

General culture techniques

Autoclaved seawater was used initially in test cultures but this proved both time consuming and often caused precipitation of crystals in the seawater which interfered with later microscope work.

The use of autoclaved seawater as the basis for the culture medium was compared to filtered seawater using a few simple spore cultures. Filtered seawater was found to be the most suitable for these experiments. For all further experiments seawater was filtered using a 0.22 μm Bolsten filter. The filtered seawater was tested for contaminants under the planned experimental conditions and found to be suitably clean. The medium used for growth experiments was F2 seawater preparation (see Appendix for recipe) with GeO_2 usually at 1 mg/l to inhibit diatom growth. All glassware and utensils were sterilised in an autoclave prior to use.

Carpospore release

The photoperiod experiments required a good stock of vegetative conchocelis material. Vegetative conchocelis filaments were grown by first collecting carpospores from fertile thalli.

The reproductive thalli were washed in fresh seawater and all large epiphytes were removed. Fertile sections were cut from the thalli and washed in 5 % Betadine [Polyvinylpolypyrrolidone (PVP-1) Iodine complex, Sigma number PVP-1]/seawater solution by leaving the sections in the solution for 3 minutes. The sections were then washed in seawater and left for 10 minutes in sterile seawater. Often spore release occurred during washing in the seawater so spores could be collected using a syringe. If spore release did not occur or if spore

release was poor the sections of thallus were placed between layers of moist paper towel and then placed in a refrigerator overnight. The next morning the thalli sections were placed back into freshly sterilised seawater which generally caused the liberation of large quantities of spores within the first few minutes. A spore suspension was created by placing the collected spores in a flask with a magnetic stirrer to prevent the spores settling for the duration of the inoculation procedure.

Spore cleaning

Even after the thalli had been washed and cleaned with Betadine solution contamination was often still a problem. To try to alleviate this the technique described by Birnie & Boney (1980) for the separation of contaminants from carpospores was used. A concentrated spore suspension was first produced using gentle centrifugation (1000 rpm for fifteen minutes) of the original spore suspension. Two ml of this spore suspension were placed into glass centrifugation tubes and Histopaque - 1077 (a specific density solution used for the separation of human blood cells by differential centrifugation, Sigma H8889) solution were added to the tubes. Two ml of half strength Histopaque solution were first gently added below the spore suspension, using a 5 ml syringe and needle, followed by 2 ml of full strength solution at the bottom of the tube. The tubes (with the spore suspension at the top) were then centrifuged at 1500 rpm. for 15 minutes. The *Porphyra* carpospores were separated out from the contaminants (diatoms and other spores by the differential centrifugation) at the boundary of the full strength and half strength histopaque. The resulting layer of carpospores was then drawn off using a sterile syringe and large needle and placed into 100 ml of sterile seawater to wash off the Histopaque solution.

Using a sterile pipette 1 ml of the resulting spore suspension was added to 50 ml of F2 seawater medium in a 100 ml evaporating dish. The dishes were labelled and a petri dish used as a lid. The conditions used for the initial

conchocelis growth were 12 and 15 °C and 16:8 and 8:16 photoperiod. Seawater medium in the dishes was replaced weekly and dishes were checked daily for contamination.

Approximately 7 days after germination individual filaments of conchocelis were removed from the dishes using sterile watchmakers forceps. Twenty filaments were placed in new sterile dishes (12 dishes in total) with 50 ml of fresh medium further to reduce any contamination that may still have been present.

Stock conchocelis cultures were also kept in flasks with aerated moving seawater which produced conchocelis balls. These were cultured in green light (30 µmol $m^{-2}s^{-1}$ at 10 °C) to reduce green algal contamination. The seawater medium used was half strength F2 and was changed every 2 weeks. When the conchocelis balls had grown to a diameter of 1 cm each was split into four equal portions and returned to flasks.

When suitable quantities of conchocelis material had been cultured (a period of 4 - 6 weeks) the photoperiod experiments were then set up.

Photoperiod experiments

Conchocelis filaments of *P. laciniata* and *P. linearis* were used in this experiment to study growth under varying photoperiods.

The culture conditions used were: continuous light; 16:8, 12:12 and 8:16 with a light level of 60 $\pm$ 5 µmol $m^{-2}s^{-1}$ and a temperature of 13 °C $\pm$ 1°C.

Conchocelis filaments were collected from the stock cultures and homogenised into small filament fragments (10 - 20 cells/fragment). The filament fragments were put into F2 seawater medium in a flask with a stirrer to prevent the filaments settling out. Using a very large needle (2 - 3 mm bore) with a 5 ml syringe, evaporating dishes containing 50 ml of media were inoculated with 2 ml of the homogenised conchocelis filaments. For each experiment three

replicates of each condition were inoculated so that dishes could be destructively sampled. In total 60 dishes were inoculated with *P. laciniata* and 48 with *P. linearis*. A further six dishes for each experiment were inoculated for the reversal of photoperiod after the final set of growth measurements. The dish positions in the growth cabinets were alternated after each medium change to cancel out slight differences in conditions.

Growth measurement

Dry weight was used as a method of growth measurement. After each photoperiod experiment had been set up the dry weights of four replicates of the initial inoculum of conchocelis were measured. This involved the use of 6 cm^2 pieces of mesh (40 µm hole diameter). The dry weight of each piece of mesh was measured after approximately 12 hours in an oven at 100 °C. Initial measurements were carried out by forcing conchocelis inoculum samples through the mesh thus retaining the conchocelis filaments. The mesh with conchocelis filaments was squeezed to remove as much seawater as possible to reduce to a minimum the effect of the weight of salt crystals when the samples were dried. Once the conchocelis samples had been collected on the mesh they were placed in an oven at 100 °C for 12 hours and then weighed on a balance. This gave initial inoculum dry weight values (Table 4.1). The same process was carried out at each destructive sample time. *P. laciniata* samples were taken at 8, 24, 41, 57 and 70 days and *P. linearis* at 32, 51, 71, 102 days.

Presence of conchosporangia

At each sampling time the dishes of growing conchocelis were examined using a stereo zoom microscope for the presence of conchosporangia. If conchosporangia were present then one dish was selected at random and the contents were gently homogenised for 20 seconds. 20 single drop samples of this

Inoculation dry weights (mg)

Species	Replicate	Initial mesh dry weight	Mesh + algae dry weight	Algal dry weight	Mean dry weight	S. Error
P. linearis	1	270.0	274.5	4.5		
	2	272.2	277.1	4.9		
	3	251.4	256.0	4.6		
	4	2658	270.2	4.4	**4.6**	0.1
P. laciniata	1	254.7	258.2	3.5		
	2	267.5	271.7	4.2		
	3	266.4	269.8	3.4		
	4	278.9	282.8	3.9	**3.7**	0.2

Table 4.1 Inoculum dry weights of *P. laciniata* and *P. linearis* for photoperiod experiments (errors are one standard error)

homogenate were examined using a stereozoom microscope and the number of conchosporangium fragments were recorded. This process was carried out for one dish from each of the photoperiods.

Induction of conchospore release

At the end of each experiment three dishes with the best conchosporangium growth were transferred from the 16:8 photoperiod to the 8:16 photoperiod and *visa versa* to try to induce conchospore release.

Another dish from each of these two photoperiods was placed at a lower temperature (6 °C) for 12 hours repeatedly over 4 days to try to induce conchospore release. These dishes were checked daily for 2 weeks to see if any spore release occurred.

Cross gradient experiment

This experiment used a light/temperature cross gradient table (Plate 4.1) to create a range of temperatures and light intensities within one culture unit (Edwards & Van Baalen, 1970). The cross gradient table consisted of an aluminium sheet approximately 1 cm thick with a cooling unit at one end and a heating unit at the other. These units created a temperature gradient from one end of the table to the other. The whole unit was placed in a constant temperature room at 10 °C. The practical working limit of the table was approximately 0 - 18 °C. In this experiment the units were adjusted to produce a gradient of 4 - 15 °C within the planned working area of the table.

A series of fluorescent tubes hanging above the table were used to create a light intensity gradient across the table perpendicular to the temperature gradient. These lights could be raised for removal of dishes and lowered to a

Plate 4.1 Cross gradient table. Lights are raised in the photograph to show the white table. The lights are lowered during culture experiments.

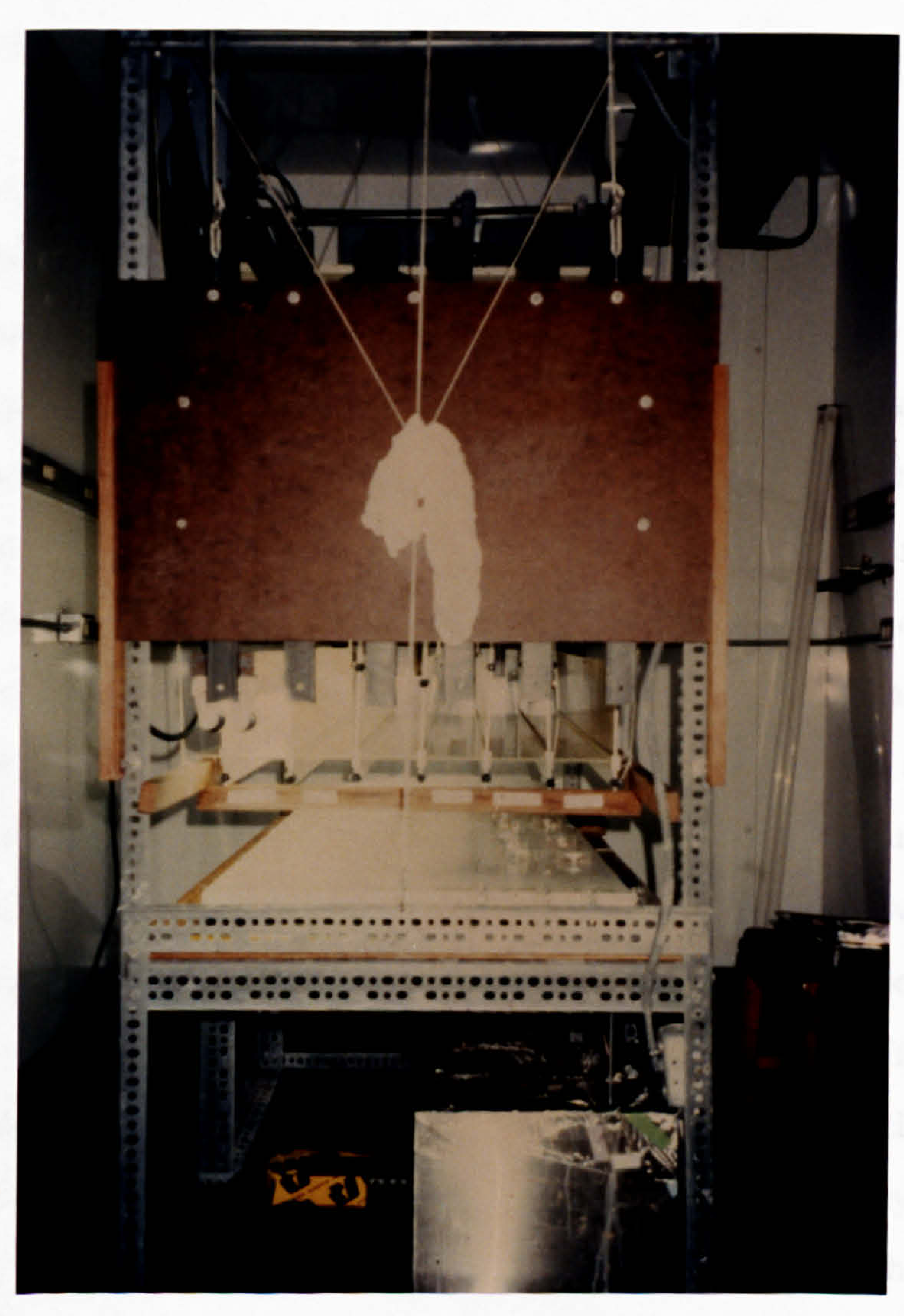


Plate 4.1 Cross gradient table. Lights are raised in the photograph to show the white table. The lights are lowered during culture experiments.

height that created the desired experimental light conditions. A series of short opaque plastic walls placed between the lights helped to produce the necessary light gradient over the relative short distance across the table.

Four light regimes, 4, 30, 55 and 100 µmol $m^{-2}s^{-1}$ and four temperature regimes, 6, 9, 12 and 15 °C were used for the experiment.

Conchosporangium material

Previous conchocelis material had been grown using the techniques described earlier for production of conchocelis stocks. A quantity of this free conchocelis had produced conchosporangium branches (hand-like in appearance). When this material was gently homogenised it produced numerous similar sized fragments (Fig. 4.1) of conchosporangium material.

The size of the conchosporangium material used in this experiment allowed small (4 cm) petri dishes to be used, thus enabling an accurate temperature to be maintained in each dish as each dish only covered a relatively small area on the table. Ten measured fragments of conchosporangium material were inoculated into each dish at the beginning of the experiment. Two replicate dishes were placed (touching) at each temperature and light condition with the position of each dish alternated daily to eliminate any small differences in conditions due to a dish's individual position. The position of each dish was accurately marked so that once material had been measured they could be returned to the same conditions. The sea water medium in each dish was changed with fresh F2 seawater medium from the same batch every two days to eliminate nutrient effects and to ensure that salinity remained constant. Measurements were carried out at approximately five day intervals using a grid graticule in a stereo zoom microscope at 50X magnification. Measurements were of the area covered by the fragment in numbers of whole squares of the graticule.

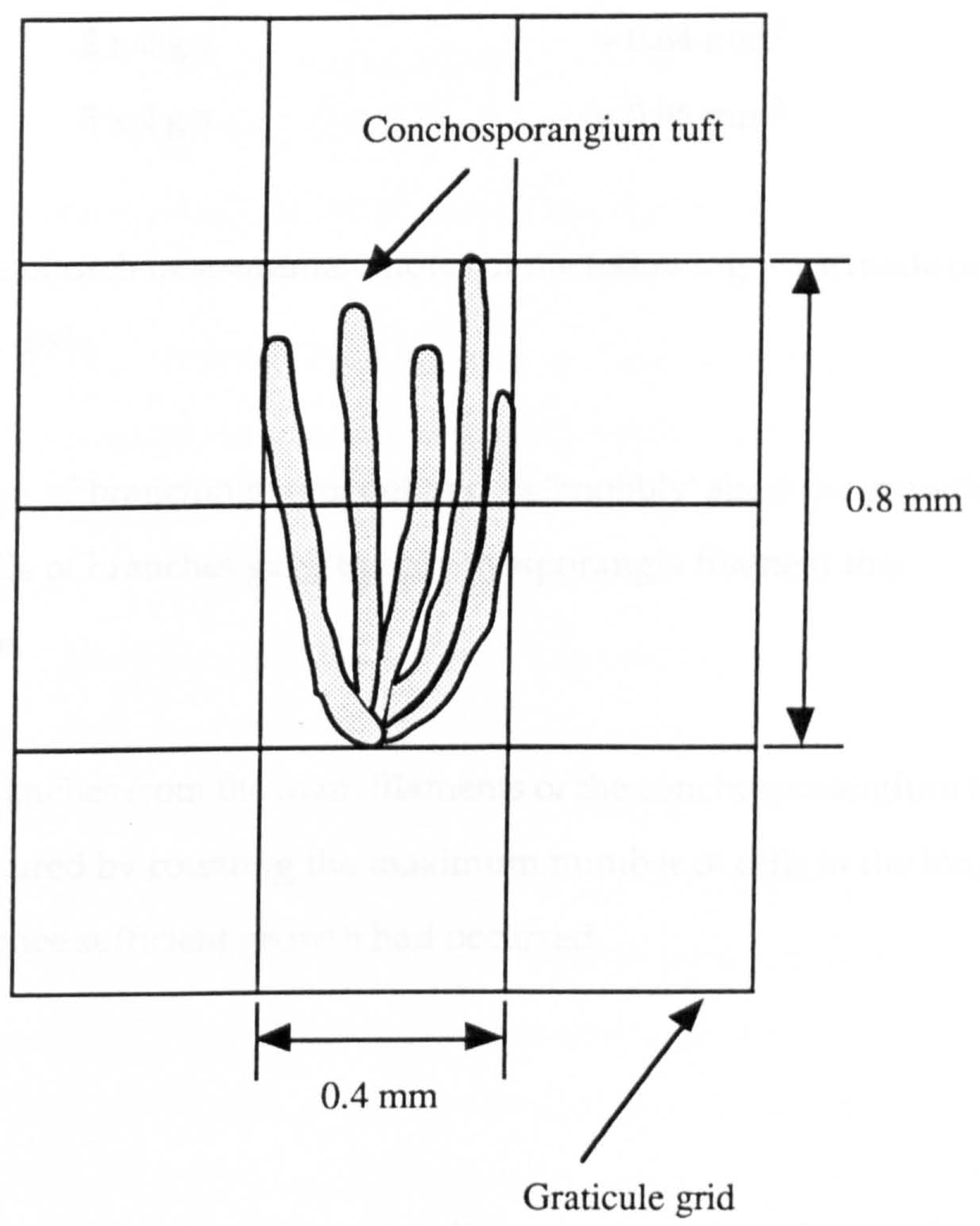


Fig. 4.1 Appearance of the conchosporangia tufts used in the cross gradient experiment. The tufts were measured using an eye piece grid graticule. All tufts selected for inoculation had the same overall length and width and most had five branches.

1 x 1 graticule squares (g.s.)	= 0.16 mm^2
2 x 1 g.s.	= 0.32 mm^2
3 x 1 g.s.	= 0.48 mm^2
2 x 2 g.s.	= 0.64 mm^2
3 x 2 g.s.	= 0.96 mm^2

At the time of each measurement notes of the following were made on the material in each dish:

Initial stages of branching were defined as 'knobbly' since the growths of the first cells of branches gave the conchosporangia filament this appearance.

The side branches from the main filaments of the conchosporangium tufts were measured by counting the maximum number of cells in the longest branches once sufficient growth had occurred.

Shell culture

The conchocelis of *P. laciniata* and *P. linearis* were cultured in shells and grown under various conditions in an attempt to induce conchosporangium growth and then conchospore production.

The seawater used to make up the culture medium was held in dark storage for 1 month prior to its use, to reduce fouling problems.

Shell inoculation

Conchocelis material was grown using the techniques described previously. A quantity of conchocelis filaments from the stock cultures was

selected and homogenised in a liquidizer for 30 seconds to produce a filament length of approximately 200 μm. These filament fragments were placed in seawater medium to await inoculation.

Shells of the king scallop *Pecten maximus* (L.) and the cultured oyster *Crassostrea gigas* Thunberg were collected and scrubbed clean. The shells were placed in sterile seawater for 2 weeks then scrubbed clean again and autoclaved (15 bar, 15 minutes).

Whole shells were placed in large flat culture dishes with the inner surface uppermost and covered with sterile seawater medium to a depth of 2 - 3 cm above the shells. Replicate dishes were then placed in culture chambers at 13 ± 1 °C with a photon irradiance of approximately 60 μmol $m^{-2}s^{-1}$ in a 16:8 and 8:16 photoperiod. The homogenised conchocelis filaments were pipetted evenly onto the water surface and thus onto the shell surface. The culture tanks were covered with a black muslin screen to reduce light to 5 - 10 μmol $m^{-2}s^{-1}$ for the first three days of culture to discourage filaments from growing towards the light and thus encourage penetration into the shells. Also if cultures are left in high light the production of small oxygen bubbles around the photosynthesising fragments causes them to float to the surface (Melvin *et al.*, 1986). After this period the screens were removed and cultures returned to the standard conditions. After the shells had been penetrated by conchocelis (2 - 3 weeks) they could then be cleaned using an artist's paint brush. Once clean the shells were returned to the culture conditions in fresh culture medium and clean dishes. If fouling was a problem the shells were cleaned in fresh water for up to 30 minutes and also a 1 % Betadine solution could be used for cleaning (for 3 minutes) with a further rinsing in seawater.

As whole shells were space consuming fragments of shells were also used and inoculated in the same way. This also facilitated transfer of replicates to various culture conditions.

After the shells and fragments were inoculated and conchocelis had penetrated the shells and shown good growth (large areas of pink coloured shell, after approximately 3 - 4 months) the culture conditions were altered from 16:8 to 8:16 and *visa versa*. The dishes were transferred to whichever condition yielded the best conchosporangium growth for further conchospore induction experiments.

Conchospore induction

The shells where conchosporangium growth occurred were kept in these conditions until adequate growth of conchosporangia had occurred. The shells and fragments were cut into approximately 1 cm^2 fragments to produce the large number of fragments necessary for the conchospore induction experiments. After the cut fragments had been maintained in the original culture conditions for 1 week after cutting, to check for damage, the culture conditions were altered to try to induce conchospore production and release.

RESULTS

Culture techniques

Initial culture procedures did not remove all contaminants and in trial experiments growth of green algae was a particular problem. Washing the thallus material carefully and selecting the areas of the thallus with the least epiphytes before collection of carpospores helped to reduce later contamination problems. Young thalli had less epiphyte development and so were selected in preference to older individuals.

The development of a clean and reliable seawater medium also proved to be important since many cultures in initial experiments were lost due to the introduction of contaminants from the medium. Filtered (2 µm) seawater stored in the dark for at least 1 month and then 0.2 µm filtered proved to be a highly successful contaminant free medium of which large quantities could be easily produced.

Porphyra thalli were able to tolerate many washing conditions e.g. freshwater washing and washing with a Betadine solution. These cleaning techniques were also useful in helping to reduce contamination problems.

Carpospore release

Carpospores were readily produced from fertile thalli of both *P. linearis* and *P. laciniata* particularly after overnight storage in the refrigerator.

The spore cleaning technique (Birnie & Boney, 1980) was particularly useful for the production of high concentration spore suspensions used for inoculation of large numbers of culture dishes. This technique also removed diatoms and other debris (which formed a layer below the spores) and also separated other spores from the suspensions.

Germination of the carpospores of both species occurred in both 8:16 and 16:8 photoperiods but the resultant growth of the conchocelis was greater in long day conditions (16:8).

Stock cultures of conchocelis were maintained in both circulating and stationary seawater medium. Growth was faster in the flowing conditions and appeared to be less susceptible to contamination, particularly when the medium and glassware were regularly changed, and some culture stocks were maintained in this way for up to 3 years.

Photoperiod experiments

Two major disasters were experienced during the running of these experiments. Firstly, after sufficient stock cultures had been set up and the photoperiod dishes inoculated, contamination with a green alga and an *Ectocarpus* species appeared at about 30 days in the photoperiod experiment. Consequently the material had to be discarded and the experiment terminated.

A repeat experiment was then set up with the addition of: improved cleaning of thalli and spore preparations and the production of contaminant free seawater medium. This second experiment was progressing successfully with no contamination and good growth when at 50 days a cooling unit overheated and the temperature in the culture chambers rose to 40 °C killing all the cultures. The third attempt was successful for the full duration of the experiment.

The graphs of the growth of *P. linearis* (Fig. 4.2) show how the log of the dry weight of the conchocelis increased with time. Growth was initially faster from the inoculation to the first recorded weight at 32 days. In the continuous, 16:8 and 12:12 photoperiods the growth appeared to follow a similar pattern. The rate of growth was fairly constant from 32 days onwards until after the measurement at 71 days when the growth rate reduced slightly. The growth rate at the short day

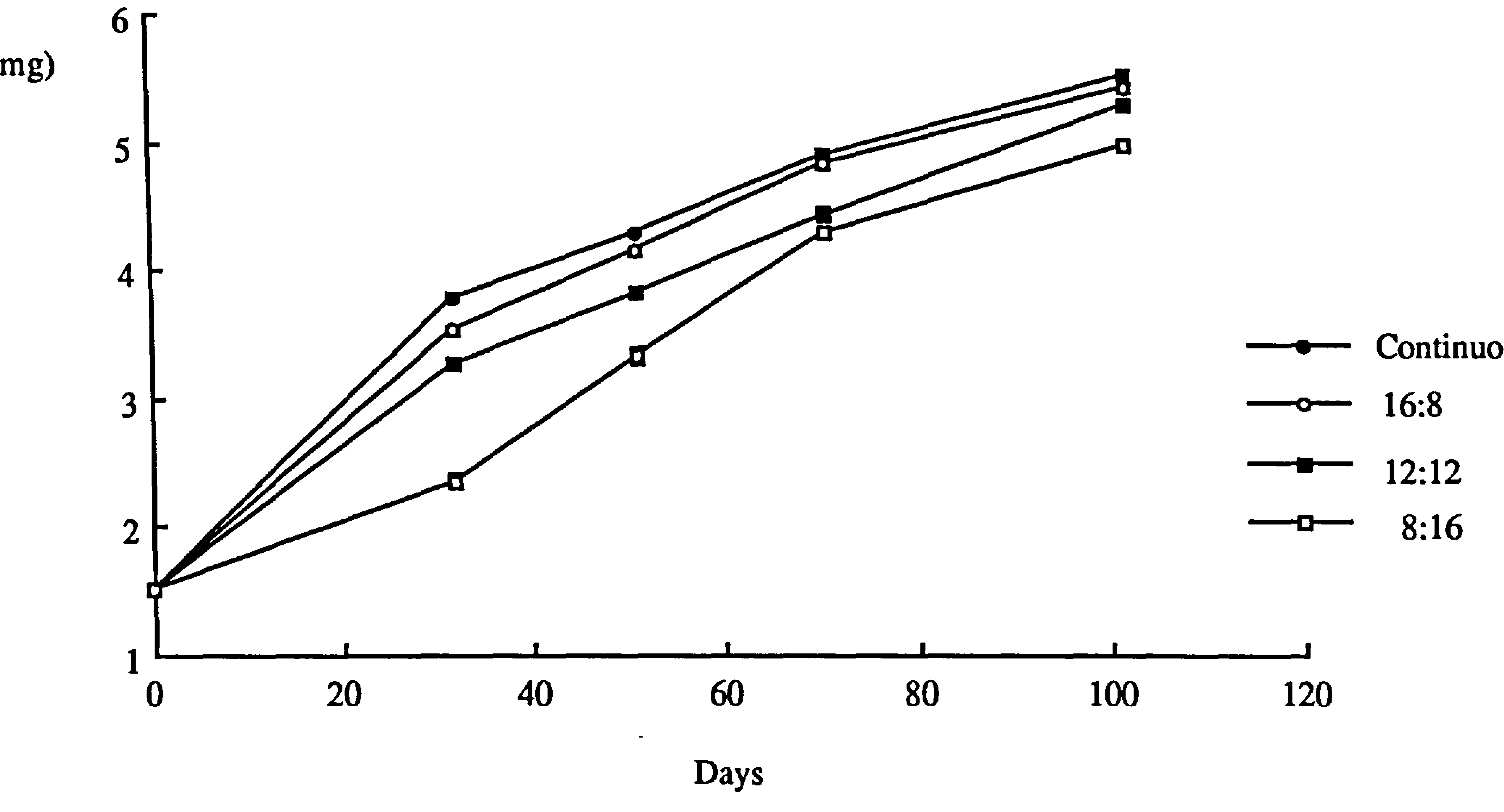


Fig. 4.2 Log dry weight (mg) of the conchocelis of *P. linearis* grown at four different photoperiods
(Error bars are one standard error of the mean)

photoperiod (8:16) was initially slower than at the other photoperiods but increased after the measurement at 32 days. After the 71 day measurement the growth rate reduced as with the other photoperiods.

The greatest biomass of conchocelis occurred in the cultures subjected to continuous light and decreased in turn in the 16:8, 12:12 and 8:16 cultures. This trend in biomass follows the trend in total quanta of light available.

Fig 4.3 shows results for growth of conchocelis of *P. laciniata* conchocelis. Growth was initially fast in all photoperiod conditions with a reduction occurring after the first measurement at 8 days. The growth rate was fairly stable from this point for the rest of the experiment. At the short day photoperiod (8:16) the growth rate appeared to reduce slightly after the 41 day measurement. At the final measurement (70 days) the greatest mean biomass was recorded in continuous light conditions with a decreasing biomass as the photoperiod decreased to short day conditions (8:16). The final biomass recorded in the short day conditions (0.0790 g) was noticeably lower than at the other three light periods.

When the filaments of conchocelis had covered the base of the culture dishes the filament density increased further and on examination it appeared that filaments grew more often in the horizontal plane than vertically even when the covering density in the dish was high.

The number of conchosporangia recorded in cultures of *P. linearis* (Fig. 4.4) was relatively low when first measured after 30 days and material was found in all photoperiod conditions except for the short day. After 51 days more conchosporangium material was evident, occurring in all the photoperiod conditions. After 71 days the amount of conchosporangium material had increased particularly in short day conditions. At 102 days the number of fragments recorded had increased dramatically in all photoperiods.

During these observations it was noted that as well as characteristic conchosporangium growth the conchocelis filaments had areas of thickened

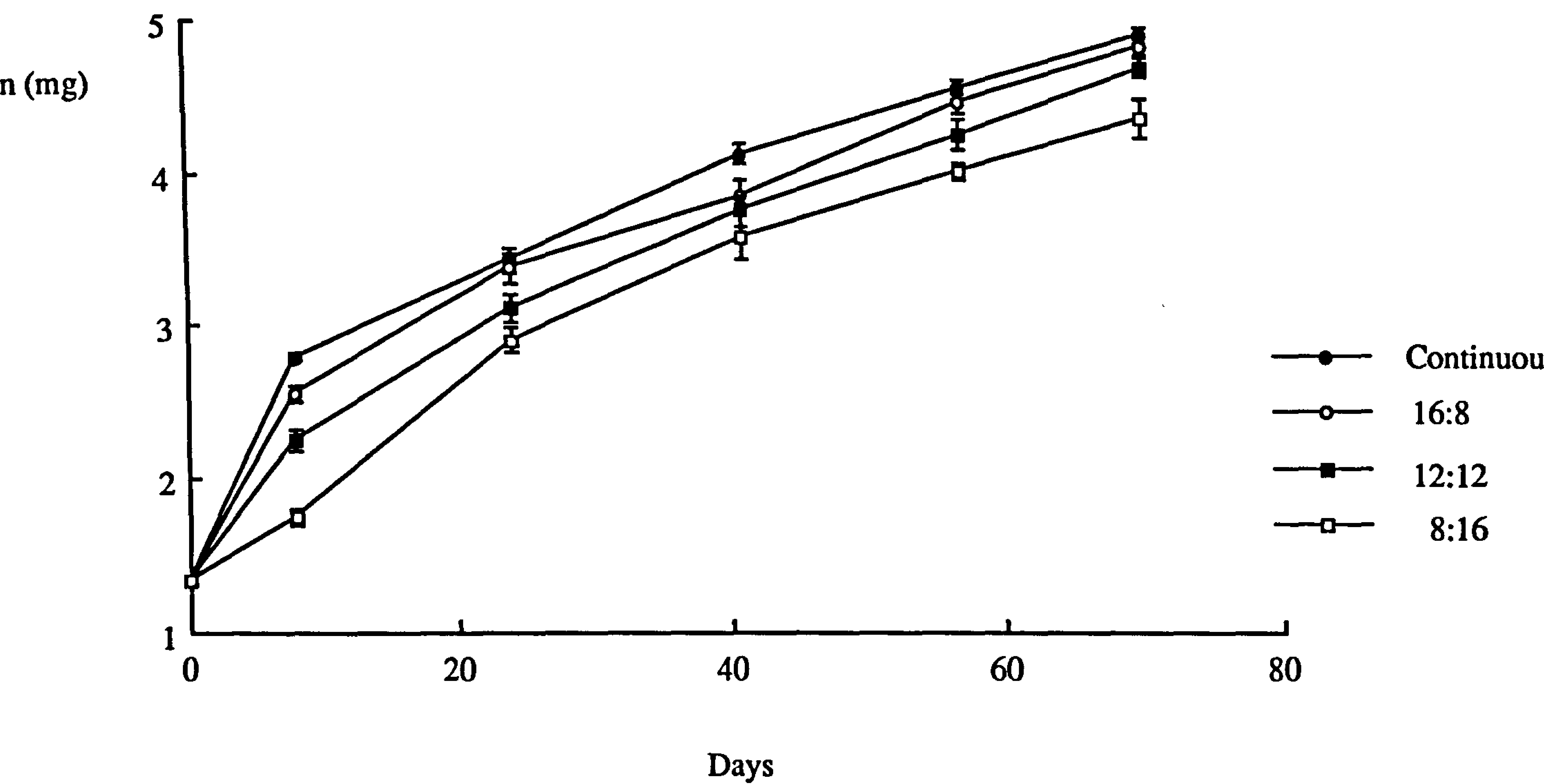


Fig. 4.3 Log dry weight (mg) of the conchocelis of *P. laciniata* grown at four different photoperiods (Error bars are one standard error of the mean)

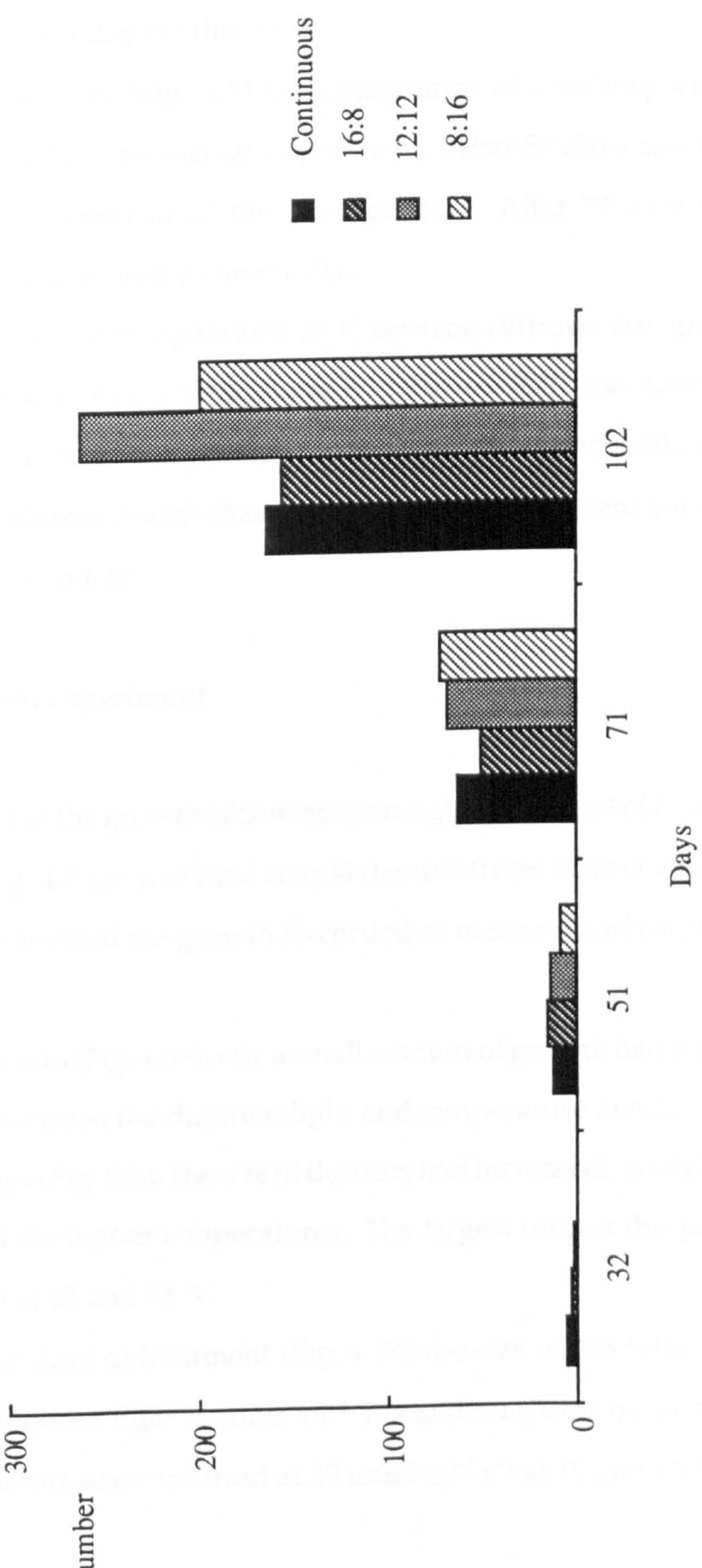


Fig 4.4 Number of conchosporangia of *P. linearis* in a 20 drop sample taken from cultures grown at different photoperiods

filaments with characteristic constrictions. These thickened filaments appeared prior to conchosporangium growth and were present throughout the experiment. Once apparent they did not alter in shape and were noticeably abundant in short day conditions.

For *P. laciniata* (Fig. 4.5) the appearance of conchosporangia occurred approximately 20 days into the experiment. After 57 days conchosporangium growth was apparent in all the photoperiods. After 70 days the number of fragments had increased dramatically.

Growth of conchosporangia in *P. laciniata* cultures was greater than that seen in *P. linearis*. In other preliminary experiments it was also noted that this species produced conchosporangia in free culture more readily than *P. linearis*. The swollen filaments with characteristic constrictions were not apparent in the cultures of *P. laciniata*.

Cross gradient experiment

The plot of the growth of conchosporangium material of *P. laciniata* (Fig. 4.6 a, b, c, see Fig. 4.7 for standard errors) demonstrates how changes in light and temperature affected the growth (recorded as mean area of conchosporangium tufts).

After 3 days (Fig. 4.6a) only a small amount of growth had taken place small differences between the different light and temperature levels.

At 8 days (Fig. 4.6a) the size of the tufts had increased. A slight increase was noticeable at the higher temperatures. The largest tufts at this point were in 30 μmol m^{-2} s^{-1} at 12 and 15 °C.

After 15 days of treatment (Fig. 4.6b) the size of the tufts was noticeably lower at the lowest light (4 μmol m^{-2} s^{-1}) and temperature (6 °C) levels. The largest mean tuft sizes occurred at 30 μmol m^{-2} s^{-1} at 12 and 13 °C.

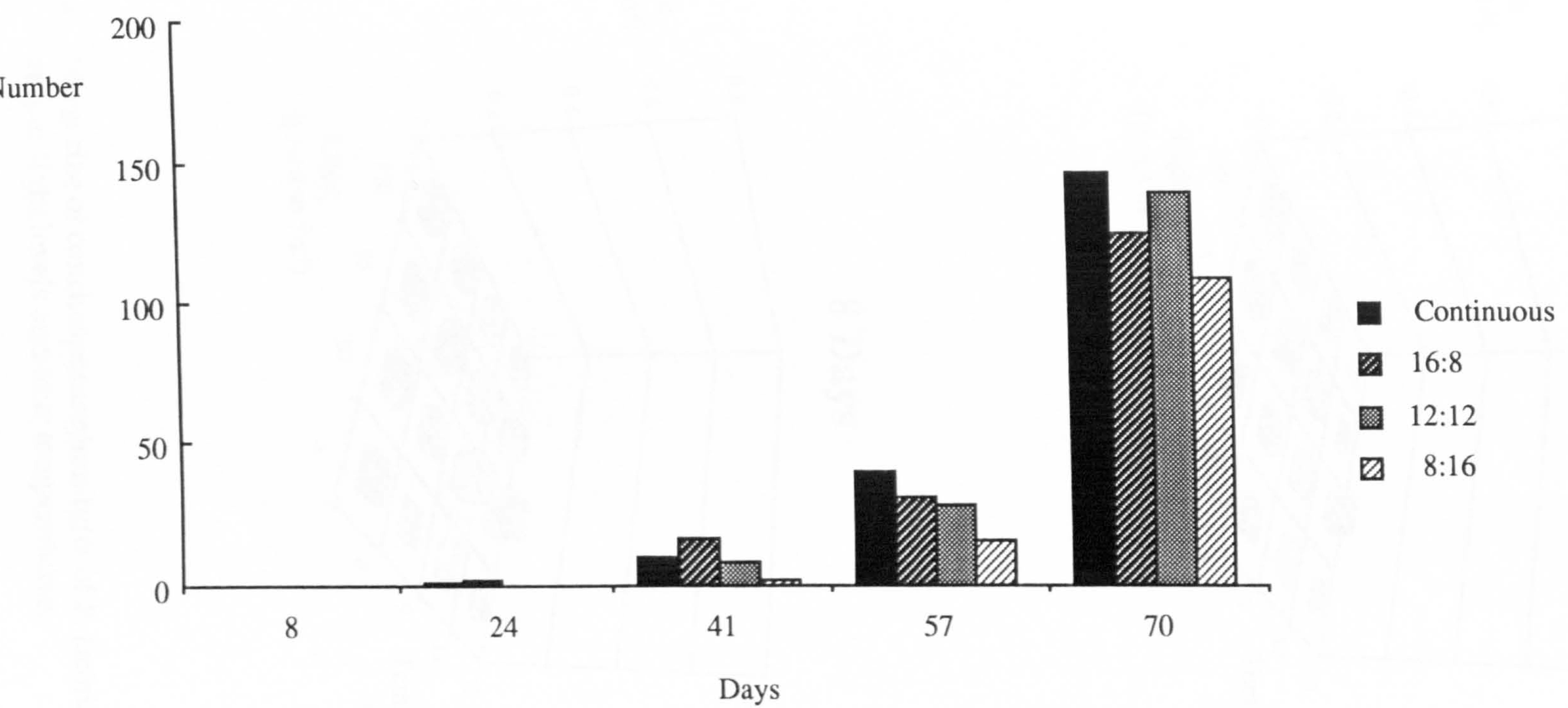
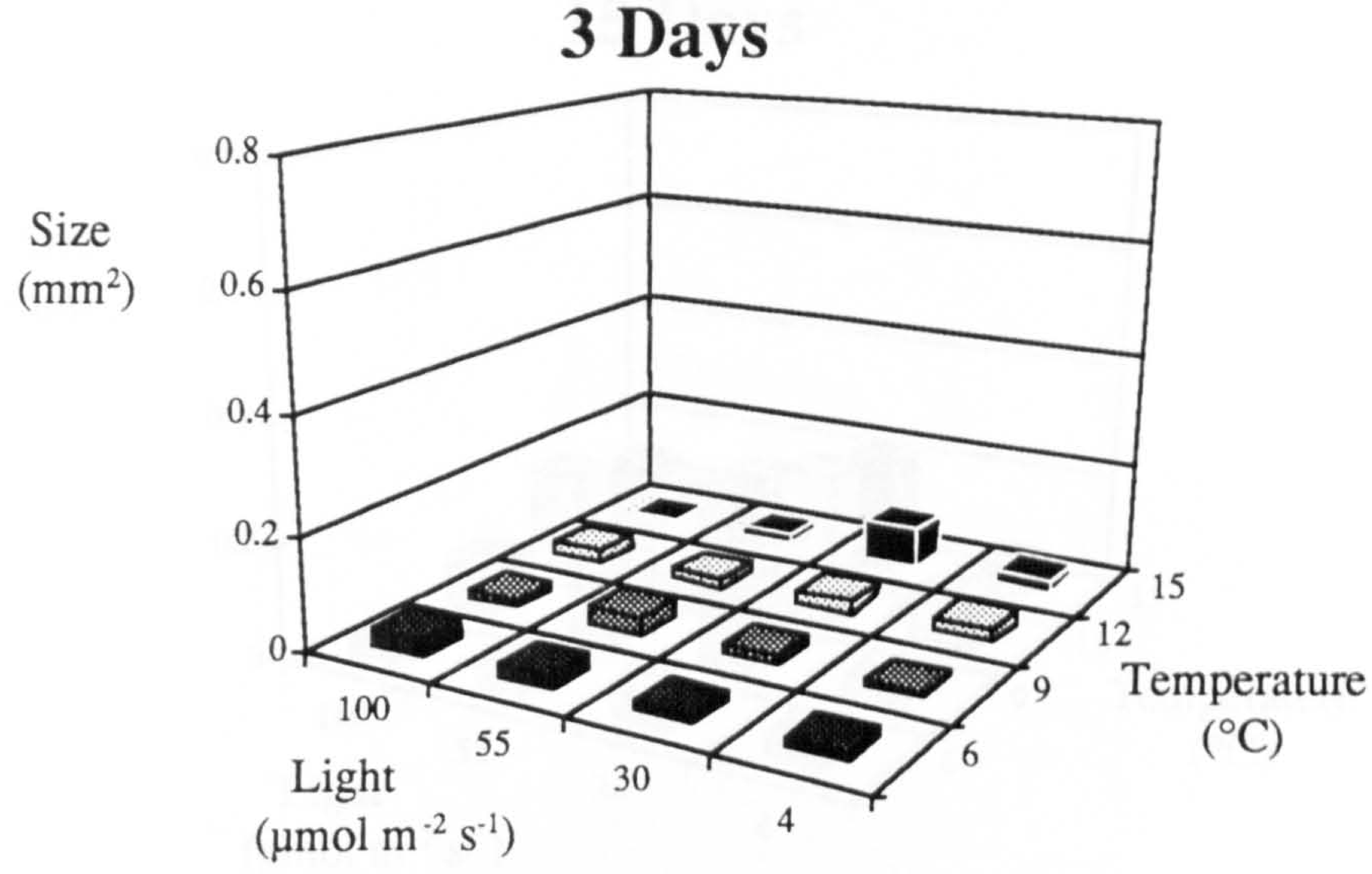


Fig 4.5 Number of conchosporangia of *P. laciniata* in 20 drop samples taken from cultures grown at different photoperiods



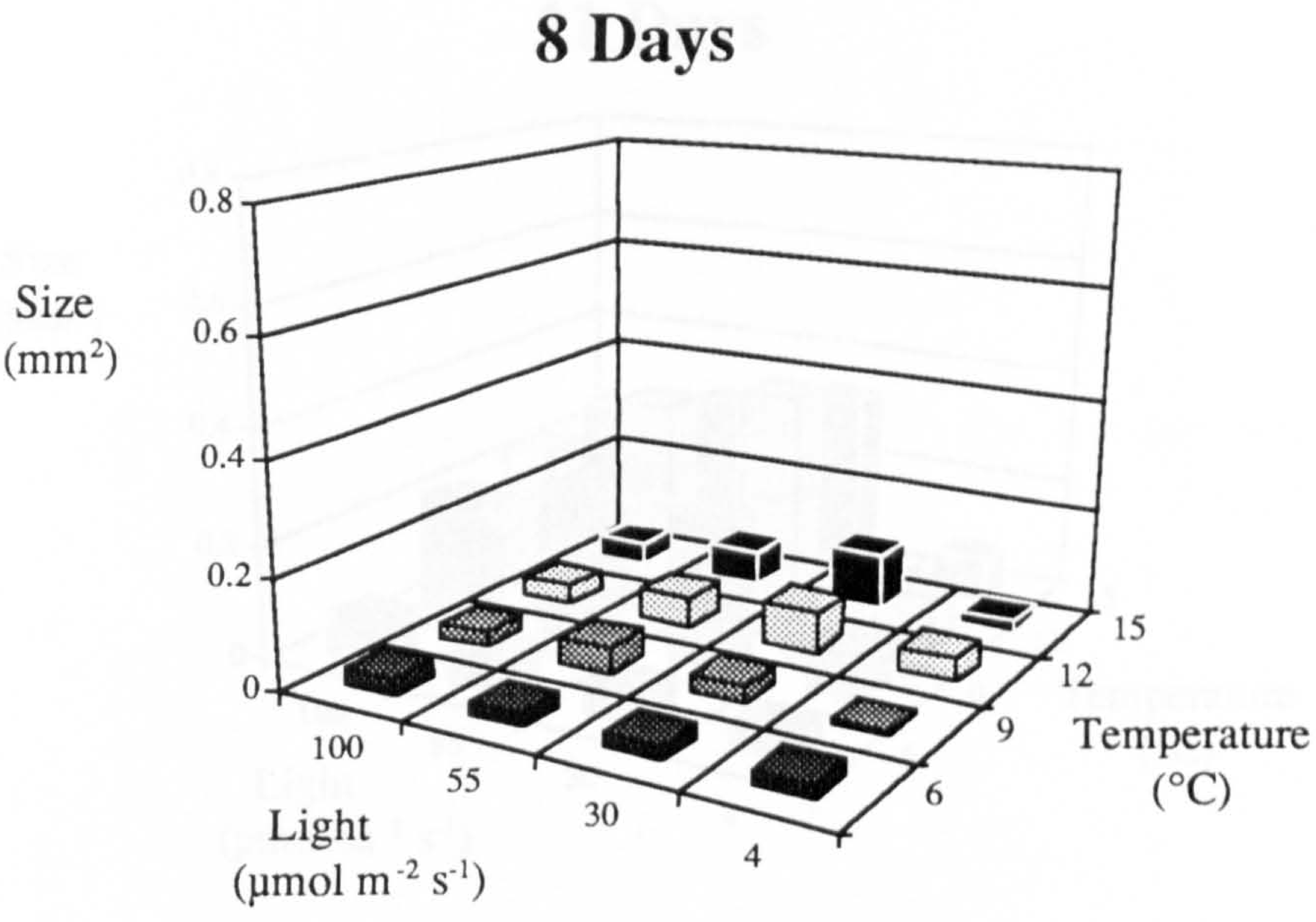
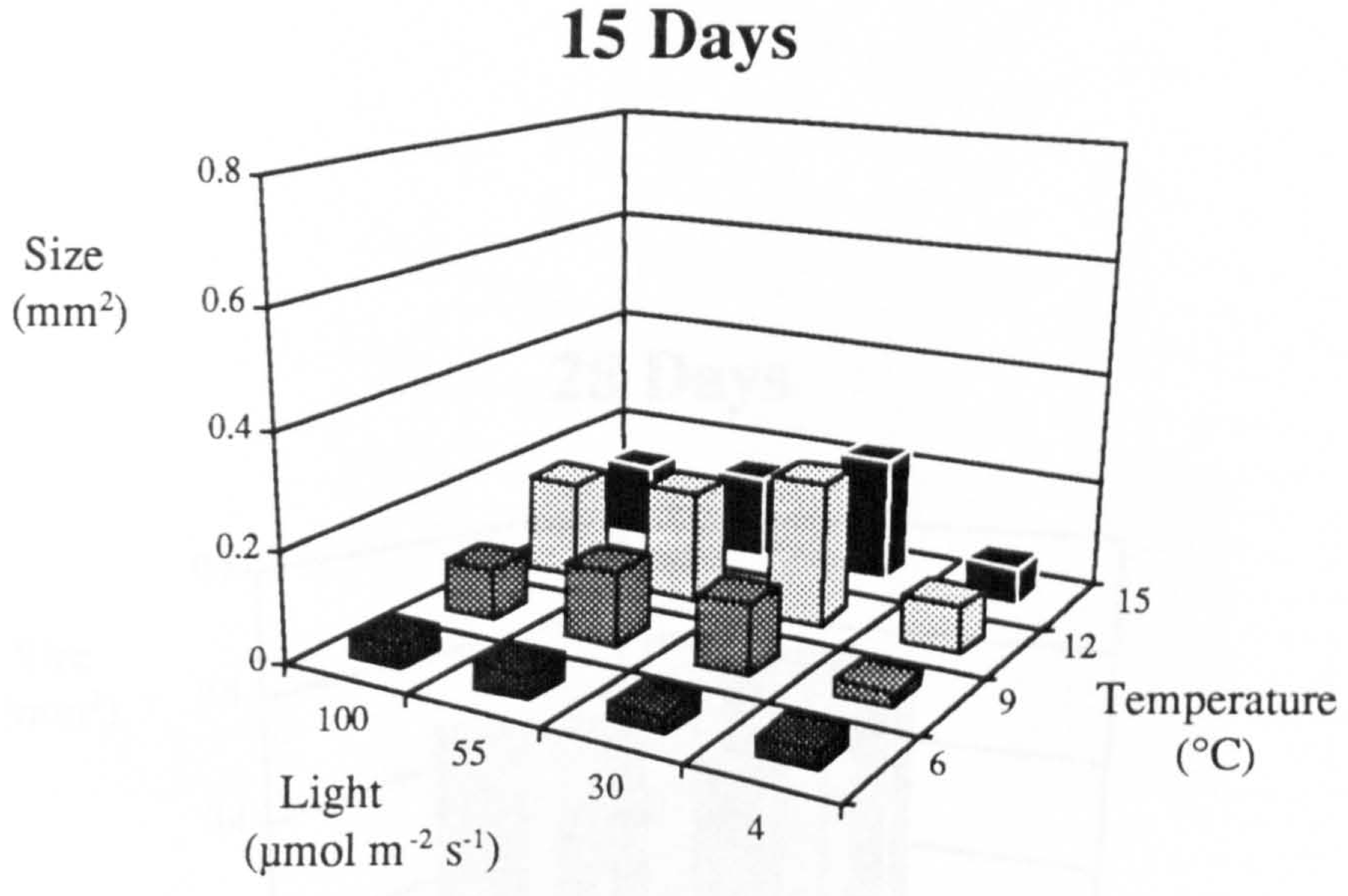


Fig 4.6a Mean size of conchosporangium tufts of *P. laciniata* at four light levels and four temperatures.



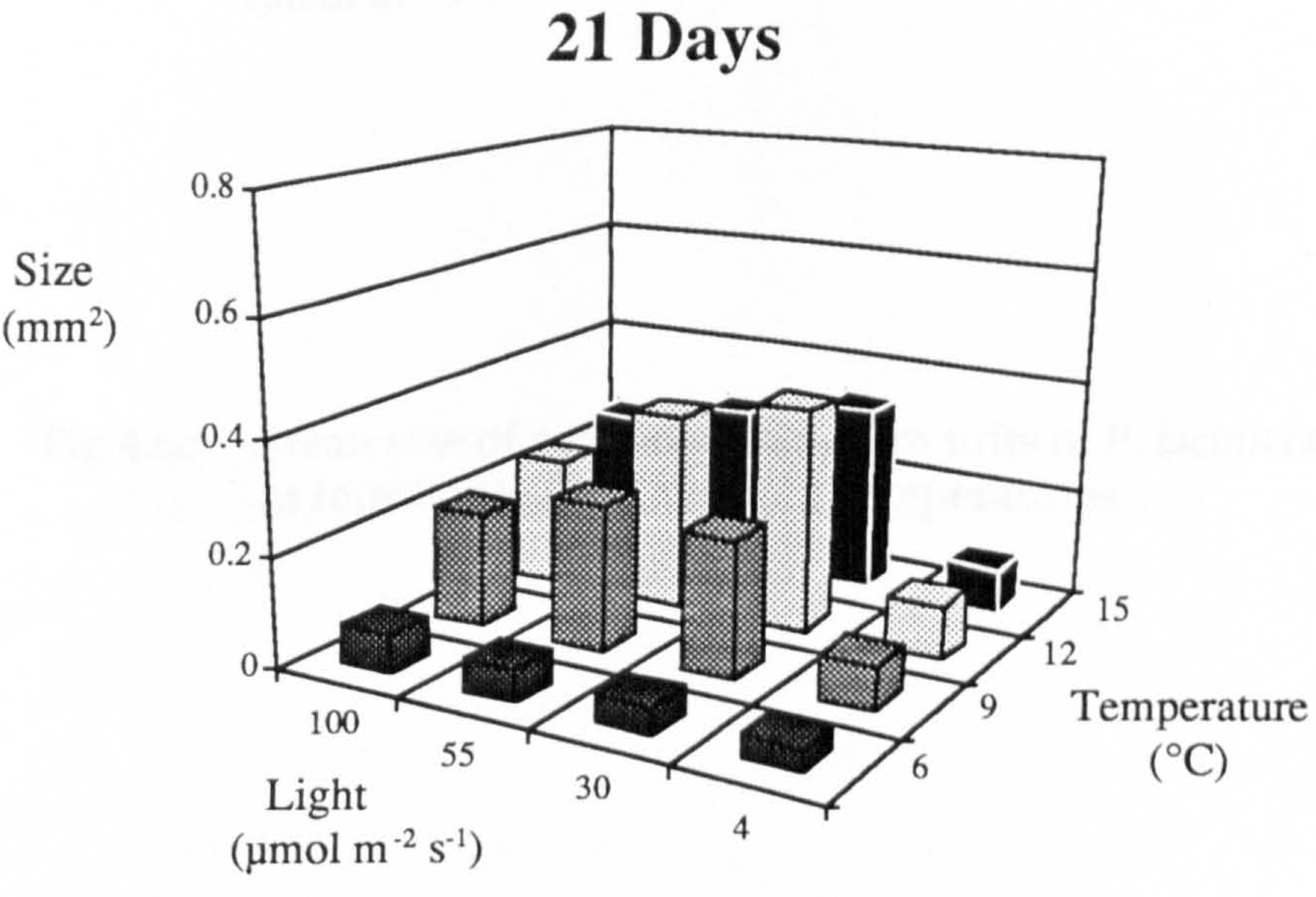


Fig 4.6b Mean size of conchosporangium tufts of *P. laciniata* at four light levels and four temperatures.

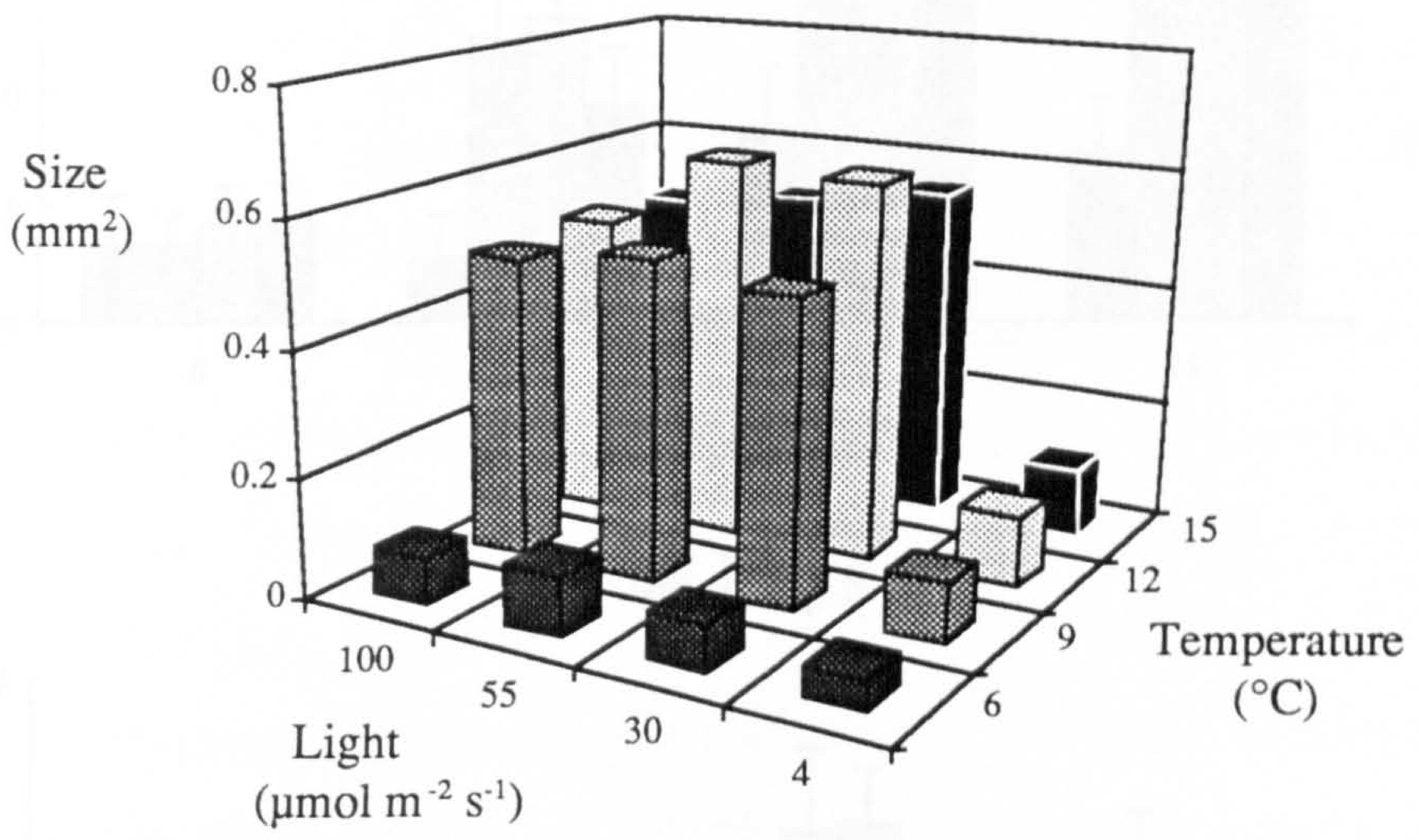
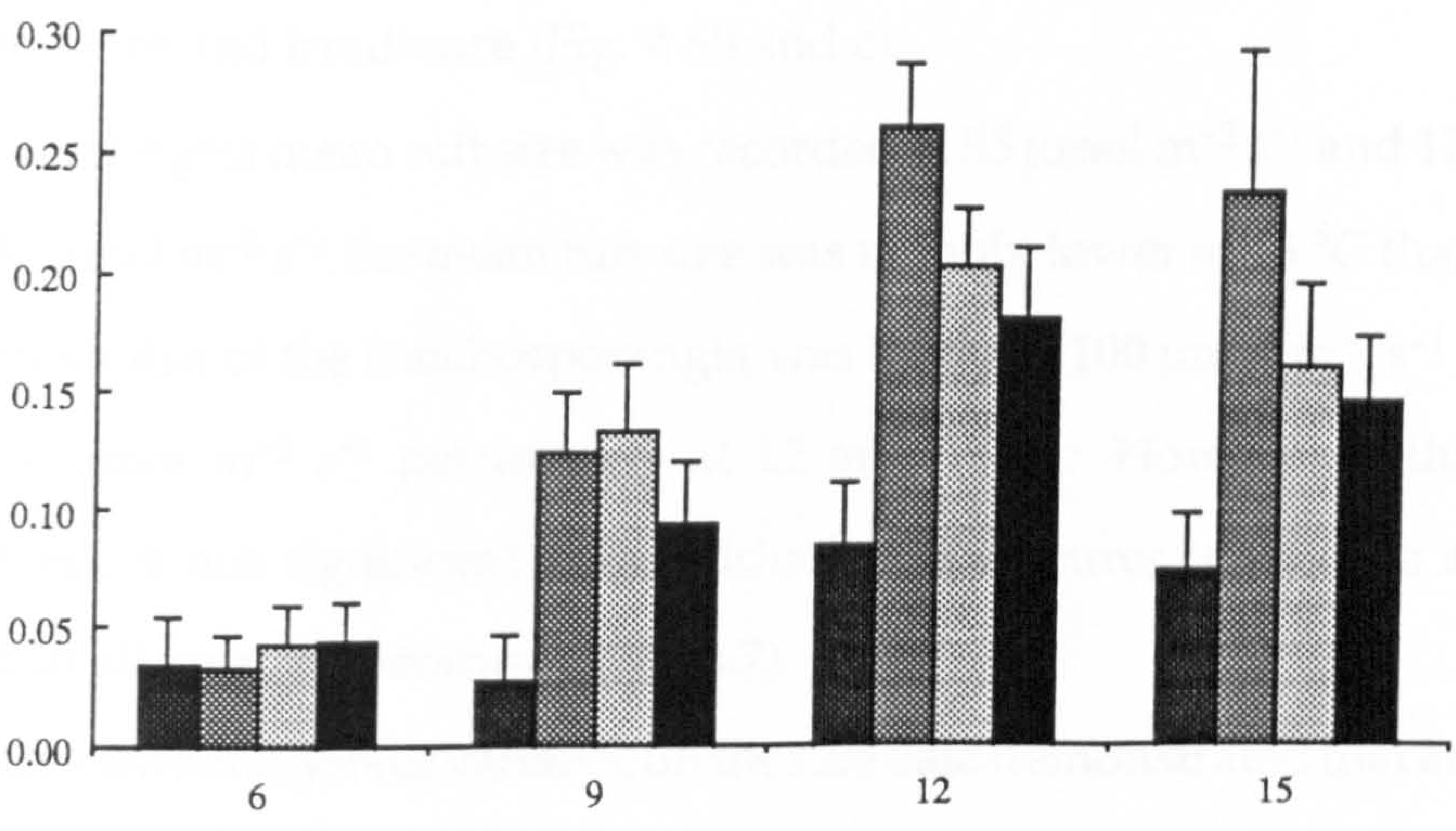


Fig 4.6c Mean size of conchosporangium tufts of *P. laciniata* at four light levels and four temperatures.



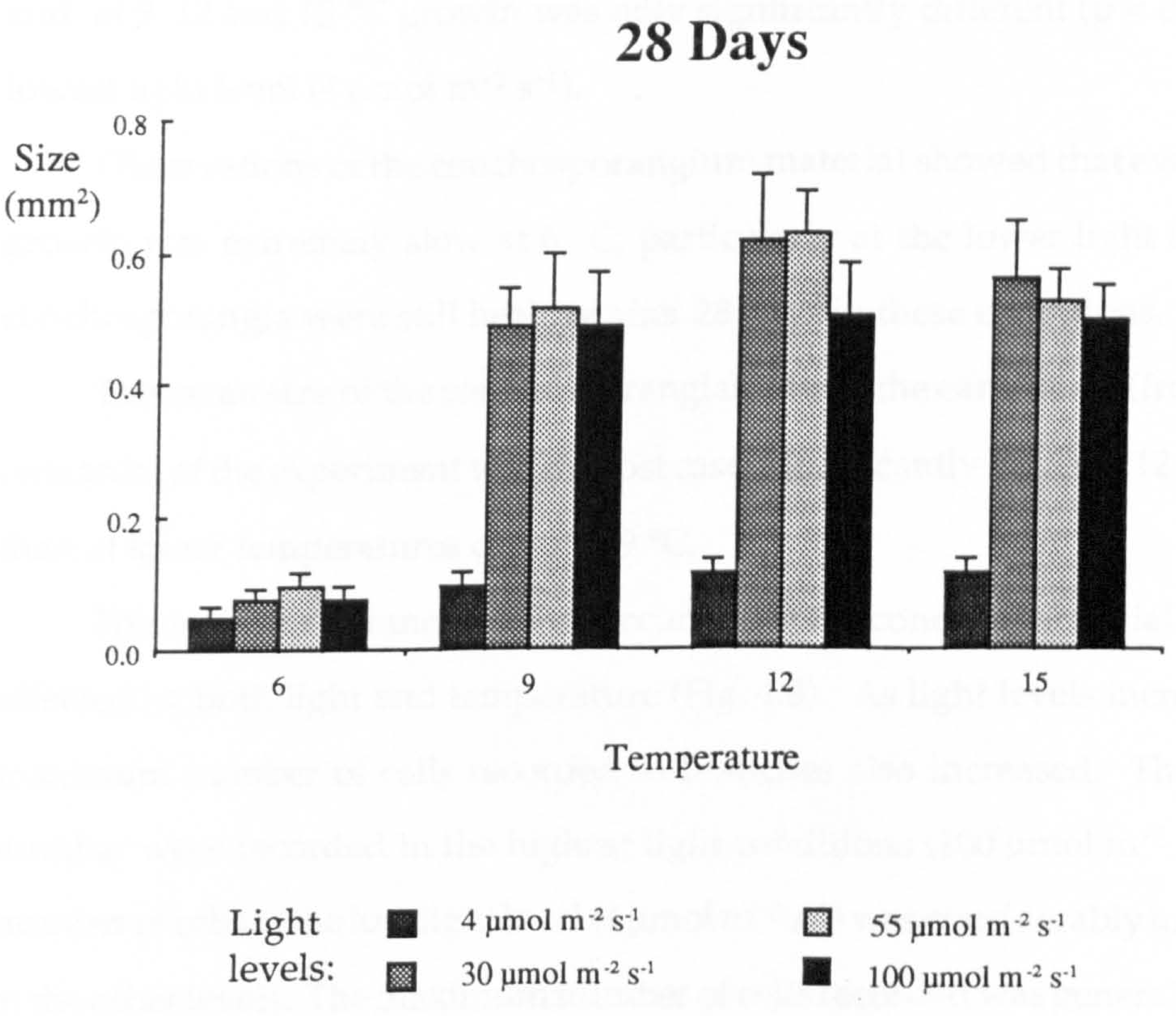


Fig. 4.7 Showing one standard error of mean sizes of conchosporangium tufts at four light levels and four temperatures.

There was no change in the relative sizes in the different conditions at 21 and 28 days, though growth had clearly continued in all except the lowest temperature and irradiance (Fig. 4.6b and c).

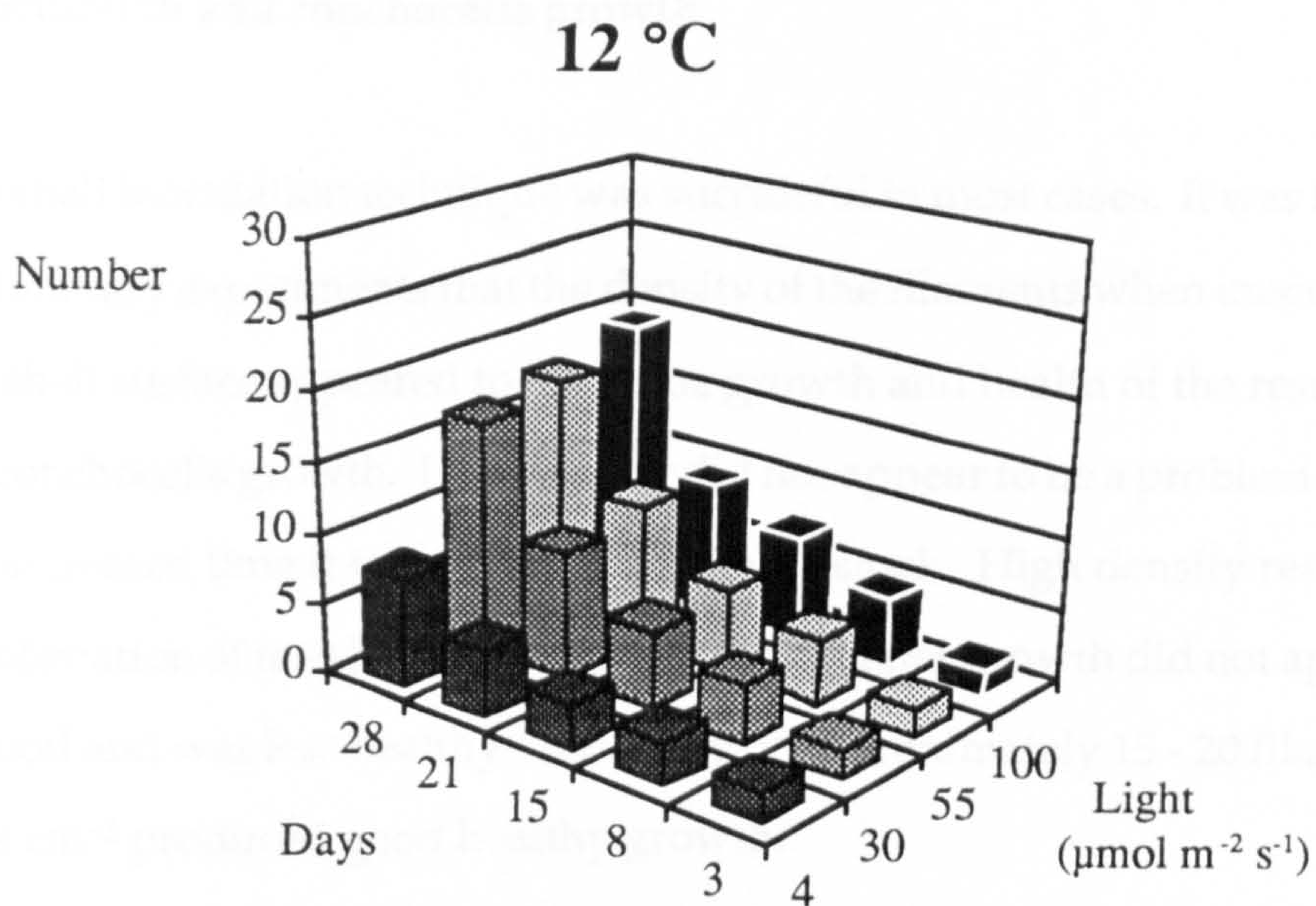
The largest mean tuft size was recorded at 55 µmol m^{-2} s^{-1} and 12 °C. At 55 and 30 µmol m^{-2} s^{-1} the mean tuft size was notably lower at 15 °C than at 12 °C. The mean size of the conchosporangia was lower at 100 µmol m^{-2} s^{-1} than at 55 and 30 µmol m^{-2} s^{-1} particularly at 12 and 15°C. However, although this difference is not significant at individual temperatures it was the same way round at all four temperatures (Fig. 4.7).

Oneway analysis of variance on the size data demonstrated that at 6°C there was no significant difference ($p < 0.05$) in growth at all the different light levels and at 9, 12 and 15 °C growth was only significantly different ($p < 0.05$) at the lowest light level (4 µmol m^{-2} s^{-1}).

Observations of the conchosporangium material showed that even though growth was extremely slow at 6 °C, particularly at the lower light levels, the conchosporangia were still healthy after 28 days in these conditions.

The mean size of the conchosporangial tufts at the early stage (from 8 days onwards) of the experiment was in most cases significantly larger at 12 and 15 °C than at lower temperatures of 6 and 9 °C.

The amount of branching that occurred on the conchosporangial tufts was affected by both light and temperature (Fig. 4.8). As light levels increased the maximum number of cells recorded in branches also increased. The highest number were recorded in the highest light conditions (100 µmol m^{-2} s^{-1}). The number of cells at the low light level (4 µmol m^{-2} s^{-1}) was considerably lower than at the other levels. The maximum number of cells recorded was generally higher at 15 °C than at 12 °C for a given time and light level. Some branching growth may not have been observed using the area measurements but since branching was recorded as the number of cells in the longest branches this data should still give some indication of the degree of branching.



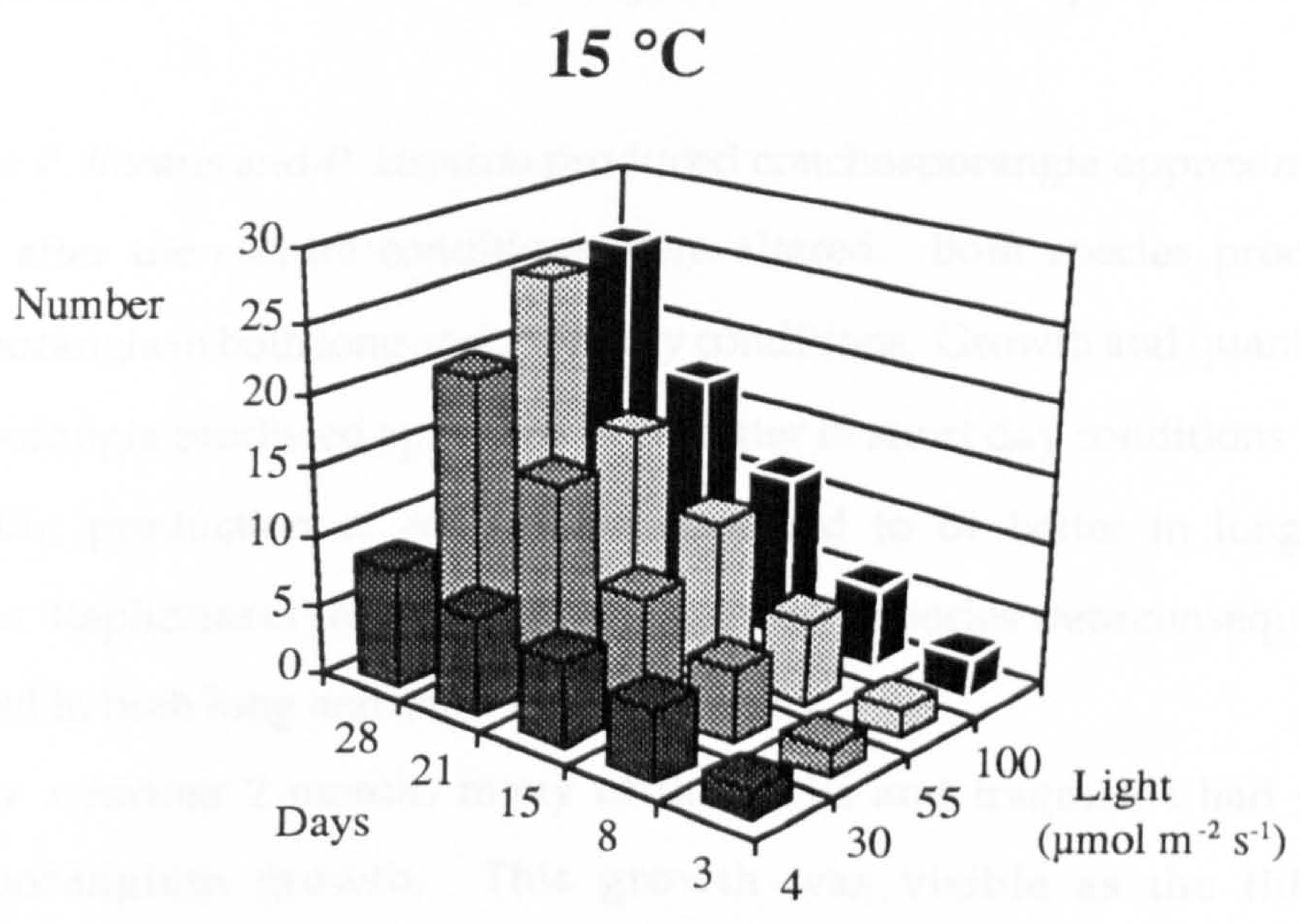


Fig. 4.8 The maximum number of cells in the side branches of the conchosporangium tufts of *P. laciniata* at 12 and 15 C.

Shell inoculation and conchocelis growth

The shell inoculation technique was successful in most cases. It was found from preliminary experiments that the density of the filaments when inoculated onto the shell surface appeared to affect the growth and health of the resulting 'in shell' conchocelis growth. Low density did not appear to be a problem apart from the increased time it took to grow a suitable stock. High density resulted in rapid infestation of the shell with conchocelis but later growth did not appear to be as good and was less healthy. A density of approximately 15 - 20 filament fragments cm^{-2} produced good healthy growth.

The conchocelis was easily visible growing in the shells after approximately 1 month as small (2 - 3 mm diameter) pink patches. The colour of the patches helped in the assessment of the health of cultures. After 3 - 4 months the shells and fragments were almost completely covered with the pink conchocelis growth.

Both *P. linearis* and *P. laciniata* produced conchosporangia approximately 1 month after the culture conditions were altered. Both species produced conchosporangia in both long and short day conditions. Growth and quantity of conchosporangia produced appeared to be better in short day conditions for *P. laciniata* but production of conchocelis appeared to be better in long day conditions. Replicates of shells and fragments of each species were consequently maintained in both long and short day conditions.

After a further 2 months many of the shells and fragments had good conchosporangium growth. This growth was visible as the thicker conchosporangium filaments emerged from the shell surface (Plate 4.2a, b) to form a mat covering extensive areas of the shell. It was noted in some back up cultures where light levels were lower (20 - 25 $\mu mol\ m^{-2}\ s^{-1}$) that conchosporangium filaments all grew in the same direction, this being towards areas with higher light levels.

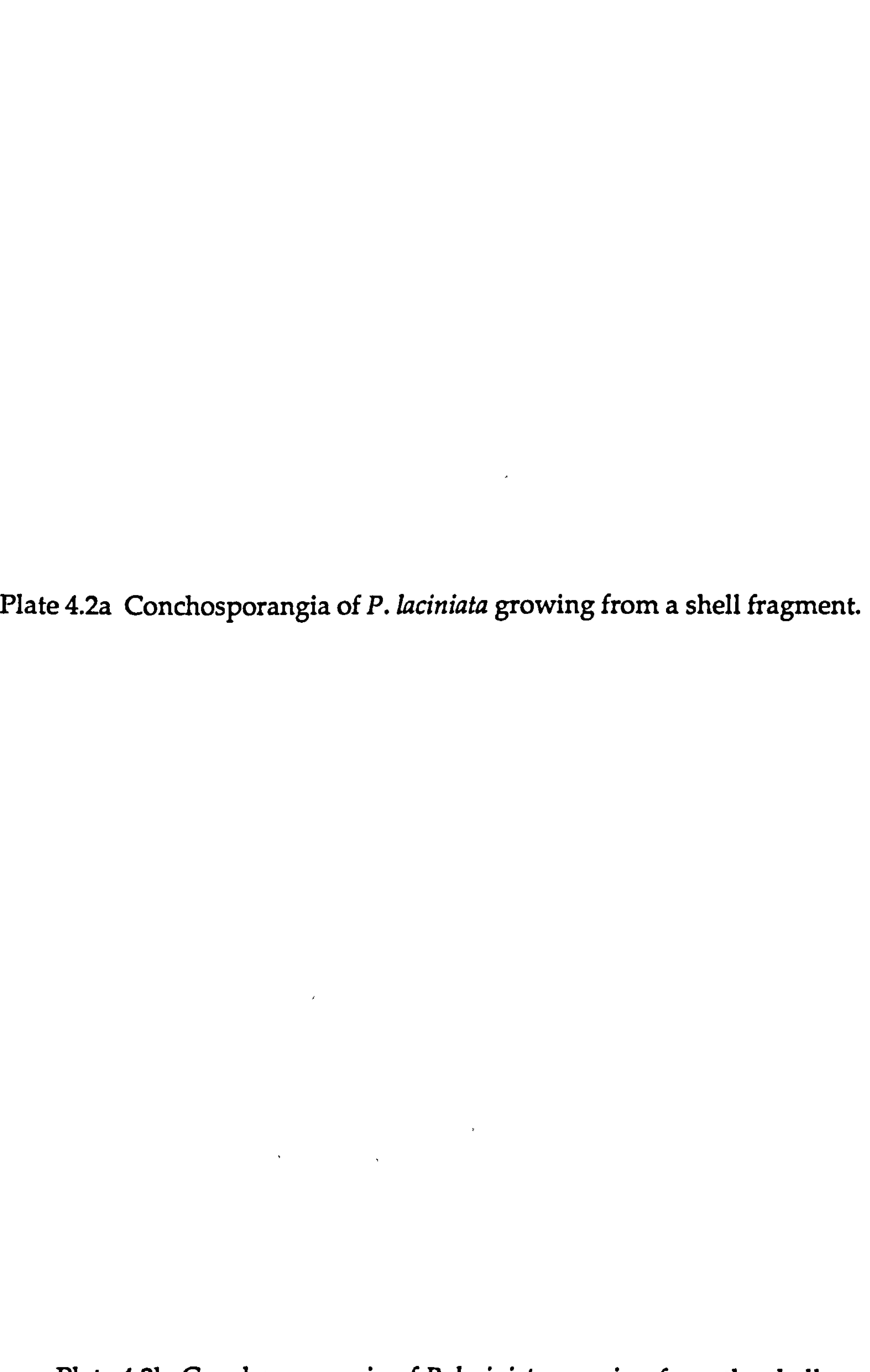


Plate 4.2a Conchosporangia of *P. laciniata* growing from a shell fragment.

Plate 4.2b Conchosporangia of *P. laciniata* growing from the shell.
(Tufts approx. 1 - 1.5 mm height)

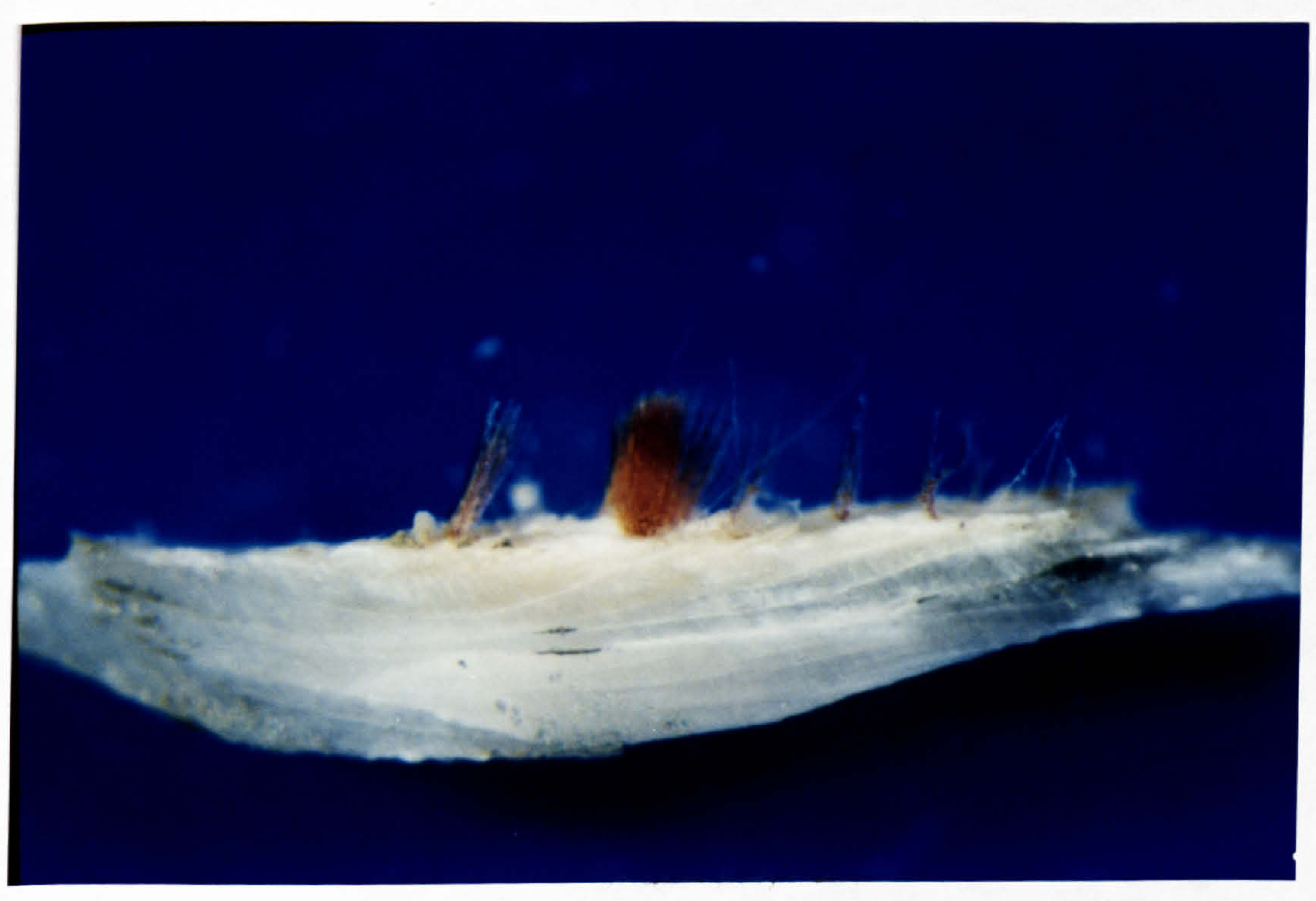


Plate 4.2a Conchosporangia of *P. laciniata* growing from a shell fragment.

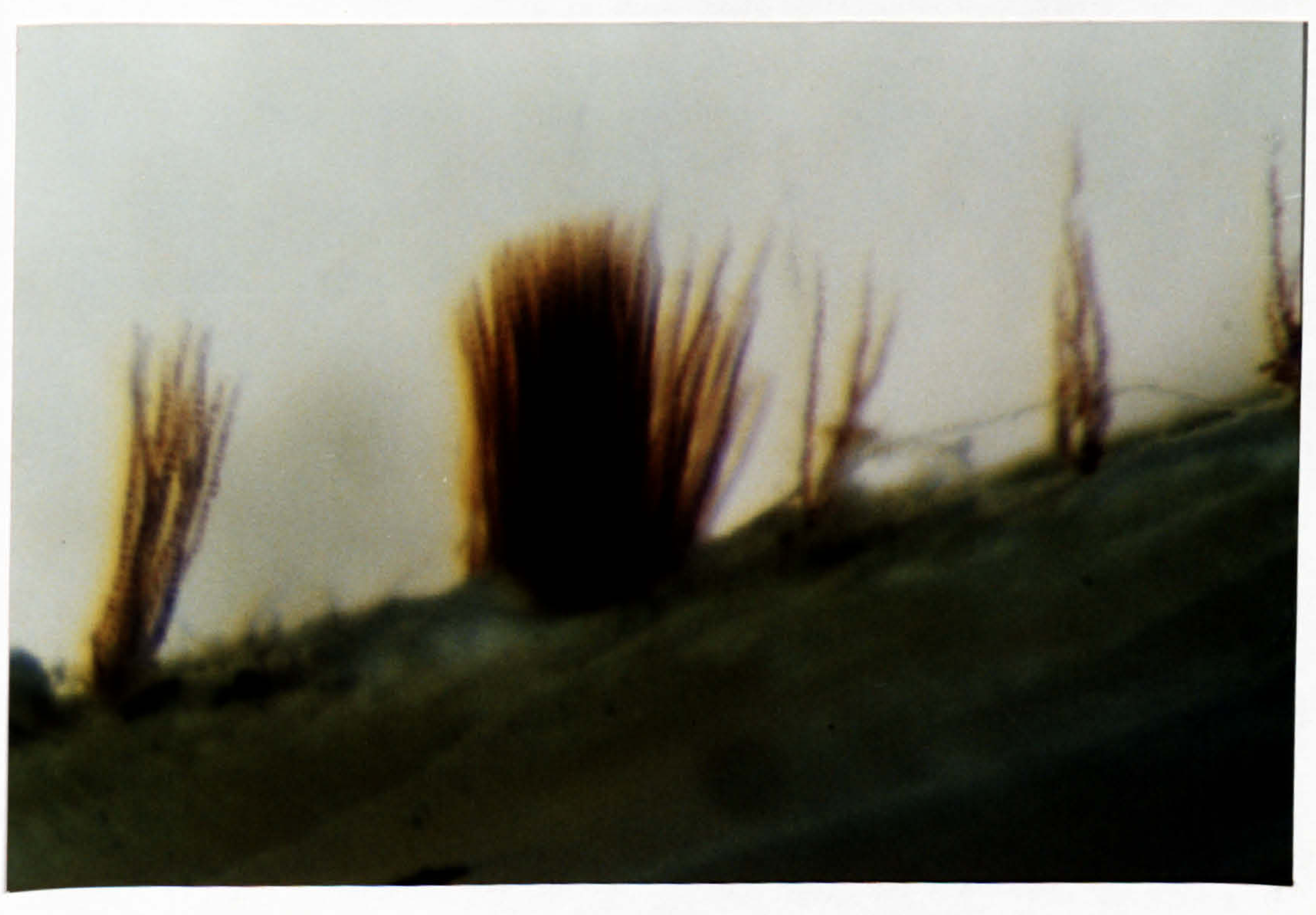


Plate 4.2b Conchosporangia of *P. laciniata* growing from the shell.
(Tufts approx. 1 - 1.5 mm height)

Conchospore induction

The conchospore induction experiments (Table 4.2a, b) were unsuccessful in all cases with one exception (Table 4.2c). When *P. laciniata* cultures were transferred from a short to a long day photoperiod with a cold shock the production of conchospores was not observed. However, three small thalli growing on the base of the culture dish near to the shell fragment with conchosporangium were noted after 2 weeks. These shell fragments in the culture dish containing the germinating thalli were observed for an extra month but no further new germinating thalli were seen.

	CULTURE CONDITIONS					RESULTS
	Light (mol $m^{-2}s^{-1}$)	Temp. (C)	Photoperiod (Light:Dark)	Salinity (‰)	Cold shock	Conchospore Induction ?
Initial conditions :	60	13 and 15	8:16	34		
New Conditions	60	13	8:16	34	-	No
	60	6	8:16	34	-	No
	20	13	8:16	34	-	No
	20	6	8:16	34	-	No
	60	6	8:16	15	-	No
	10 60 60	1 C 12 hr. 13 C 12 hr. 13	12:12 12:12 8:16	34	12 hrs. at 1 C followed by 12 hrs at 13 C. Repeat for 4 days	No

Table 4.2a Culture conditions tested for the initiation of conchospore production in *P. laciniata*. Each set (row) had 3 replicate shell fragments in 3 replicate dishes. Photoperiod numbers in bold = hours dark

	CULTURE CONDITIONS					RESULTS
	Light (mol $m^{-2}s^{-1}$)	Temp. (C)	Photoperiod (Light:Dark)	Salinity (‰)	Cold shock	Conchospore Induction ?
Initial conditions	60	13	16:8	34		
New conditions :	60	13	8:16	34	-	No
	20	6	8:16	34	-	No
	20	6	8:16	15	-	No
	60	6	8:16	15	-	No
	10 60 60	1 C 12 hr. 13 C 12 hr. 13	12:12 12:12 8:16	34	12 hrs. at 1 C followed by 12 hrs at 13 C. Repeat for 4 days	No

Table 4.2b Culture conditions tested for the initiation of conchospore production in *P. laciniata*.
Each set (row) had 3 replicate shell fragments in 3 replicate dishes.
Photoperiod numbers in bold = hours dark

	CULTURE CONDITIONS					RESULTS
	Light (mol $m^{-2}s^{-1}$)	Temp. (C)	Photoperiod (Light:Dark)	Salinity (‰)	Cold shock	Conchospore Induction ?
Initial conditions :	60	13	8:16	34		
New conditions :	60	13	16:8	34	-	No
	20	13	16:8	34	-	No
	20	6	16:8	34	-	No
	60	6	16:8	15	-	No
	10 60 60	1 C 12 hr. 13 C 12 hr. 13	12:12 12:12 16:8	34	12 hrs. at 1 C followed by 12 hrs at 13 C. Repeat for 4 days	Possibly spores as 3 small thalli noted in dish near to conchosporangia

Table 4.2c Culture conditions tested for the initiation of conchospore production in *P. laciniata*. Each set (row) had 3 replicate shell fragments in 3 replicate dishes. Photoperiod numbers in bold = hours dark

DISCUSSION

The culture of any algal species requires a great deal of knowledge and patience since not only does one have to learn about the life history and culture characteristics of a particular species but also the many varied culture techniques available, in order to produce adequate material with which to start and then run an experiment. This can often be both time consuming and frustrating as was the case with the loss of cultures in this study. Therefore the removal of as many known pitfalls as possible in culture techniques during long term culture studies is somewhat of a priority.

The production of both unialgal and axenic cultures requires removal of micro-algal contaminants (Birnie & Boney, 1980). Chapman (1973) used various mechanical methods for the removal of surface contaminants prior to spore release. There is relatively little difficulty in obtaining spores from fertile thalli of *Porphyra* but the removal of contaminants can prove to be a problem if large quantities of spores are required. The application of the technique described by Birnie & Boney (1980), combined with previous thallus cleaning, were successful in this study for producing unialgal cultures of *Porphyra*.

Growing conchocelis cultures in shells facilitates removal of many of the common contaminants as the shells themselves can be washed relatively vigorously to remove fouling organisms while the conchocelis is protected inside the shell. Shell cultures may also be treated with various cleaning and decontaminating solutions to maintain clean cultures (Melvin *et al.*, 1986). There has been evidence that conchocelis growing in shells can survive approximately one month in fresh water (Umbayashi, 1961) demonstrating how growth in shells helps to protect conchocelis from adverse environmental conditions. Therefore the potential exists for using particular cleaning techniques to remove contaminants from shell cultures which would be unfeasible for use in free living cultures.

Growth

The appearance of the thallus phase of a species is likely be triggered by environmental stimuli or triggers that affect growth and maturity of the conchocelis phase and subsequent conchospore release.

Previous studies on the growth of conchocelis indicate that the exact conditions for optimal growth are species specific (Waaland *et al.*, 1987). However, the optimum light levels and temperatures for *P. linearis* and *P. laciniata* found in this study were similar to optimum conditions found for *P. torta* (Waaland *et al.*, 1987), *P. miniata* (Chen *et al.*, 1970) and *P. linearis* (Bird *et al.*, 1972). The conditions are often those that would be expected to occur naturally at the times of conchocelis growth (Waaland *et al.*, 1987).

The experiments on growth of conchocelis at various photoperiods showed rapid initial growth in both *P. laciniata* and *P. linearis.* This is somewhat surprising since at the start of growth a lag phase often occurs with rapid growth occurring after this lag phase. The initial rapid growth may arise from the fact that the original conchocelis material was taken from growing 'tufts' or 'balls' of filaments and then homogenised. The process of homogenisation consequently may produce a higher proportion of apical cells leading to the initial rapid growth. It is also possible that filaments that were previously sheltered in the centre of the 'balls' and as a result only received low light irradiance would then be exposed to an increase in light and possibly nutrients. If these filaments had storage products available then the initial rapid growth may be possible under their new improved culture conditions.

The biomass of conchocelis of both *P. linearis* and *P. laciniata* increased when subjected to progressively longer light periods in the photoperiod experiments.

This is presumably as a result of the increased quanta of light available. The growth of the conchocelis phase of *P. tenera* was also found to be directly proportional to daylength within the range of 8 to 16 hours of light (Dring, 1967).

Bird *et al.* (1972) worked on *P. linearis* from Nova Scotia and found that rather than a photoperiodic response it was a critical temperature of 13 °C which controlled conchospore liberation and was also possibly involved in the production of viable conchosporangia. This temperature occurred in the intertidal prior to the appearance of the thallus phase.

The conchocelis of both *P. laciniata* and *P. linearis* were shown to produce conchosporangia in both long and short days. Waaland *et al.* (1987) demonstrated that the high shore winter species *P. torta* also produced conchosporangia under a wide range of conditions. The conchocelis of *P. kuniedai* required a short day photoperiod for the production of conchosporangia but also produced conchosporangia under long day conditions however, to a lesser degree (Kurogi *et al.*, 1962; Kurogi & Sato, 1962). There appeared to be a greater number of conchosporangia present in the 12:12 and 8:16 photoperiods than in longer day length after 102 days of growth of *P. linearis* while in *P. laciniata*, the greatest number of conchosporangia appeared in long day cultures.

Once conchosporangial material has been produced in a culture later cultures may have a greater number of conchosporangia as a result of further photoperiodic induction. Additionally, increased growth of existing conchosporangia, due to the greater quantity of light available to cultures grown in long days, may also be an influencing factor.

A further experiment using cultures grown similarly in different photoperiods but with equal daily quantities of light would have demonstrated whether production of conchosporangia was in fact a photoperiodic response. A critical light period for the plants must occur to produce a photoperiodic response. During a change from long to short day a response (formation of conchosporangia) would thus be triggered. If at this time the long night is

interrupted by a short light period and the subsequent production of conchosporangia is inhibited then a photoperiodic response has been demonstrated (Waaland *et al.*, 1987). The effectiveness of such a light break in the dark period is generally diagnostic of a genuine photoperiodic response (Luning, 1981; Dring, 1984).

The photoperiodic control of conchosporangium formation has been demonstrated in *Porphyra spiralis* var. *amplifolia*. Conchosporangia were abundant in an 11-13 hour photoperiod, but almost completely inhibited when a 0.5 hour light break of approximately 0.2 µmol m^{-2} day^{-1} was introduced during the dark period (Kapraun & Lemus, 1987).

Photoperiodic control can thus restrict the initiation of certain developmental stages to a particular season of the year but this may be further restricted if temperature also acts as a limiting factor. The observed seasonality of a species may also be said to be indicative of an environmental trigger (photoperiod or temperature) during the production of the reproductive phase of the conchocelis and in the maturation and liberation of the conchospores.

The use of the cross gradient table enabled the effect of light intensity and temperature on the growth of conchosporangia of *P. laciniata* to be demonstrated. Conchosporangia survived well at low light intensities (4 µmol m^{-2} s^{-1}) with the observation of substantially reduced growth and darker pigmentation of conchosporangium tufts presumably as a response to the low light level. As photon irradiance increased the growth of the conchosporangia increased with optimum growth at 30 - 55 µmol m^{-2} s^{-1}. However, at 100 µmol m^{-2} s^{-1} growth of the tufts was reduced. Conchocelis has been found to occur naturally in dead shells in the subtidal (Clokie & Boney, 1980; Clokie *et al.*, 1981) where it would consequently experience lower light intensities than if growing free without the protection of the shell matrix. The shell matrix itself would also reduce light levels as a result of scatter. The depth of water would also reduce incident light levels. Increasing evidence, however, indicates that conchocelis can and does

grow in the intertidal (Drew, 1954; Ogata, 1955, 1961; Dixon & Richardson, 1969; Mumford, 1975; Martinez, 1990) where light levels would be expected to be higher than in the subtidal. Consequently growth of conchocelis may be limited at times of high light levels and grow vigorously at times of the year when light is at an optimum (autumn for a winter species).

It has been observed (Melvin *et al.*, 1986) that the light intensity affected the depth at which the conchosporangia grow in the shell matrix. If light levels are high the conchosporangia will grow deeper in the shell. Light levels are of consequent importance since the sporangia must burrow out of the shell to release conchospores (Melvin *et al.*, 1986). It is possible that a reduction of light levels may be an important factor of seasonal growth of the conchosporangia in bringing them to the surface of the shell when the conchosporangial filaments can then be triggered by other environmental conditions to induce conchospore release.

An increase in temperature improved growth of the conchosporangia with the optimum being 12 °C for *P. laciniata* in this study. However, growth was only slightly reduced at 15 °C. An improvement in the percentage of conchocelis that developed conchosporangia in *P. angusta* with increased temperature was noted by Chiang & Wang (1980). The shape and abundance of the conchosporangia was also affected in the present work. Increased temperature and light produced a rapid increase in the amount of branching of the conchosporangial filaments and thus affected measured growth of conchosporangia. Conchocelis branching was found to be more dense at higher temperatures for several species of *Porphyra* (Kurogi & Akiyama, 1966).

Shell culture

The growth of conchocelis in shells was successful. It was found from preliminary tests that the inoculation density of conchocelis fragments affected

the subsequent growth of the conchocelis in the shell. If the density was too high the plants developed quickly but did not appear to be as healthy or to produce conchosporangia as readily as cultures grown from a lower inoculation density. Similar observations were noted by Melvin *et al.* (1986).

Stock cultures maintained in lower irradiance had healthier, deeper pigmentation and growth was slower as expected.

P. laciniata appeared to grow in shells more vigorously and produced conchosporangia more readily than *P. linearis.* This may have been as a result of the condition of conchocelis of each species at the time of inoculation which can affect the further development and growth of conchocelis in the shells (Melvin *et al.*, 1986). Conchocelis of *P. torta* which is only germinating directly from carpospores or is very young (less than 4 to 6 weeks after carpospore germination) and still consists of only vegetative filaments will successfully penetrate and grow into shells (Waaland *et al.*, 1987).

In stock cultures of conchocelis growing in shells, the conchosporangial branches visible above the shell surface grew perpendicularly to the surface in the initial light conditions. Light conditions were reduced in the stock cultures by placing a screen over the top of the dish. As a result of one of the screens moving, a light gradient was accidentally produced from one side of the dish to the other. This was noticed as the conchosporangia showed unusual growth. The filaments on all (six) shell fragments in the dish had turned and grown towards the side of the dish with the higher irradiance. A negative phototropism was demonstrated by van Tussenbroek (1984) for *P. umbilicalis* vegetative conchocelis cultures that were two weeks old. A positive phototrophic response was observed for five day old conchocelis of *P. tenera* and *P. yezoensis* (Migita & Kim, 1971). The negative response noted by van Tussenbroek (1984) may be a response necessary for the shell boring habit of the conchocelis (ie. turning away from the light and penetrating the shell). The positive phototrophic response of the conchosporangia noted in this study may have occurred as a result of light

intensities being too low for optimal growth. Alternatively, it may be a demonstration of the positive phototrophic reaction necessary for conchosporangia to grow out from inside the shell in order to produce and release conchospores.

Conchospore induction

Considerable diversity in the conditions necessary to induce conchospore release has been observed for *Porphyra* species. Conchospore release only occurred with *P. miniata* in an 8:16 photoperiod between 2 and 7 °C (Chen *et al.*, 1970). Short day photoperiods were noted for conchospore induction in the species *P. tenera, P. angusta, P. kuniedai* & *P. pseudolinearis* (Kurogi *et al.*, 1962) and long day photoperiods in *P. nereocystis* (Dickson & Waaland, 1985). For *P. perforata* an increase in both photoperiod and light was necessary for conchospore induction. *P. kuniedai* and *P. pseudolinearis* spores were more abundant under lower light intensities whilst few occurred under higher light intensities (4 - 14 and to 80 μmol m^{-2} s^{-1} respectively) (Kurogi & Akiyama, 1965). Conchospore release in *P. carolinensis* appeared to be controlled by a combination of short photoperiod and low temperature (Freshwater & Kapraun, 1986) while *P. linearis* would only release conchospores at 13°C and photoperiod had little effect (Bird *et al.*, 1972). Clearly the conditions necessary for conchospore induction vary greatly and are often species specific.

The conchospore induction experiments on *P. laciniata* and *P. linearis* did not produce any conchospore release. This may have been a result of a number of factors. Cultures used in this study may have been affected by earlier experimental conditions which could have prevented conchosporangium becoming mature at a later stage under different conditions. Alternatively, the conditions tested in the experiments may have been incorrect for these particular species (from the examples above it can be seen that they vary greatly from

species to species) so not triggering the necessary maturation and subsequent conchospore release. Kornmann & Sahling (1991) noted that the results of culture experiments on *P. linearis* by Bird *et al.* (1972) were not uniform. In some cases the full life history could be carried out five times while other cultures formed conchocelis with sporangia which did not release conchospores. Kornmann & Sahling (1991) therefore hypothesised that the basic thallus material in the study by Bird (1972) was not uniform but contained thalli with carposporangia and thalli with asexual sporangia. It is now thought that there are in fact two species of *P. linearis* in the north west Atlantic (Lindstrom, pers. comm.). Kornmann (1991) also found no conchospore release from conchosporangia of *P. linearis* when cultures had originated from the asexual blades of this species. This may have been the case with the cultured material in this study which showed no conchospore release.

The possible conditions for the culture of any stage in the conchocelis phase of *Porphyra* species clearly varies greatly. The environmental triggers and responses for specific stages which are necessary for the formation of the subsequent phase in the life history also show great variation. There is also the added complexity created by triggers often being an interaction/combination of environmental conditions. Therefore, careful thought and planning of culture experiments is important if the maximum amount of information is to be obtained from what can be very time consuming experiments.

CHAPTER 5

THE USE OF ACIDIFIED SEAWATER AS AN ALGAL CULTURE CLEANING TECHNIQUE

INTRODUCTION

The removal of contaminants from preliminary stages of culture processes can be successfully achieved using techniques such as those used for cleaning spore cultures (Birnie & Boney, 1980) and removing surface contaminants prior to spore release with mechanical methods (Chapman, 1973).

The production of unialgal cultures from the beginning of culture experiments is by far the most desirable situation. However, if cultures become contaminated during later stages and the use of a cleaning technique is impractical then cultures will have to be discarded. This was the case in this study which resulted in the destruction of many months work. Therefore, it would be worthwhile to devise a technique for cleaning cultures once experiments had begun thus reducing lost time when cultures would have to be restarted from original stocks.

The material in this experiment originated from cultures prepared for experiments in Chapter 4. Conchosporangia first appeared in conchocelis cultures when culture conditions had been altered to a short day photoperiod after approximately three months treatment at initial conditions (long day). It was planned to use the conchosporangium material for a photoperiod experiment. However, cultures became contaminated with a brown alga (*Ectocarpus* sp.) and also some with a filamentous green alga. The *Ectocarpus* sp. was the most common contaminant observed in cultures. The origin of this contamination was unknown but may have arisen from original spore suspensions. Alternatively, it may have been added to cultures at a later stage as a result of contaminated culture medium.

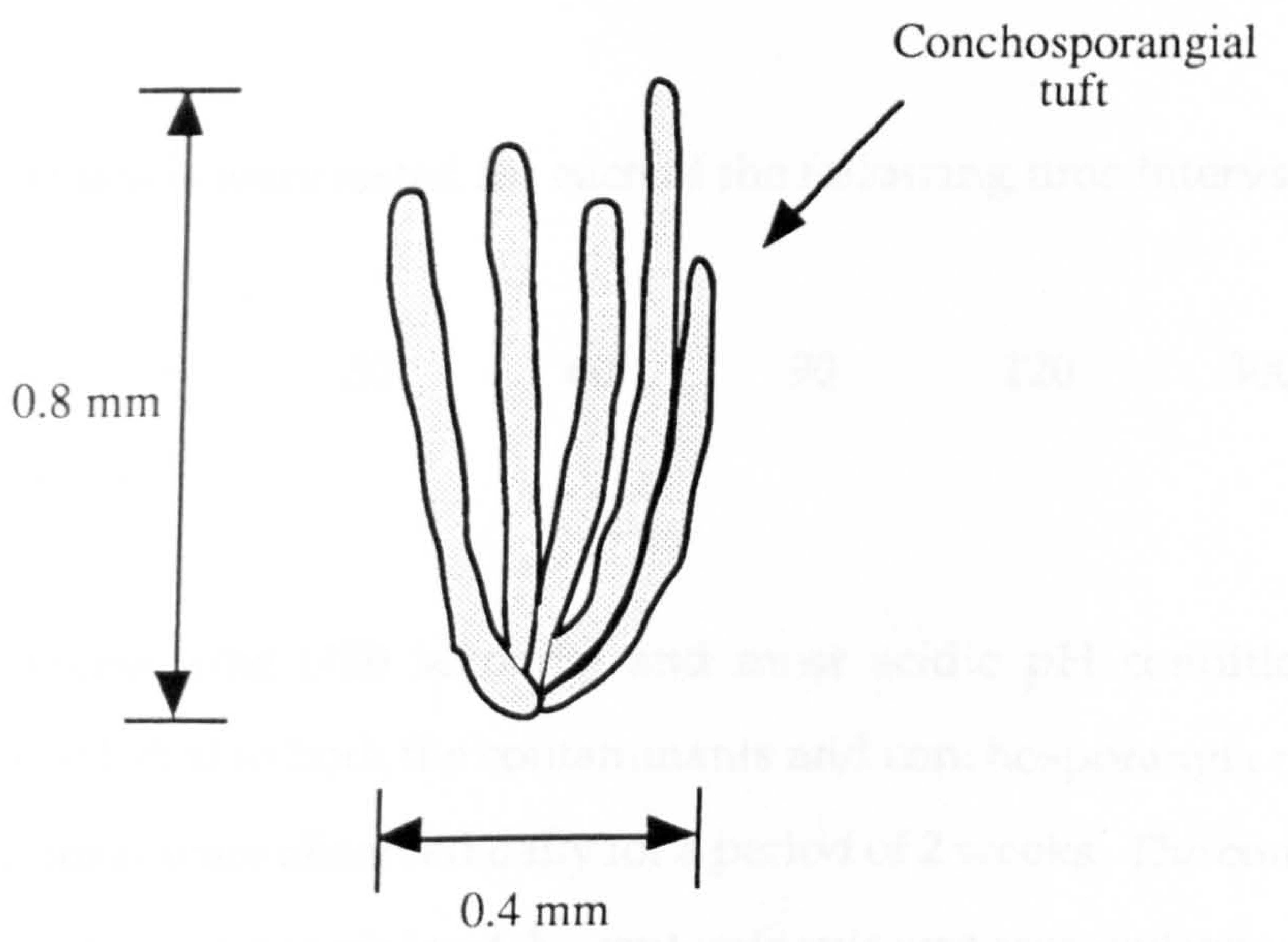
The conchosporangial material appeared healthy and unaffected by the contamination and so an experiment was designed (Waaland, pers. comm.) using various pH levels of acidified seawater to attempt to eliminate the contaminants.

METHODS

Conchocelis of *P. laciniata* were previously cultured for growth and photoperiod experiments under standard stock culture conditions of 16:8 light/dark period, 30 to 40 µmol $m^{-2}s^{-1}$ light and a temperature 12±1 °C. Cultures were free living and maintained in dishes with 100 ml of F2 seawater medium (see Appendix for recipe).

For this experiment F2 seawater medium was acidified using hydrochloric acid (HCl) to produce 5 pH levels. The effect of treatment on contaminated conchosporangial material with 5 different pH solutions and 6 different time intervals was tested.

Conchosporangial material of *P. laciniata* consisted of tufts of filaments with no vegetative material (see figure below).



The filaments were of approximately equal size and shape. Ten contaminated tufts were selected at random from cultures and placed in 4 cm diameter petri

dishes with culture medium of normal pH. A petri dish placed on a rotating stirrer was half filled with a pH treatment medium and the 10 contaminated tufts were added. The stirrer was set to approximately 100 r.p.m. The tufts remained in the dishes for a set time, were then removed and placed in another petri dish on the stirrer containing normal pH seawater medium. This seawater medium was used to wash the acid from the tufts for a standard 1 minute at 100 r.p.m. This washing process was repeated 3 times with the seawater being replaced with fresh medium between each washing.

The 10 conchosporangia tufts were then placed in fresh medium in 4 cm petri dishes and cultured at a room constant temperature (13 °C) with a photon irradiance of 30 μmol $m^{-2} s^{-1}$ and 16:8 photoperiod. Three replicate dishes were used for each treatment.

The following pH treatments were tested:

pH	—	6	5	4	3	2

The pH levels were tested for each of the following time intervals:

Time (seconds)	—	30	60	90	120	300	480

The longest time (480 seconds) and most acidic pH conditions were designed to be lethal to both the contaminants and conchosporangia material.

The cultures were observed daily for a period of 2 weeks. The condition of the conchosporangial material and the contaminants was assessed using various methods: the general health of the tufts was noted; the condition of cells of the tufts was studied for any abnormalities and where possible, an estimation of the

percentage number of dead or damaged cells was recorded. The condition of the contaminants growing on and around the conchosporangia was similarly recorded.

At the end of the experiment the number of live conchosporangia tufts in the dishes, the number of contaminated tufts and the general condition of the contaminants was recorded.

RESULTS

The treatment of the contaminated conchosporangia with pH/time treatments was, in general, successful although in some cases loss and damage to conchosporangia tufts occurred.

The treatment affected conchosporangia as well as the contaminating algae. It can be seen from Fig 5.1 that at pH 6 and 5 all conchosporangia tufts survived all time treatments. At pH 4 all conchosporangia survived until the two longest treatments of 300 and 480 seconds. All time treatments caused some loss of tufts at pH 3 with approximately 80 % survival after 30 and 60 seconds. After 300 seconds survival was reduced to 50 % and at 480 seconds to 47 %. pH 2 affected survival at all time treatments with 20 % survival at 300 seconds and only 7 % survival after 480 seconds, the lowest survival of all treatments.

The pH/time treatments also affected survival of contaminants (Fig. 5.2). At pH 6 all conchosporangia were still contaminated after treatment. At pH 5 and 4 some contamination was removed from tufts with 73 % of tufts remaining contaminated at pH 5 and 23 % at pH 4 after 480 seconds of treatment. The first treatment that removed all contamination was pH 3 for 120 seconds. All contamination was removed at pH 2 but as can be seen in Fig. 5.1 the survival of conchosporangia was noticeably affected.

The optimum treatment for survival of tufts and contaminant removal for cultures of conchosporangia material was pH 3 for 120 seconds. Some conchosporangia tufts were lost but 70 % survived this treatment.

The conchosporangia tufts that survived were often damaged (to varying degrees) by the treatments. This damage was apparent within one day of the treatment and was noted as damage/death of conchosporangia cells, particularly at extremities of the tufts. The damaged cells appeared as transparent skeletons many of which were lost after the first few days following treatment.

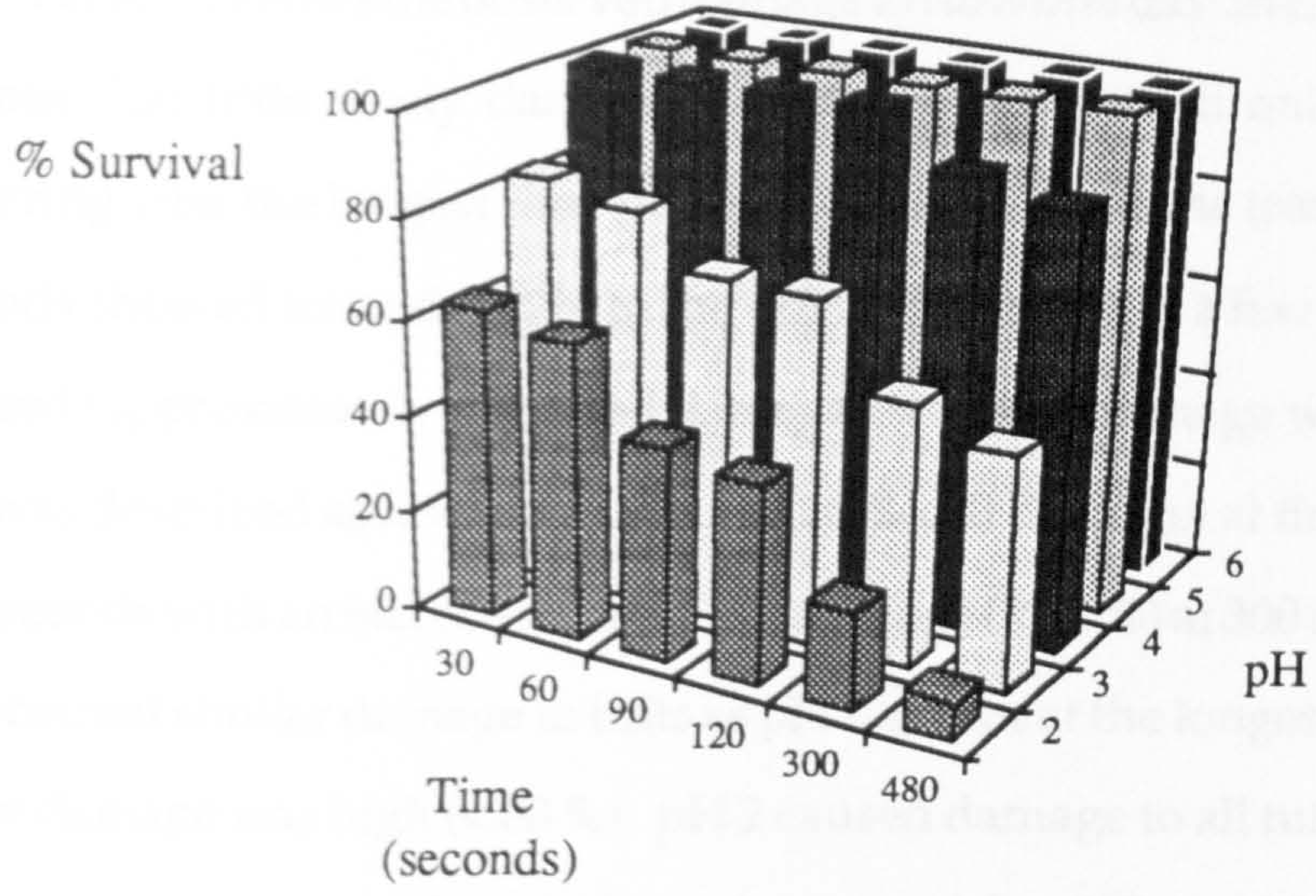


Fig. 5.1 The survival (%) of conchosporangia of *P. laciniata* in various acid and time treatments.

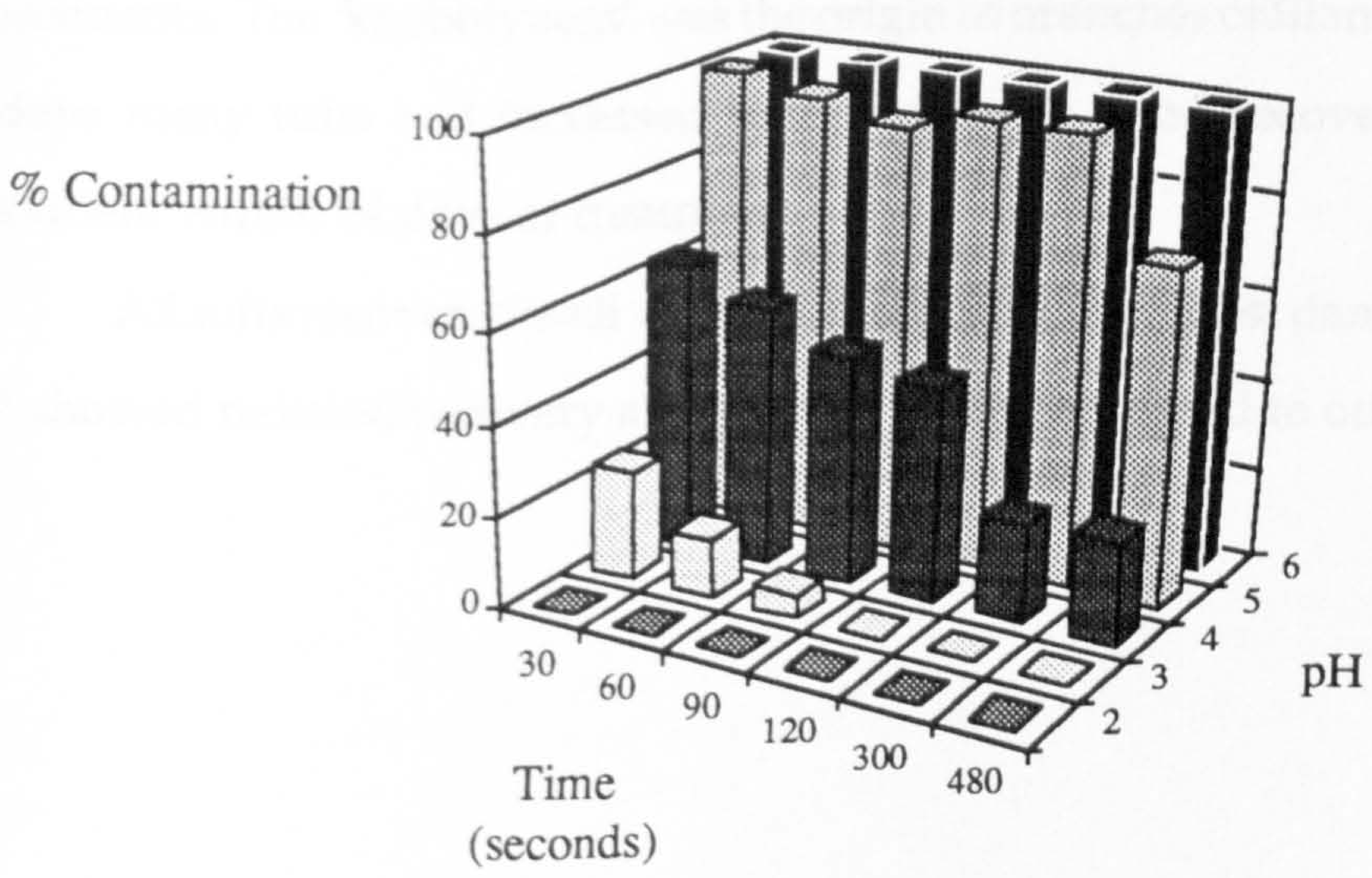


Fig 5.2 The percentage of contaminated of conchosporangia survivors remaining after treatments.

Table 5.1 shows the observed damage to tufts one day after treatment. It can be seen that little if any damage occurred at pH 6 with only slight damage occurring after the longest time treatment. At pH 5 all time treatments above 90 seconds showed some damage to the cells although only a few of the tufts were affected (approximately < 5 % cell damage). Greater damage was noted at pH 4 and was described as low level (approx. 20 % cell damage) at time periods up to 120 seconds with an increase to medium damage (< 40 %) at 300 and 480 seconds. pH 3 caused similar damage to tufts as pH 4 except at the longest time treatment where damage was high (< 80 %). pH 2 caused damage to all tufts with even the shortest treatments causing medium damage. After 300 and 480 seconds at this pH cell damage had become extreme (> 90 %).

From the observations of cell damage/death it can be seen that the optimum treatment described for removal of contaminants (pH 3 for 120 seconds) caused relatively low damage (~ 20 %) to the conchosporangia material.

Recovery of conchosporangial filaments was noted at approximately four days after treatment. This recovery was evident as 'knobblyness' of tuft filaments. The 'knobblyness' was the origin of branches of filaments. After eight days many tufts had increased branching with good recovery in most tufts evident within 14 days of treatment.

All tufts recovered well with the exception of the most damaged tufts at pH 2 showed reduced recovery and growth when compared to other treatments.

Time (seconds)

pH	30	60	90	120	300	480
6.0	0	0	0	0	0	+
5.0	0	0	+	+	+	+
4.0	++	++	++	++	+++	+++
3.0	++	++	++	++	+++	++++
2.0	+++	+++	++++	++++	+++++	+++++

Key: Level of cell damage:

0	-	No damage	+++	-	Medium
+	-	Present	++++	-	High
++	-	Low	+++++	-	Extreme

Table 5.1 The levels of cell damage observed in conchosporangia of *P. laciniata* after various pH/time treatments

DISCUSSION

The use of acidified seawater in this experiment was mostly successful in killing contaminating algae at pH levels where the conchocelis was able to survive. It is difficult to be conclusive as to the reasons why conchocelis material was able to withstand lower pH conditions for longer periods than the contaminating species. A possible explanation involves consideration of the type of substratum in which conchocelis of *Porphyra* grows. The conchocelis in most *Porphyra* species 'bores' readily into shells and other calcified substrates (Ogata, 1955, 1961; Migita & Kim, 1971; Suigiyama *et al.*, 1971; Matamala *et al.*, 1985; Martinez, 1990). The process the conchocelis uses for boring is not well known. However, in light of the fact that conchocelis bores into calcified substratum a possible hypothesis is that the boring process involves the use of some kind of acid to dissolve calcium in shells or other substrata thus creating a 'bore' hole along which to grow. Ogata (1961) estimated that the hydrogen ion concentration in the cell contents of conchocelis was pH 5 to 6 for *P. tenera* and also that the calcareous substances in which conchocelis bore were easily dissolved by such a weak acid. Hence the use of acidic 'secretions' by *Porphyra* may provide an explanation as to why conchosporangia tufts can withstand these conditions when compared to contaminating species.

The conchosporangia in this experiment were apparently unaffected by a pH of 5 or 6. The use of seawater medium of pH 2 with 8 minutes treatment was designed to be lethal to both contaminants and conchosporangia and although this pH/time treatment caused a great deal of damage to cells and the death of many conchosporangia tufts it must be noted that some conchosporangium managed to survive this severe treatment.

The removal of all algal contaminants at the earliest stage possible in culture experiments is by far the most desirable solution. However, if necessary,

contaminants can be removed as demonstrated in this experiment. The use of this technique would be beneficial if the cultured material was of great value or if time was a limiting factor in the event of production of fresh cultured material.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

This investigation examined various aspects of the biology of species of *Porphyra* from the Isle of Man. These aspects ranged from general ecology, seasonality and taxonomy of the blade phase to the culture of the conchocelis phase.

The mapping work of *P. linearis* demonstrated the reoccurrence of patches within the same areas from year to year. This would appear to be indicative that the conchocelis populations occur within close proximity to the adult thallus populations. The conchocelis populations may even occur within (either in barnacles or in the limestone rock) the sites of the mapped patches of the blade phase.

The detailed mapping work demonstrated how the population of *P. laciniata* changes with the seasons. This work also demonstrated how it is possible to use maps of this nature to obtain quantitative data on populations of this type. The substratum of the mapped areas in this rock to sand transition zone changed dramatically over the period of the study and appeared to be related to the seasons. The movement of sand must cause abrasion problems to many species of algae as well as species of grazing animals. *Porphyra* appears to be able to withstand the abrasion and temporary covering of sand.

Some of the species found on the Isle of Man show marked seasonal occurrence, *P. linearis* occurs solely in winter and *P. leucosticta* in the summer. *P. laciniata* and *P. umbilicalis* can be found most of the year round but as was demonstrated in the detailed study of a population of *P. laciniata* a marked seasonal increase in population biomass does occur in this species.

It can be seen from examples of recent research on species of *Porphyra* in Britain (Boney, 1978; Reed, 1979; Birnie & Boney, 1980; Clokie & Boney, 1980; Reed & Collins, 1980; Reed *et al.*, 1980a, b; Clokie *et al.*, 1981; Reed *et al.*, 1981;

Akpan & Farrow, 1984; van Tussenbroek, 1984; Malkin *et al.*, 1990) that their number is limited with no studies carried out on the ecology or taxonomy since the 1960's by Conway (1964a, b, c, 1967).

As a result of doubts on the taxonomy of some species the technique of electrophoresis was used. This demonstrated the existence of six species of *Porphyra* around the coast of the Isle of Man rather than the five species previously assumed (Conway, 1964a). It is likely that the unknown species *P.* sp. is the species described as *P. purpureo-violacea* by Kornmann & Sahling (1991). In light of the discovery of this additional species and other recent studies on the taxonomy of *Porphyra* species (Lindstrom & Cole, 1990; Kornmann & Sahling, 1991; Lindstrom & Cole, 1992a, b) it is possible that other, as yet undescribed, species exist around the coast of Britain. The application of electrophoresis in the present study as an aid to the taxonomy of this morphologically similar genus demonstrated the effectiveness of this technique.

Further work on the taxonomy of species around the British coasts is needed. This kind of study could then be expanded into a study of European species. The application of electrophoresis proved successful in the recent work by Lindstrom & Cole (1990, 1992a) which helped to resolve taxonomic problems in species of *Porphyra* from the Pacific coast of North America and Canada. It has also been successfully used in the study of relationships between species from the North Atlantic and North Pacific coasts of North America (Lindstrom & Cole, 1992b).

Initial understanding of aspects of the biology of a particular species often develops from comparisons with other studies on similar or different species from past literature. If there is uncertainty within the taxonomy of a species or genus within a study then this leads to uncertainties (when comparing results with other studies) of species characteristics, whether taxonomic or ecological. This makes any subsequent assumptions and conclusions from which useful future studies can be made somewhat difficult. The reliability of the taxonomy

of a species is thus of great importance in order to have a solid foundation on which to base any further studies.

The technique of electrophoresis could also be applied to intra-specific population studies of the blade phase to assess factors such as intra-specific genetic diversity. The degree of population diversity could be used as a possible inference of the range of sporing from parent conchocelis population to produce the population of the blade phase. The technique of electrophoresis could also be applied to studies on the conchocelis phase as it was noted in preliminary experiments testing the electrophoretic techniques that the conchocelis phase resolved well on a test gel.

The use of chromosome counts and karyotypes can also be useful in distinguishing some species. *Porphyra* species tend to have a low number of chromosomes with many having the same number but where differences do occur they can be useful in helping to distinguish species (Lindstrom & Cole, 1992a). In the present study only *P. laciniata* was distinguishable from the other species studied which all had the same chromosome number.

The work on the ecology and seasonality of species around the Isle of Man included studies of the differences in the protein content between species. The winter species *P. linearis* had the highest protein content of the four species studied. The protein content is indicative of the potential quality of an alga for food (Noda, 1971; McLachlan *et al.*, 1972; Nisizawa, 1987) and so it would appear, as noted by McGregor (1989), that *P. linearis* would have the greatest potential for the production of a valuable food crop in cultivation. McLachlan *et al.* (1972) noted that *P. linearis* from Nova Scotia was adjudged superior to other species tested in terms of taste and protein content. It would be beneficial to carry out more detailed assessments of protein, amino acids and flavour substances of the species found in Britain and Europe.

When considering the results of McLachlan *et al.* (1972) it is of note that

recent work by Lindstrom (pers. comm.) indicates that the previously recognised *P. linearis* is in fact a complex of two species. This highlights (as stated earlier) the difficulties in the taxonomy in some species and the potential problems of comparisons of species over a wide geographic range. Is the *P. linearis* in this study the same as that of McLachlan *et al.* (1972) permitting direct comparisons or is it in fact a different species ? The classification of *P. linearis* in Britain in light of Lindstrom's work would consequently be useful.

Studies by Clokie *et al.* (1981) and Akpan & Farrow (1984) on the conchocelis phase of species of *Porphyra* demonstrated the presence of conchocelis within the shells of dead molluscs in the subtidal. More recently studies have shown that the conchocelis phase exists in various substrata in the intertidal (Matamala *et al.*, 1985; Martinez, 1990). Boney (1978) noted that the production of conchospores from this habitat would facilitate the establishment of the dense and often seasonal populations of the blade phase. In this present study conchocelis was found growing in the shells of barnacles in the intertidal but the research was limited to the recording of presence or absence. Further more detailed research of the abundance, seasonality and species identification (where possible) of these conchocelis populations would be of great interest when considering relationships of these conchocelis populations with populations of the more conspicuous blade phase.

The attempts to produce conchospores in the culture experiments of *P. linearis* and *P. laciniata* were unsuccessful under the conditions tested in this study. Considerable variation in the conditions necessary for conchospore release have been observed in other *Porphyra* species. For example, conchospore release in *P. miniata* only occurred with a short day photoperiod and between 3 to 7 °C (Chen *et al.*, 1970). *P. linearis* would only release spores at 13 °C and photoperiod had little effect (Bird *et al.*, 1972). In *P. columbina* from central Chile

a temperature drop from 15 - 10 °C was necessary for conchospore release. The production, maturation and release of spores can clearly be very species specific and may consist of numerous evolving factors.

Although the culture of all phases of the life history is important when considering the cultivation of *Porphyra* the reliable production of conchospores is essential for further mariculture research with any species (Waaland *et al.*, 1986). When conchospores can be reliably produced from cultures then applied research on net cultivation of the blade phase could be carried out as with *P. torta* in North America (Waaland *et al.*, 1986). Further studies would hopefully find the necessary conditions for conchospore production in the species cultured in this study.

Seaweed cultivation is in its infancy in Europe with preliminary study occurring particularly in Britain and France where the prospects appear to be encouraging (Guiry & Blunden, 1991). The speed with which a viable cultivation system can be set up in Europe depends a great deal on the particular seaweed species used. In North America the Japanese species *P. yezoensis* was introduced in cultivation trials. As a result of the wealth of research in the Far East (e.g. Matsadaira & Iwasaki, 1953; Iwasaki & Matsudaira, 1954a; Iwasaki & Matsudaira, 1954b; Sano, 1955b; Kurogi & Hirano, 1956; Iwasaki & Matsudaira, 1958; Yoshida *et al.*, 1964; Terumoto, 1965; Kurogi & Akiyama, 1966; Yoshida, 1966; Imada & Saito, 1971; Miura, 1975, 1976; Yoshihara, 1977) many cultivation related problems have already been overcome and large amounts of selection for factors such as growth and productivity have already been carried out with this species and so a farming project could be set up relatively quickly. The use of an indigenous species in cultivation trials adds years of research onto the project time. The introduction of foreign species, however, raises many issues both biological and ethical. The ecological consequences of the introduction of alien marine species are often difficult to predict. Previous examples as in the case of

the introduced alien *Sargassum muticum* (Yendo) are striking enough (Rueness, 1989). Research on the potential for cultivation using indigenous species should be carried out to help in the assessment of whether introductions are in fact necessary.

Many interesting factors of the biology of some *Porphyra* species are presented here. Some questions have been answered and consequently further potentially exciting avenues of research have also been opened. Some useful techniques and areas for future research are presented for the resolution of at least some of these problems.

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APPENDIX

To make F/2 seawater media:

Guillards Enrichment;

2.5g	NaH_2PO_4 H_20
37.5g	$NaNO_3$
2.5g	Fe Sequestrene

O.5ml of each of the following trace metals;

$CuSO_4$ $5H_2O$	0.98g/100ml
$ZnSO_4$ $7H_2O$	2.2g/100ml
$CoCl_2$ $6H_2O$	1.0g/100ml
$MnCl_2$ $4H_2O$	18.0g/100ml
$NaMoO_4$ $2H_2O$	0.63g/100ml

2.5 ml Biotin	0.1mg/ml
0.25ml VitB12	1.0 mg/ml
50 mg Thiamine	

Make upto 1750 ml and store frozen in 250 ml aliquots.

Stand for 24 hours at room temperature prior to use

USE 3.5 ml/l OF THE ABOVE ENRICHMENT TO MAKE F/2 MEDIA

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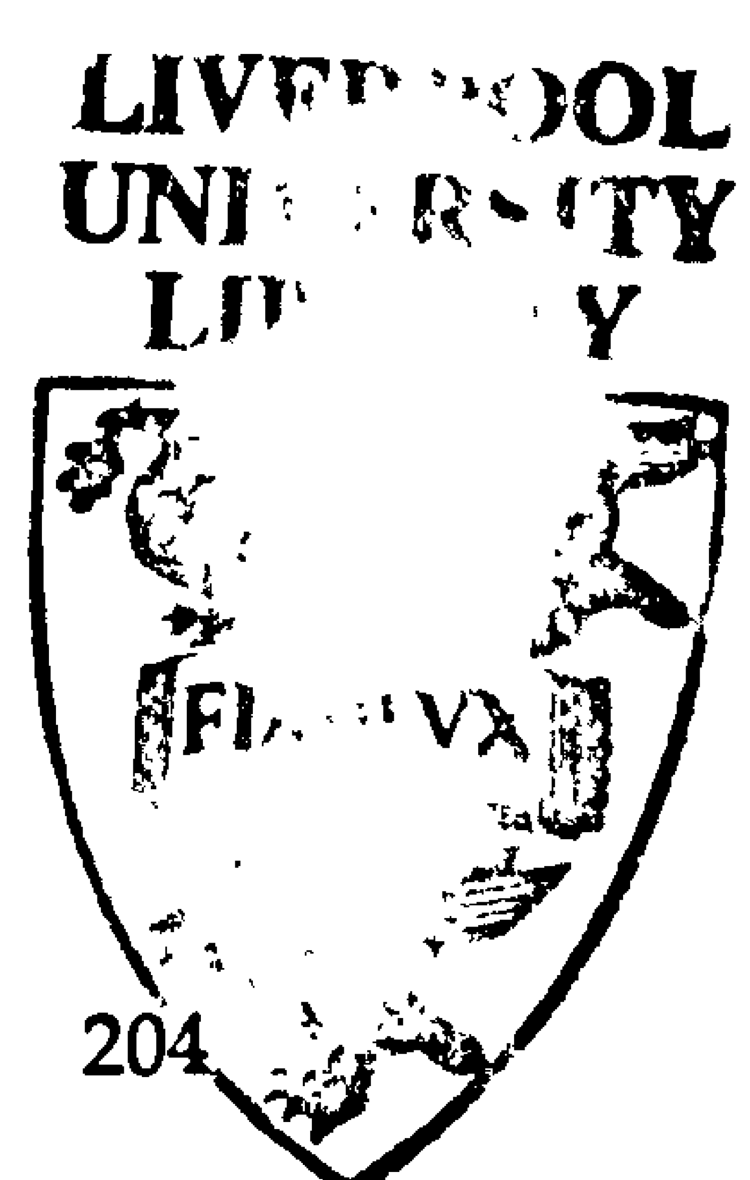

ABSTRACT

Various aspects of the biology of intertidal *Porphyra* species present in the Isle of Man were investigated. This involved: observational ecology of four species linked with a detailed seasonality study of a population of *Porphyra laciniata*; assessments of protein levels of four species; the use of electrophoretic techniques as an aid to a study of the taxonomy of Manx *Porphyra* species, and aspects of the culture of the conchocelis phase.

Ecological observations demonstrated a marked seasonality of occurrence with the species *P. linearis* occurring in winter and *P. leucosticta* in summer to autumn. *P. umbilicalis* and *P. laciniata* were present all year round although peaks in occurrence and biomass were observed in spring. The detailed study of the population of *P. laciniata* showed a significant biomass increase in March to April for two consecutive years. The area of shore where *P. laciniata* was studied was shown to be dynamic with dramatic changes at times in the substratum due to the movement of sand in and out of these areas. Mapping techniques proved to be successful in demonstrating the seasonal changes in patch cover and the dynamic nature of the population of *P. laciniata* studied.

P. linearis patches were mapped for two consecutive years and found to occur in almost exactly the same positions from year to year. This is indicative of a population of conchocelis (the previous stage in the life history) being in close proximity or even occurring within the area of the patches of the blade phase. The conchocelis phase (not identified to species level) was found growing in intertidal barnacles close to these *P. linearis* patches and also within the populations of *P. laciniata* in Port Erin.

P. linearis had the highest protein level of all the species measured with a peak level in January of 42.63 % and a mean seasonal level of 35.95 %. *P. leucosticta* had the lowest recorded mean seasonal level (25.07 %).

Only one specimen of the subtidal species *P. miniata* was found during the course of the study and so this species was not involved in the course of the research.

Enzyme electrophoretic techniques were successful in distinguishing between the four species investigated and also demonstrated the presence of a fifth unknown species similar in morphology to *P. laciniata*. Further work using electrophoretic techniques coupled with other morphological observations clearly separated these two species. It is believed (although is not yet conclusive) from comparisons with work carried out by Kornmann (1991) that this unknown species is *P. purpureo-violacea*. Electrophoresis also demonstrated that three morphotypes of *P. umbilicalis* were the same species.

Haploid chromosome numbers were found to agree with those in previous studies. *P. laciniata* had 5 chromosomes and *P. umbilicalis*, *P. linearis* and *P. leucosticta* had 4.

This part of the study demonstrated that the taxonomy of this genus in Britain requires further research in order to be certain of species identity and also of the total number of species present around British coasts.

The culture of the conchocelis phase of *P. laciniata* and *P. linearis* was successful in both free cultures and cultures grown in shells. Some free living cultures were grown for up to three years. The production of conchosporangia did not appear to be a result of a photoperiodic response. Attempts to produce conchospores from *P. laciniata* in both shell and free living cultures was unsuccessful in all conditions tested.

As a result of contamination difficulties the removal of algal contaminants from cultures of conchosporangia of *P. laciniata* using various pH treatments with acidified seawater was tested. This was successful in some treatments and also demonstrated the ability of the conchosporangia to survive acidic conditions (10 % survival in pH 2.0 for 8 minutes).