THE SPECIFICITY OF TEMPERATE ANTHOZOAN-DINOFLAGELLATE SYMBIOSES

A thesis submitted for the degree of Doctor of Philosophy

by

Simon K. Davy, B.Sc. (University of Wales)

School of Ocean Sciences, University of Wales, Bangor,
Menai Bridge, Anglesey, Gwynedd, U.K.

February 1994.

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ABSTRACT

The aim of this study was to investigate the specificity of temperate dinoflagellate - anthozoan symbioses and the influence of any specificity on the percentage contribution of photosynthetically fixed carbon to the host's daily respiratory carbon budget (CZAR).

The British symbiotic Anthozoa Cereus pedunculatus (Pennant), Anthopleura ballii (Cocks), Anemonia viridis (Forskal) and Isozoanthus sulcatus (Gosse) and the sub-tropical anthozoan Aiptasia pallida (Verrill) and their algal symbionts were investigated.

The characteristics of freshly isolated and cultured symbionts were studied to determine whether the algal cells residing in each host species appeared different and how symbiont characteristics may be determined by the host environment. Morphology, division behaviour and photosynthetic pigment composition were examined using SEM, TEM, LM, TLC and HPLC respectively. The symbionts of each host species were identified as dinoflagellates of the genus Symbiodinium, even though there was an absence of a motile phase in the cell cycle of the British hosts. Differences between symbionts of different host species and conspecific hosts were evident with respect to thylakoid arrangement, cell size and cell division rate, and possibly pigment concentrations. Host determined phenotypic plasticity was observed with respect to the absence of a "pellicle".

Host-symbiont recognition and specificity were investigated by reinfecting aposymbiotic C. pedunculatus with symbionts from different host species and measuring the density and biomass of symbionts at different time intervals. The presence of different strains or species of Symbiodinium was evident, with recognition of these different symbionts being predominantly post-endocytotic. 'Homologous' symbionts were the most 'successful' at repopulating C. pedunculatus. After 9 months, the population densities and biomasses, and division rates of the different 'strains' of symbionts were the same, suggesting a greater symbiotic integration with 'heterologous' 'strains' with time.

The reflection of specificity by the photosynthetic fixation of carbon in vivo was measured using an O_2 electrode. 'Homologous' symbionts photosynthesized at a greater rate than 'heterologous' symbionts in vivo, despite the higher photosynthetic rates of some of these 'heterologous' symbionts in their original hosts. Photosynthate translocation by symbionts in response to different host environments was investigated using in vivo and in vitro ^{14}C labelling and the 'growth rate method'. The percentage translocation was host-symbiont specific and appeared to be controlled by different mechanisms in different host species and conspecific hosts from different locations. The CZAR in 'natural' and reestablished symbioses was calculated. Values ranged from 25.7 to 112.2 % in the 'natural' symbioses and 58.1 to 76.7 % in the reestablished symbioses when measured using ^{14}C. When measured using the 'growth rate method' values were 73.2 to 188.4 % and 88.3 to 146.5 % respectively. The CZAR in the reestablished symbioses and consequently their ability to survive autotrophically reflected the specific host-symbiont relationship. Symbiosis specificity and the reduced availability of carbon to host growth and reproduction in 'heterologous' symbioses may limit 'novel' symbioses in the field.

CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Drs. John Turner and Ian Lucas, for their patience, support and encouragement during the course of this project. I am also indebted to Dr. Gay Kennaway for her assistance with the pigment analysis and radioisotopic work. I am very grateful to Dr. Angela Douglas, who encouraged me to investigate the relationship between symbiosis specificity and the CZAR, and to Dr. Elaine Robson for her continued and enthusiastic interest in my work. I am also indebted to Dr. Andy Yule, for his invaluable advice on statistics. I would like to thank all those technicians who have assisted me over the past 3 years. In particular, I am grateful to Mr. Gareth Williams for his expert advice on the use of ^{14}C, and to Mrs. Ann Buckland for the preparation of TEM sections and grids. I would also like to thank Mr. David Roberts and Mr Bill Rowntree, whose superb photographic skills are responsible for the photographs of anthozoans and the quality of the plates that appear in this thesis. I am also very grateful to my mother, Mrs. Hazel Davy, for her company and help whilst collecting sea anemones from the south coast of England, and to all the members of the 1992 Lough Hyne expedition for collecting material on my behalf.

My sincerest thanks go to my parents, whose unquestioning support during the whole of my academic career has enabled me to achieve all that I have. This project was funded by the S.E.R.C..

CHAPTER 1

BROAD AIM, BROAD INTRODUCTION AND SPECIFIC OBJECTIVES

1.1 Broad Aim

This work aimed to investigate whether anthozoan-dinoflagellate symbioses in British waters exhibit specificity and how any specificity is reflected in the ability of the symbionts to satisfy the respiratory carbon demands of the host.

1.2 Broad Introduction and Underlying Rationale.

1.2.1 The definition of symbiosis.

The term symbiosis was originally introduced by de Bary in 1879 to describe the "living together of differently named organisms" (Smith, D.C. and Douglas 1987). This phrase encompasses both mutualistic symbioses, where all the partners are 'fitter' together than apart (Law and Lewis 1983), and parasitism, where an organism in the symbiosis benefits at the expense of the 'fitness' of the other organisms in the association (Smith, D.C. and Douglas 1987). The inclusion of both mutualistic and parasitic associations within the concept of symbiosis is still widely supported (Smith, D.C. et al. 1969; Roughgarden 1975; Starr 1975; Trench 1979; Smith, D.C. 1980; Trench 1981; Smith, D.C. and Douglas 1987). This definition of symbiosis takes account of the possible shift between mutualism and parasitism in an

association. Douglas and D.C. Smith (1983) stressed that any benefit a host may derive from a symbiosis is the result of a net balance between gross benefit and cost. Therefore, symbioses which are normally considered as mutualistic may become parasitic under certain developmental and environmental conditions (Douglas and Smith, D.C. 1983; Steen 1986; Tytler and Spencer Davies 1986; Turner 1988; Suharsono et al. 1993).

1.2.2 The classification and distribution amongst host taxa of photosynthetic symbionts in marine invertebrate symbioses.

Most symbioses involve two coexisting organisms with distinctly different evolutionary and genetic histories, the larger organism being referred to as the host and the smaller organism being referred to as the symbiont (Smith, D.C. and Douglas 1987). In the aquatic environment, photosynthetic symbionts have been identified in symbiosis with several species of protist and species of a number of animal phyla (eg. Porifera, Ctenophora, Cnidaria, Platyhelminthes, Mollusca and Chordata) (Droop 1963; Trench 1979). These photosynthetic symbionts have been classified using the terms 'zooxanthellae', 'zoochlorellae' and 'cyanellae'. The 'cyanellae' are all prokaryotic cyanobacteria (blue-green algae), however the terms 'zooxanthellae' and 'zoochlorellae' refer to algae from several taxa, simply referring to yellow/brown and green algal symbionts respectively. Consequently, although these

terms are derived from names originally proposed for generic taxa by Brandt in 1882, no precise taxonomic connotation can be attached to them (Blank and Trench 1986).

The commonest 'zoochlorella' belong to the genus Chlorella and class Chlorophyceae. Algae of the genus Chlorella are predominantly freshwater and found in symbiosis with a range of fresh water taxa, including the Protozoa, Porifera, Cnidaria, Mollusca and Platyhelminthes (Smith, D.C. and Douglas 1987). The most widely investigated symbioses involving Chlorella cells are with cnidarians, and in particular hydra (eg. Muscatine and Lenhoff 1965; McAuley 1982; Neckelmann and Muscatine 1983; Douglas and Smith, D.C. 1984; Taylor, C.E. et al. 1989). Chlorella-like symbionts have also been identified in symbiosis with a marine cnidarian, the temperate anemone Anthopleura xanthogrammica, in which the green alga coexists with a 'zooxanthella' (Muscatine 1971; O'Brien 1980). Chloroplasts of algae belonging to the Chlorophyceae also exist in symbiosis with marine sacoglossan opisthobranch molluscs (Taylor, D.L. 1968a; Marin and Ros 1992).

The predominant symbionts included in the 'zooxanthellae' can been placed in the phylum or division Dinophyta (in botanical nomenclature) or Dinoflagellata (in zoological nomenclature), and the class Dinophyceae ("dinoflagellates") (Dodge 1984; South and Whittick 1987; Sleigh 1989). But there are subtle differences between the classifications given by various authors, even within botanical and

zoological systems. The assignment of the dinoflagellates to both botanical and zoological classification schemes illustrates the difficulty in classifying a group of organisms of which only 40-60 % are photosynthetic (Taylor, F.J.R. 1987). However, because of the photosynthetic nature of non-parasitic symbiotic dinoflagellates, the Dinophyceae will be considered here as algae.

Dinoflagellates belonging to the orders Gymnodiniales, and most recently the Peridiniales, Phytodiniales and Prorocentrales, have been identified in symbioses with invertebrates (Trench 1992; Banaszak et al. 1993). Dinoflagellate symbionts belonging to the order Gymnodiniales are by far the best known of these taxa and in particular symbionts of the genera Amphidinium and Symbiodinium have been extensively studied.

D.L. Taylor (1974) recognized three species of Amphidinium as symbionts: A. chattonii in the 'By-the-Wind-Sailor' Velella velella, A. klebsii in the flatworm Amphiscolops langerhansi, and Amphidinium sp., which is also known as Zooxanthella nutricola (Blank and Trench 1986), in the radiolarians Collozoum inerme and Sphaerozoum punctatum. But recently, Banaszak et al. (1993) have identified the symbiont of Pacific and Mediterranean V. velella as Scrippsiella velellae or Scrippsiella chattonii (order Peridiniales) respectively and the symbiont of C. inerme as Scrippsiella nutricola. The symbionts of the flatworm Haplodiscus sp. have been identified as A. belauense (Trench

and Winsor 1987).

Without doubt, the most important gymnodinioid symbionts, both with respect to the numbers of associations with which they are involved and their ecological importance, belong to the genus Symbiodinium. Symbiodinium cells have been identified in association with hundreds of host species representing 3 phyla distributed from the Caribbean, through the warmer waters of the Atlantic, to the Indo-Pacific (Taylor, D.L. 1974). By far the most common hosts of Symbiodinium cells are the Cnidaria, although species of the Mollusca (particularly Tridacna spp. and nudibranchs) (Taylor, D.L. 1969a; Kempf 1984; Hoegh-Guldberg et al. 1986; Klumpp et al. 1992) and Porifera (Smith, D.C. and Douglas 1987) have also been identified as hosts. Of the Cnidaria, the Anthozoa (zoanthids, sea anemones, stony corals and octocorals) act as the most frequent hosts. In these animals, contrary to the conclusion of Kawaguti (1964), the algal symbionts are held singly within vacuoles of the host endodermal cells (Glider et al. 1980; Van-Praet 1985; Trench 1987; Shick 1991; Gates et al. 1992). In contrast, Symbiodinium cells in the tridacnid clams are located intercellularly (Fitt and Trench 1981).

Originally believed to be a single, pandemic species, Symbiodinium microadriaticum (Freudenthal) (Kevin, M.J. et al. 1969; Taylor, D.L. 1969a), different strains or species of Symbiodinium have now been indicated (Kinzie and Chee 1979; Schoenberg and Trench 1980a, b and c; Blank and Trench

1985; Trench and Blank 1987; Rowan and Powers 1991b). 4 species and 1 sub-species have been specifically identified in published work, in spite of the lack of data illustrating sexual recombination between Symbiodinium cells (cf. Freudenthal 1962; Taylor, D.L. 1974). These species are S. microadriaticum in the 'Upside-down Jellyfish' Cassiopeia sp. (Freudenthal 1962), S. goreauii in the Caribbean sea anemone Ragactis lucida, S. kawagutii in the Hawaiian stony coral Montipora verrucosa and S. pilosum in the Caribbean zoanthid Zoanthus sociatus (Trench and Blank 1987), and S. microadriaticum subsp. condylactis from the Caribbean sea anemone Condylactis gigantea (Blank and Huss 1989). In addition to these species, Trench has also named 6 other species of Symbiodinium (Banaszak et al. 1993) but has not published the explanation behind the allocation of these symbionts to different species. The specific identity of the Symbiodinium cells in other host species has not been resolved. Therefore, these symbionts are referred to simply as Symbiodinium sp., clearly stating the host species and origin (Blank and Trench 1986).

The distribution of different species or strains of Symbiodinium amongst different host species has also been a matter of much speculation. Suggestions have ranged from a different strain or species of symbiont in each host species (Loeblich and Sherley 1979), to symbionts of identical genetic 'make-up' inhabiting host species of different classes of Cnidaria (Rowan and Powers 1991a). However, there

is no evidence at present that the symbionts of the same host species differ between individual hosts (Rowan and Powers 1991a and b).

1.2.3 The geographic distribution of Symbiodinium-invertebrate symbioses and the advantages of the symbiotic habit in these environments.

Anthozoan-Symbiodinium symbioses are common in tropical seas. There is concensus that the dramatic rise in the importance of hermatypic (as defined by Schuhmacher and Zibrowius (1985)) Scleractinia as major contributors to carbonate accretions that occurred during the Triassic was the result of the initiation of the symbiosis between the corals and dinoflagellates (Trench 1981; Trench 1987). The Symbiodinium-invertebrate symbiosis in tropical shallow seas enables both partners to inhabit an environment which is low in nutrients, due to the symbiont induced ability of the host to deplete exogenous inorganic nutrients that would otherwise be unavailable for host assimilation (Miller and Yellowlees 1989) and the cycling of nutrients (particularly nitrogen, phosphorus and sulphur) between host and symbiont. This cycling conserves the nutrients and maintains nutrient levels within the symbiosis at concentrations far greater than those in the ambient water (Muscatine 1980; Cook 1983; Miller and Yellowlees 1989). In addition, the presence of dinoflagellate symbionts in scleractinian corals increases the rate of skeletogenesis (Buchsbaum Pearse and Muscatine

1971; Chalker and Taylor, D.L. 1975; Goreau, T.J. 1977), possibly by photosynthetic utilization of CO_2 enhancing the removal of carbonic acid (H_2CO_3) and hence shifting the equilibrium of other reactions in a direction favourable to calcium carbonate ($CaCO_3$) deposition (Goreau, T.F. 1959; Goreau, T.F. 1961). Alternatively, the translocation of photosynthate from the symbionts to host may supply the energetic demands of skeletogenesis (Taylor, D.L. 1977), in addition to supplementing the associated tissue growth (Spencer Davies 1984; Edmunds and Spencer Davies 1986). The higher growth and calcification rates of corals possessing algal symbionts, compared to corals permanently without symbionts, has been suggested as the reason for the virtual absence of corals lacking dinoflagellate endosymbionts from open reef environments where coral-Symbiodinium symbioses predominate (Wellington and Trench 1985).

In temperate seas, anthozoan-dinoflagellate symbioses are comparatively uncommon (Droop 1963; Taylor, D.L. 1974; Turner 1988). This scarcity may in part be the result of the physiological limitations of the symbionts (Clark and Jensen 1982), but may also reflect the eutrophic nature of temperate waters and hence the reduced selective advantage for a symbiotic existence. Due to inhabiting nutrient rich waters the benefit to temperate symbionts and hosts are less clear than in the tropics. But, as in tropical symbioses, the presence of algal symbionts does present a further route by which the host can obtain nutriment. Fixed carbon

translocation has been implicated in the enhanced calcification of temperate ahermatypic corals (Szmant-Froelich 1981; Jacques et al. 1983) and increased growth rates of temperate sea anemones (Taylor, D.L. 1969c; O'Brien 1980; Tytler and Spencer Davies 1986). Tytler (1982) revealed that the enhanced growth efficiency of the temperate sea anemone Anemonia viridis is comparable to that of higher invertebrates, perhaps allowing the host to compete more effectively with higher invertebrates in the tidal and subtidal communities. Van-Praet (1985) suggested that the predominant benefit of the symbiosis to A. viridis was a nutritional one during seasonal periods of reduced food availability. This same benefit was suggested by Marin and Ros (1992) for the mollusc Elysia timida, which retains chloroplasts of the green alga Acetabularia acetabulum. Turner (1988) suggested that despite anthozoan-Symbiodinium symbioses alternating between mutualism in summer and parasitism in winter, the net benefit derived from the symbiosis increases the reproductive 'fitness' of the association. But whether the reproductive 'fitness' of the symbiont population itself is increased by the symbiosis and hence whether the association is truly mutualistic remains to be investigated in any Symbiodinium-invertebrate symbiosis. The fact that free-living Symbiodinium cells have never been reported in temperate waters (in contrast to one tentative identification in the tropics (Taylor, F.J.R. 1983)) could indicate that this alga is an ecologically

obligate symbiont whose reproductive 'fitness' must therefore be enhanced by being in symbiosis (Law and Lewis 1983).

The predominant benefit of both temperate and tropical symbioses appears to be a nutritional one. The extent to which the Symbiodinium spp. populations can satisfy the nutritional requirements of the hosts, particularly with respect to carbon, has been investigated extensively (Muscatine et al. 1981; Spencer Davies 1984; Muscatine et al. 1984; Tytler and Spencer Davies 1986; Farrant et al. 1987b; Klumpp et al. 1992). In addition to environmental influences on carbon fluxes within Symbiodinium-invertebrate symbioses (Dykens and Shick 1984; Hoegh-Guldberg et al. 1986; Farrant et al. 1987b), the level of contribution from the Symbiodinium cells to the host's organic carbon requirements may reflect symbiont strain or species specific differences in net photosynthetic capacity and translocation of photosynthates. Alternatively, different contributions of carbon to the host may depend on the symbiont standing stocks, photosynthetic rates and translocation levels that arise as a result of varying degrees of symbiosis integration ie. the degree to which the symbiosis is greater than the sum of its parts (Starr 1975).

1.2.4 The specificity and recombination of dinoflagellate-invertebrate symbioses.

It has become clear that specificity, the unique affinity of one entity for another (Trench et al. 1981a), exists between a strain or species of Symbiodinium and its host (Kinzie and Chee 1979; Schoenberg and Trench 1980c; Trench et al. 1981a; Hinde 1988). The poor correlation between host taxa and the presence or absence of algal symbionts (Trench 1981), the fact that Symbiodinium spp. taxonomy is largely independent of host taxonomic status (Rowan and Powers 1991a), and the inability of some strains or species of Symbiodinium to persist when infected into temporarily symbiont-free ('aposymbiotic') hosts (Schoenberg and Trench 1980c) have all suggested a specific symbiont-host relationship. This specificity has also been indicated by records of indistinguishable isoenzyme patterns and genetic sequences in Symbiodinium populations from different individuals of the same host species from up to 1600 km apart (Schoenberg and Trench 1980a; Rowan and Powers 1991a and b).

All investigations of anthozoan-Symbiodinium specificity have been conducted using tropical hosts and symbionts, with the exception of the infection of the tropical scyphozoan Cassiopeia xamachana with freshly isolated symbionts of the temperate North American anemone Anthopleura elegantissima (Colley and Trench 1983). The work presented in this thesis therefore aimed to investigate whether the little studied

anthozoan-algal symbioses in British waters exhibit specificity and whether host-symbiont recognition, which leads to specificity (Trench et al. 1981a; Smith, D.C. and Douglas 1987), is pre- or post-endocytotic. More importantly, with much wider implications, it was aimed to investigate the influence of specificity on fixed carbon flux within the symbiosis. Under certain circumstances (chapter 4, section 4.1 (p.167)) hosts may become aposymbiotic, rendering them susceptible to reinfection by different strains or species of Symbiodinium than that usually associated with the host. Rowan and Powers (1991a) suggested that following such reinfections the host and symbiont metabolisms would interact to their mutual benefit and, owing to the predominantly clonal reproductive strategy of cnidarians, these 'novel' symbioses would propagate. Therefore, using a temperate anthozoan as the 'model' polyp, it was proposed that the ability of Symbiodinium sp. from different host species to satisfy the respiratory carbon budget of the host be measured. Conclusions regarding the potential for the propagation of the 'novel' symbiosis could then be inferred.

1.3 Specific Objectives

The specific objectives of the project were:

(a)To characterize freshly isolated and cultured symbionts of the British Anthozoa with respect to their morphologies, pigment profiles and division patterns in culture, and to compare these characteristics with those of a sub-tropical strain of Symbiodinium sp. and previously published details of Symbiodinium spp. from other host species.

(b)To measure the extent to which symbionts of different British and sub-tropical host species are endocytosed by and persist in reinfected aposymbiotic individuals of a host anthozoan.

(c)To relate any observed specificity to the ability of the reinfected symbionts to translocate carbon and contribute to the host's daily respiratory carbon budget (CZAR). Thus to infer whether excess carbon is potentially available to supplement the requirements of host growth and reproduction.

CHAPTER 2

COLLECTION AND MAINTENANCE OF SYMBIOTIC ANTHOZOA AND DINOFLAGELLATES

2.1 GENERAL INTRODUCTION

The specific aims of this chapter are to describe: (a) The characteristics of the anthozoan hosts under investigation; (b) the collection of symbiotic Anthozoa; (c) the maintenance of the symbiotic anthozoans in the laboratory; and (d) the isolation and culture of the symbionts.

In contrast to the common occurrence of anthozoan - dinoflagellate symbioses in tropical waters, such symbioses are rare in temperate waters. Of the 74 species of Anthozoa inhabiting British waters only 5 have been reported to possess endosymbiotic dinoflagellates: The actiniarians Aiptasia mutabilis (= couchii) (Gravenhorst), Anemonia viridis (= A. sulcata) (Forskal), Anthopleura ballii (Cocks) and Cereus pedunculatus (Pennant), and the zoanthid Isozoanthus sulcatus (Gosse). With the exception of A. viridis (eg. Taylor, D.L. 1967; Tytler and Spencer Davies 1986; Stambler and Dubinsky 1987; Harland et al. 1992; Rands et al. 1992b) the involvement of these host species with an endosymbiotic alga has been little recognised and studied. Schoenberg and Trench (1980a) claimed that A. viridis was the only British symbiotic anthozoan and in the last substantial list of symbiotic anthozoans, D.L. Taylor (1974) noted only A. viridis and C. pedunculatus despite Yonge

(1968) earlier recognising A. ballii as being symbiotic. The C. pedunculatus and A. ballii symbioses have only been explored in any detail with respect to their ecology and symbiont acquisition (Turner 1988). However, the close American relative of A. ballii, Anthopleura elegantissima, has been studied extensively (eg. Franker 1971; Buchsbaum Pearse 1974; Smith, B.L. and Potts 1987; Dykens et al. 1992). The symbiotic nature of I. sulcatus and A. mutabilis was first recorded by Manuel (1981). The symbiotic relationships involving these two host species have not been studied at all, as is reflected by their absence from the exhaustive reviews of Trench (1987) and Shick (1991). Therefore, the initial aim was to incorporate all 5 of the British symbiotic Anthozoa into the proposed project, although logistical reasons prevented the inclusion of A. mutabilis.

Most work involving symbioses between marine invertebrates and dinoflagellates of the genus Symbiodinium has been conducted using tropical and sub-tropical organisms. The most widely employed host species of actiniarian have been Aiptasia pulchella (eg. Chang et al. 1983; Wilkerson et al. 1983; Rowan and Powers 1991b; Gates et al. 1992) and Aiptasia pallida (= A. tagetes) (Verrill) (eg. Steele 1977; Clayton and Lasker 1984; Cook et al. 1988; Rowan and Powers 1991b). It was therefore proposed to incorporate the subtropical A. pallida into the research as a comparison with the temperate symbioses.

TABLE 2.1 CLASSIFICATION OF SYMBIOTIC ANTHOZOA UNDER INVESTIGATION

Species	Family	Order
Anemonia viridis (Forskal)	Actiniidae	Actiniaria
Anthopleura ballii (Cocks)	Actiniidae	Actiniaria
Cereus pedunculatus (Pennant)	Sagartiidae	Actiniaria
Isozoanthus sulcatus (Gosse)	Parazoanthidae	Zoanthiniaria
Aiptasia pallida (Verrill)	Aiptasiidae	Actiniaria

2.2 THE COLLECTION AND MAINTENANCE OF ANTHOZOA

2.2.1 DESCRIPTION OF THE ANTHOZOAN HOSTS USED

2.2.1.1 Introduction

The British symbiotic Anthozoa employed in this work and the sub-tropical *A. pallida* are classified (table 2.1 (p.16)) and described with respect to their morphologies, reproductive strategies, habitat preferences and distributions. This information is essential in the identification, collection and successful maintenance and culture of these anthozoans in the laboratory.

2.2.1.2 Species Descriptions

(a)*Anemonia viridis*

(plate 2.1 A (p.23)).

A. viridis, or the snakelocks anemone, grows to approximately 70 mm across the base with a tentacle span of up to 180 mm (Manuel 1981). *A. viridis* possesses a lightly adherent basal disc which is wider than the column. The smooth column is often completely contracted, however it may extend to be much taller than its width. The long, thick but highly flexible tentacles are irregularly arranged and number up to 200 (Manuel 1981). The tentacles are usually extended, but they may be retracted into the column on occasion, perhaps for cleaning or protection from predators (Turner 1988; Dr. J.R. Turner, UCNW, pers. comm.). The

animals are either green, brown or grey in colour, often with purple tips to the tentacles and a reddish tinge to the column and base. White radial lines are also frequently seen on the oral disc.

Reproduction is oviparous or, more frequently, asexual by longitudinal fission (Shick 1991).

A. viridis is locally abundant around the south and west coasts of Britain, being found from the mid-shore to approximately -20m (Manuel 1981). Its northern limit is the mid-west coast of Scotland (Manuel 1981) with its eastern limit in Britain being Brighton on the south coast of England (Turner pers. comm.). A. viridis is abundant on all south-west coasts of Europe and in the Mediterranean where it favours well lit, low to mid-energy sites. The anemones are usually attached to rocks or weed (particularly leaves of the seagrass Zostera sp.) (Manuel 1981; Turner 1988).

(b)Anthopleura ballii

(plate 2.1 B (p.23)).

A. ballii possesses a moderately adherent base which is marginally wider than the column. In full extension the column is trumpet shaped, however it may be completely retracted and therefore not visible below the oral disc. The column possesses 48 longitudinal rows of distinctive verrucae, with each row terminating on the parapet with a large, conical or rounded acrorhagus. The oral disc is wide and flat with the stout tentacles arranged towards the outer edge. The tentacles are in groups of 6 in 5 cycles, number

up to 96 and are usually curled at the tips. The maximum tentacle span is 120mm. The animals are brown to yellow/brown in colour, often with a purple or pink flush to the column. The whole body is normally flecked with white markings and dark stripes may join the bases of the tentacles (Manuel 1981).

Reproduction is via oviparity (Turner 1988; Turner 1989) or very rarely transverse fission (Turner pers. comm.).

Manuel (1981) reported that A. ballii inhabited holes and crevices in rocks or was buried in sand or mud attached to a hard substrate, from the mid-shore down to -25m. A. ballii is a common species in the Mediterranean but is locally abundant around Britain. It is confined to the south and west, from the Isle of Wight to the Isle of Man in the north and south-west Ireland in the west (Manuel 1981; Turner 1988).

(c)Cereus pedunculatus

(plate 2.2 A (p.24)).

C. pedunculatus, the daisy anemone, possesses a strongly adherent but fragile base which is just wider than the column. The column is trumpet-shaped with small suckers, often with attached pieces of debris. Small perforations (cinclides) are present in the column through which acontia (typical of the family Sagartiidae) are readily emitted upon disturbance. The oral disc is comparatively wide with over 200 small tentacles positioned around the undulating edge. The tentacles are arranged in multiples of 6 in up to 9

cycles. The diameter of the oral disc reaches 150mm, but more normally 30-70mm, and the animal may be up to 120mm in height. C. pedunculatus is generally buff brown to grey/brown in colour with the column being paler than the oral disc. The oral disc is flecked with white and dark brown/black to greatly varying degrees. The column suckers are white or grey and pale endocoelic stripes may also be visible on the column (Manuel 1981).

C. pedunculatus reproduces sexually by oviparity or asexually by parthenogenesis or hermaphroditism and viviparity (Rossi 1975; Shick 1991). The brooding of many juveniles in the coelenteron results in adults often being surrounded by their offspring (Turner 1988).

C. pedunculatus inhabits rocky shores, but is especially numerous buried in sand or mud attached to underlying rocks and shells. It is found in pools from the mid-shore to -25m. This anemone is abundant around the south-west coasts of the British Isles from west Scotland to the eastern end of the English Channel. It is also common throughout south-west Europe and the Mediterranean (Manuel 1981).

(d)Isozoanthus sulcatus

(plate 2.2 B (p.24))

I. sulcatus is the only species of European zoanthid known to possess endosymbiotic dinoflagellates. Like all Zoantharia it is colonial, with the coenenchyme forming an irregular basal network. The polyps, which are up to 2mm in diameter and 4mm in height, arise from the coenenchyme at

irregular intervals or in clusters. The polyps possess 19-22 tentacles which are arranged in 2 equal cycles. The column is smooth and has sand grains embedded in it. The distal end of the column terminates in marginal teeth which, unique for a British zoanthid, correspond to each tentacle rather than just the tentacles of the inner circle. I. sulcatus is olive or purple/brown in colour with white tentacle tips (Manuel 1981).

Reproduction occurs asexually by proliferation of the coenenchyme. No information is available on the method of sexual reproduction.

I.sulcatus inhabits pools on the shore amongst calcareous algae and detritus or shallow coastal waters down to -25m where it is found attached to sediment covered horizontal rock faces (Manuel 1981; Turner, pers. comm.). This zoanthid appears to be rare in British waters. However the inconspicuous nature of the animal and the habit of the polyps withdrawing upon the slightest disturbance may result in colonies being hidden by sediment and overlooked. I. sulcatus is present from the Mediterranean to south-west Britain. Confirmed identifications around the British Isles come from south Devon and south Wales (Manuel 1981), south west Ireland (Turner 1988) and Anglesey and north Wales (Turner, pers. comm.).

(e)Aiptasia pallida

(plate 2.3 A (p.25))

The subtropical A. pallida is similar in structure to the temperate A. mutabilis. The basal disc of A. pallida is marginally wider than the column and lightly adherent. The column is smooth and when extended tapers gradually towards the oral end, reaching a maximum height of approximately 60 mm. Cinclides are present in a narrow band in the middle of the column, although these are only apparent when the acontia are discharged. The long, smooth tentacles, of which there may be 50-100, are arranged in groups of 6 and are rarely retracted; on disturbance the animal rapidly contracts its column pulling the oral disc onto or into the substrate. The oral disc is concaved with the tentacles positioned on the outer edge. The maximum tentacle span is approximately 50 mm. The animal is a uniform pale yellow/brown in colour, occasionally with tiny white spots on the column. A. pallida is transparent, enabling the observation of the pharynx and mesenteries (pers. obs.).

A. pallida reproduces asexually via frequent pedal laceration (Clayton 1985) or sexually by oviparity.

A. pallida is a very common anemone throughout the subtropics where it inhabits lagoon beds and well lit pools, particularly around mangroves (B. Walters, Bermuda Biological Research Station, pers. comm.).

Plate 2.1: British Symbiotic Anthozoa.

(A) *Anemonia viridis* (scale bar = 1.5 cm).

(B) *Anthopleura ballii* (scale bar = 1.5 cm).


A

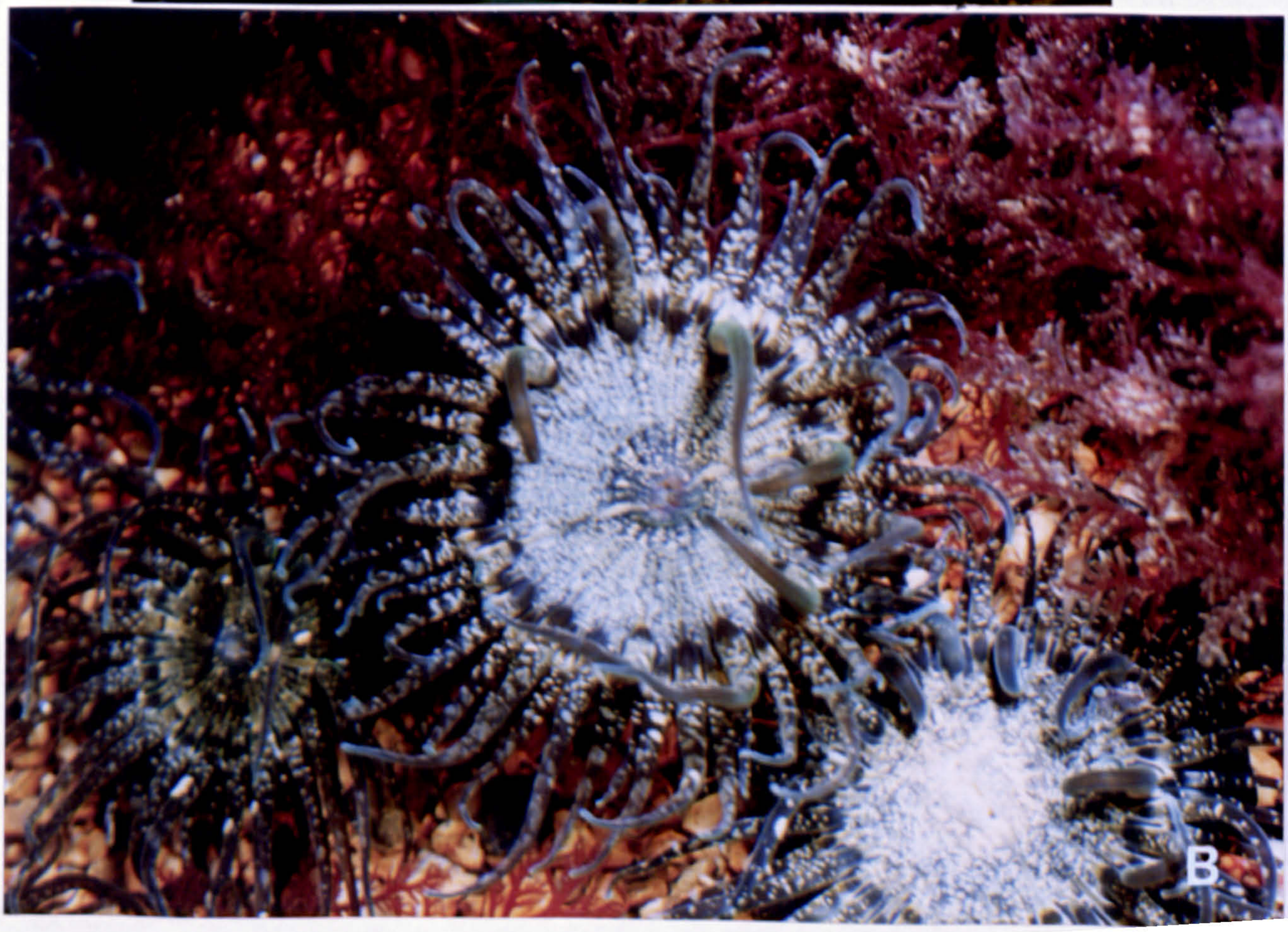

B

Plate 2.2: British Symbiotic Anthozoa Contd..

(A) **Cereus pedunculatus** (scale bar = 0.7 cm).

(B) **Isozoanthus sulcatus** (scale bar = 0.6 mm).


A

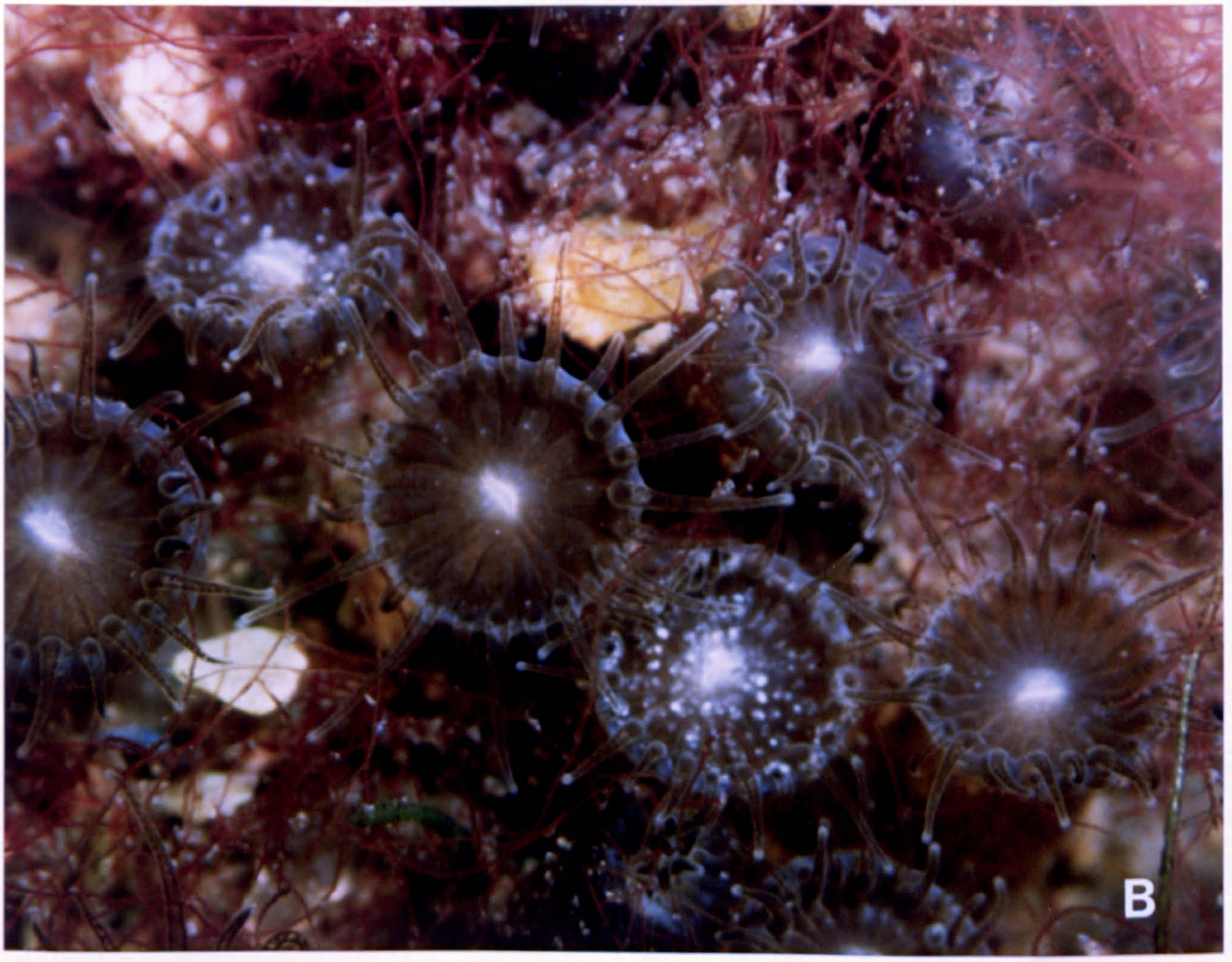

B

Plate 2.3: Subtropical Symbiotic Anthozoa.

(A) *Aiptasia pallida* (scale bar = 1 cm).

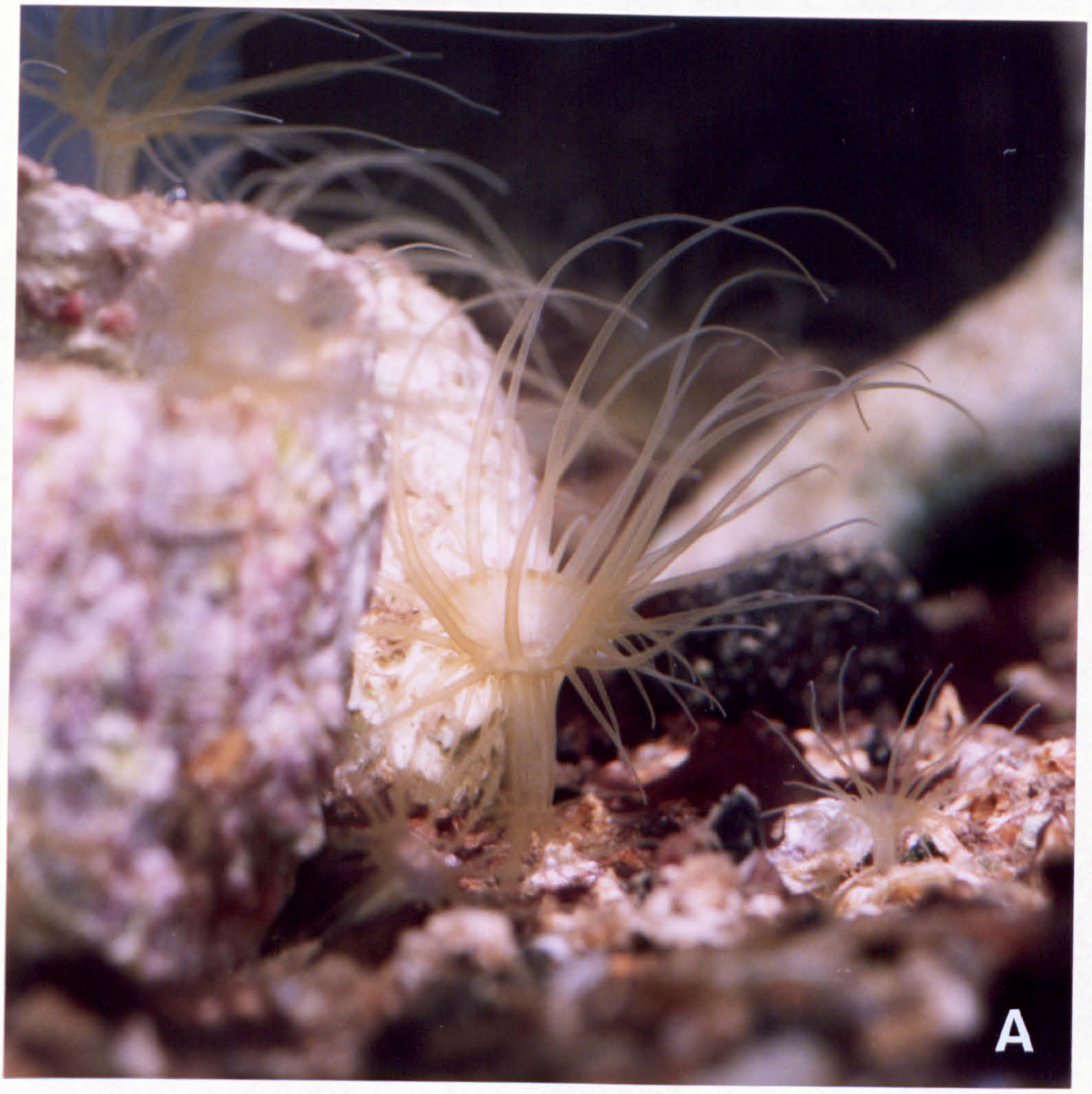

A

TABLE 2.2 LOCATIONS AND MAP REFERENCES OF ANTHOZOAN COLLECTION SITES

Location and Date	Map Reference	Species Collected
Lough Hyne, Co. Cork, Eire (August 1992)	51° 29' N, 9° 18' W	A. viridis, C. pedunculatus, A. ballii, I. sulcatus
Shell Island, Gwynedd, Wales (November 1990, July 1991)	52° 47' N, 004° 06' W	A. viridis
Trearddur Bay, Gwynedd, Wales (January 1992)	53° 16' N, 4° 4' W	A. viridis
Weymouth Harbour, Dorset, England (February 1992)	50° 30' N, 1° 30' W	A. viridis
Bembridge, Isle of Wight, England (February 1992)	50° 37' N, 1° 04° W	A. viridis
Loch Sween, Strathclyde, Scotland (July 1992)	55° 58' N, 005° 38' W	A. viridis
Netley Abbey, Hampshire, England (February 1992)	50° 48' N, 1° 23' W	C. pedunculatus
Bermuda (received January 1991	32° 20' N, 64° 50' W	A. pallida

2.2.2 COLLECTION OF THE ANTHOZOA

2.2.2.1 Introduction

Specimens of A. viridis, A. ballii, C. pedunculatus and I. sulcatus collected from Lough Hyne Marine Nature Reserve in 1988 and 1989 by Dr. J.R. Turner were initially available for research purposes. But further collections were required at later dates. All species of British symbiotic anthozoans are confined to the southern and western coasts of Europe and collections therefore focussed on corresponding British shores, most of which have been described in detail by Turner (1988).

2.2.2.2 Location of collection sites and dates of collection

The anthozoan collection sites and their map references, the dates of collection and the animals collected at each site are shown in table 2.2 (p.26). Maps showing the collection sites are given in figure 2.1 (pp.30-32). Further details of the collection of each species are given below.

(a)A. viridis

Collections of between 50 and 200 A. viridis were made from 6 different sites encompassing the extremes of its distribution around the British Isles. Animals from Loch Sween were collected by R. Beaver, University of Glasgow. A. viridis were collected by gently removing them from rocks on the lower shore during low spring tides, except at Lough Hyne where snorkelling and diving were employed. All diving

work was carried out by a qualified team.

(b)A. ballii

200 specimens of A. ballii were removed from rocks in the subtidal zone in Lough Hyne by snorkelling and diving. This anthozoan has also been reported from the Weymouth collection site (Turner 1988) however no specimens were observed here on the lower shore in February 1992.

(c)C. pedunculatus

C. pedunculatus were collected from 2 sites. The Netley collection was made on the shore at low spring tide whereas the Lough Hyne collection employed snorkelling. Because of the fragile nature of the animal's pedal disc, specimens of C. pedunculatus were collected complete with the rocks or shells to which they were attached. Approximately 30 animals were collected at Netley and 500 animals were collected from Lough Hyne. No sightings of C. pedunculatus were made at Weymouth despite it having been recorded at the site previously (Turner 1988).

(d)I. sulcatus

I. sulcatus was collected subtidally in Lough Hyne by diving. Due to this zoanthid growing on large rocks and cliff faces it was necessary to remove the encrusted pieces of rock using a chisel. The difficulty of locating and removing this animal resulted in the collection of only 6 colonies, each consisting of less than 20 polyps.

(e)A. pallida

30 specimens of A. pallida were obtained from Dr. C. Cook (Bermuda Biological Research Station). These animals were clones of one individual.

All the anemones collected from British waters were immediately placed in plastic bags, 1/4 full of sea water, and deposited in cool boxes for transportation. To prevent oxygen stress occurring to the animals during long journeys the water was changed every day and the bags occasionally aerated using a battery operated pump.

Figure 2.1 Maps of Anthozoan Collection Sites

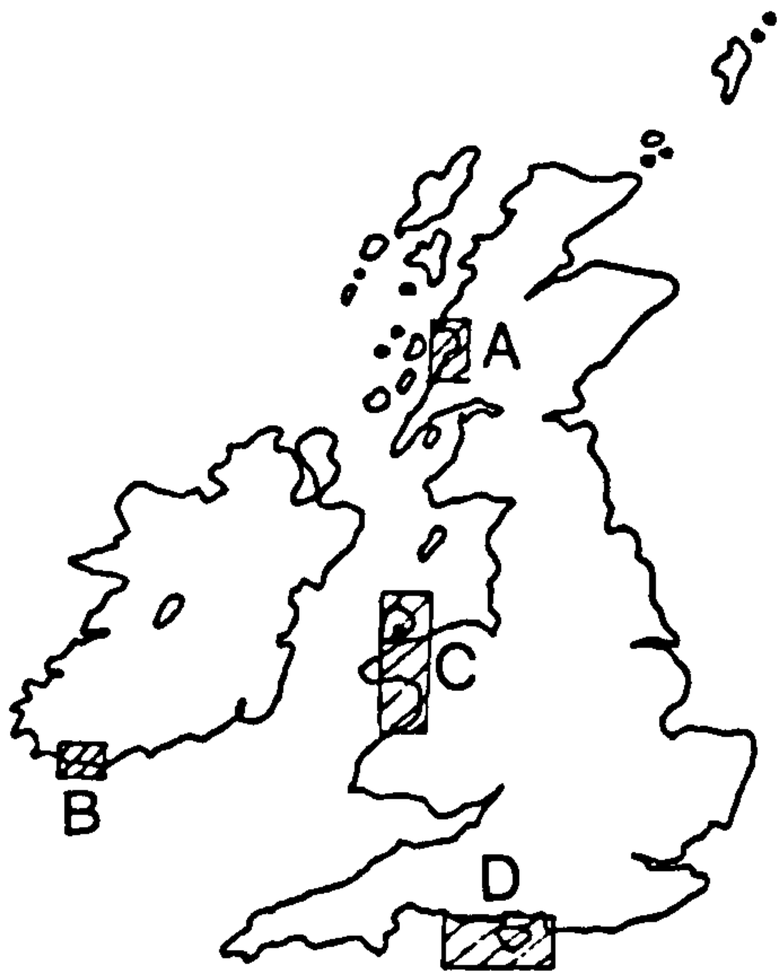


Figure 2.1.1 Location of Collection Sites in Britain

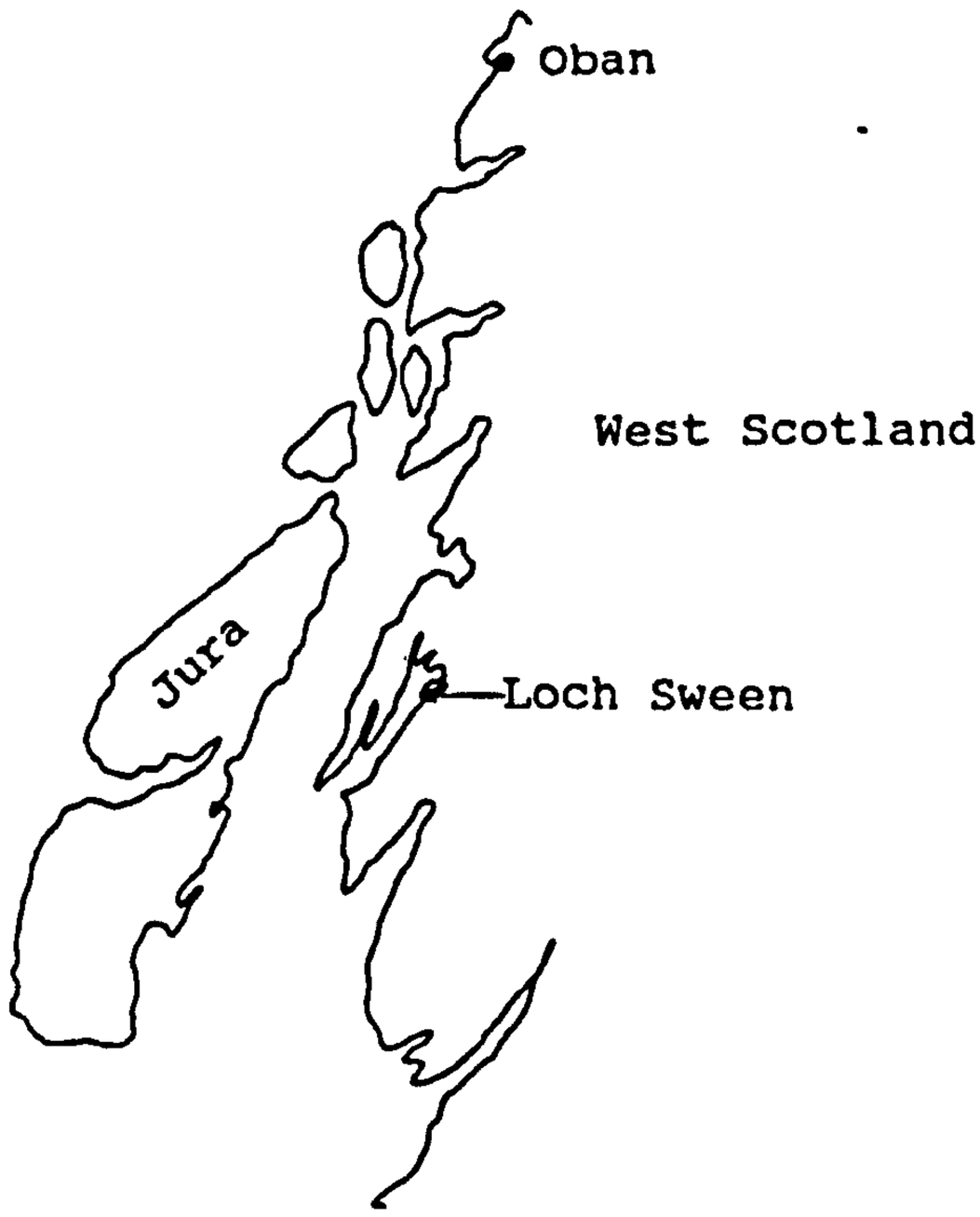


Figure 2.1.2 'A': Location of Loch Sween

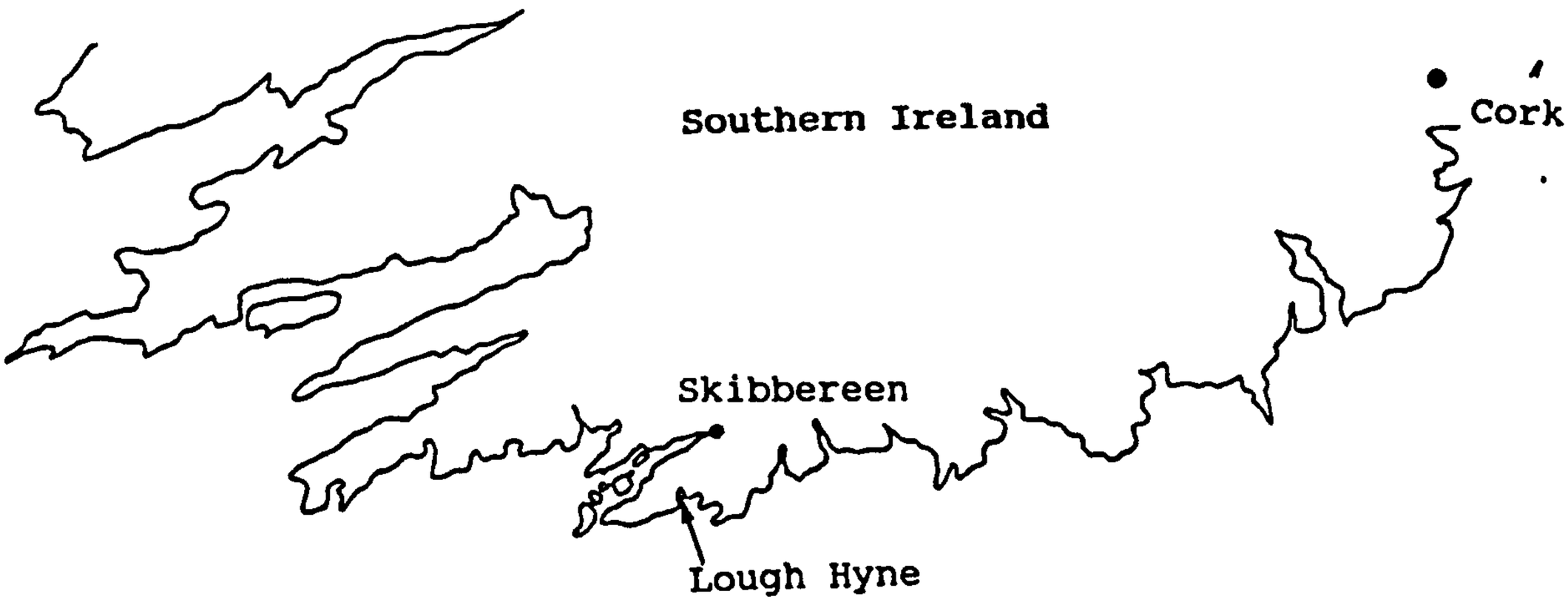


Figure 2.1.3 'B': Location of Lough Hyne

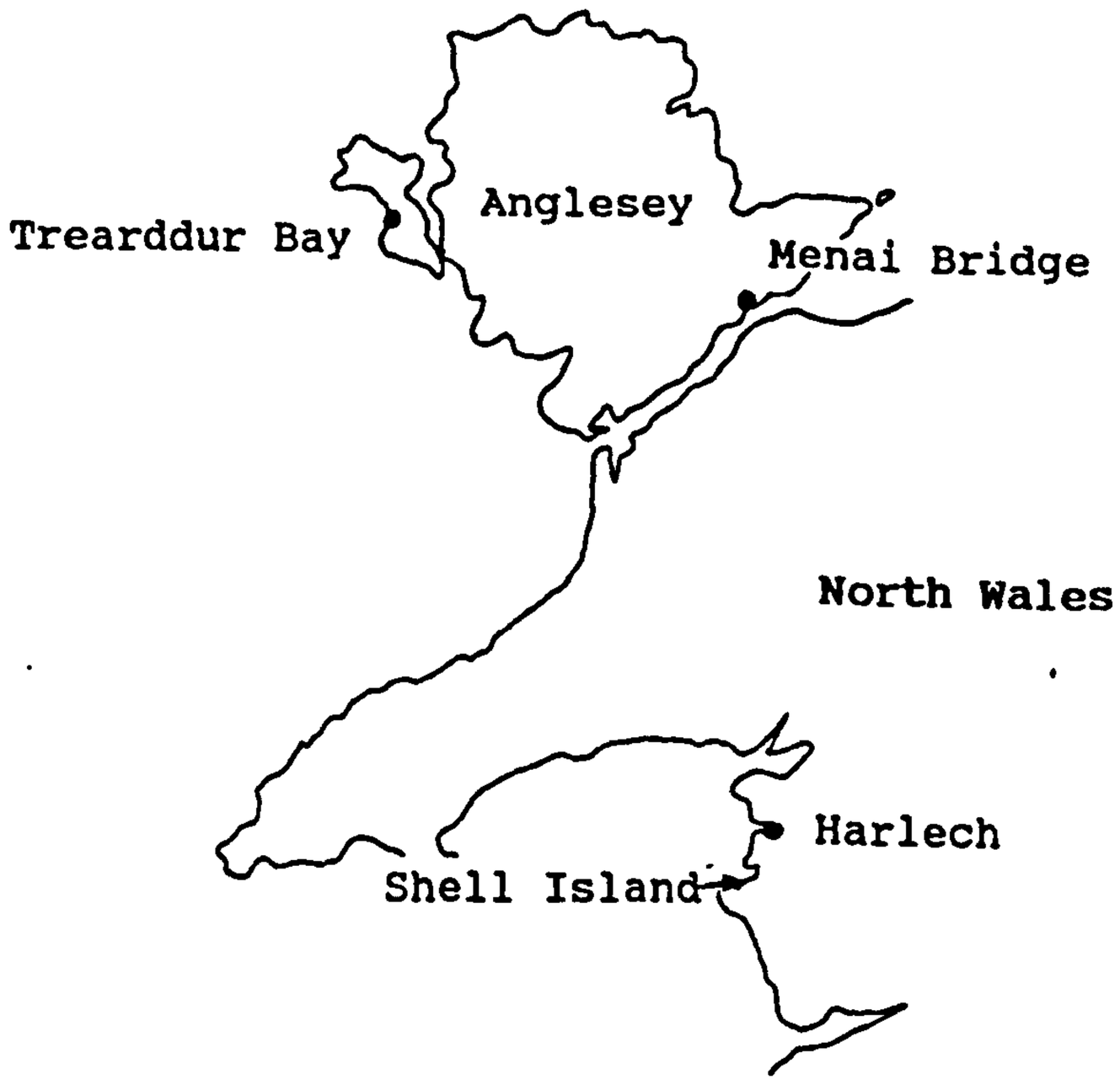


Figure 2.1.4 'C': Location of Shell Island and Trearddur Bay

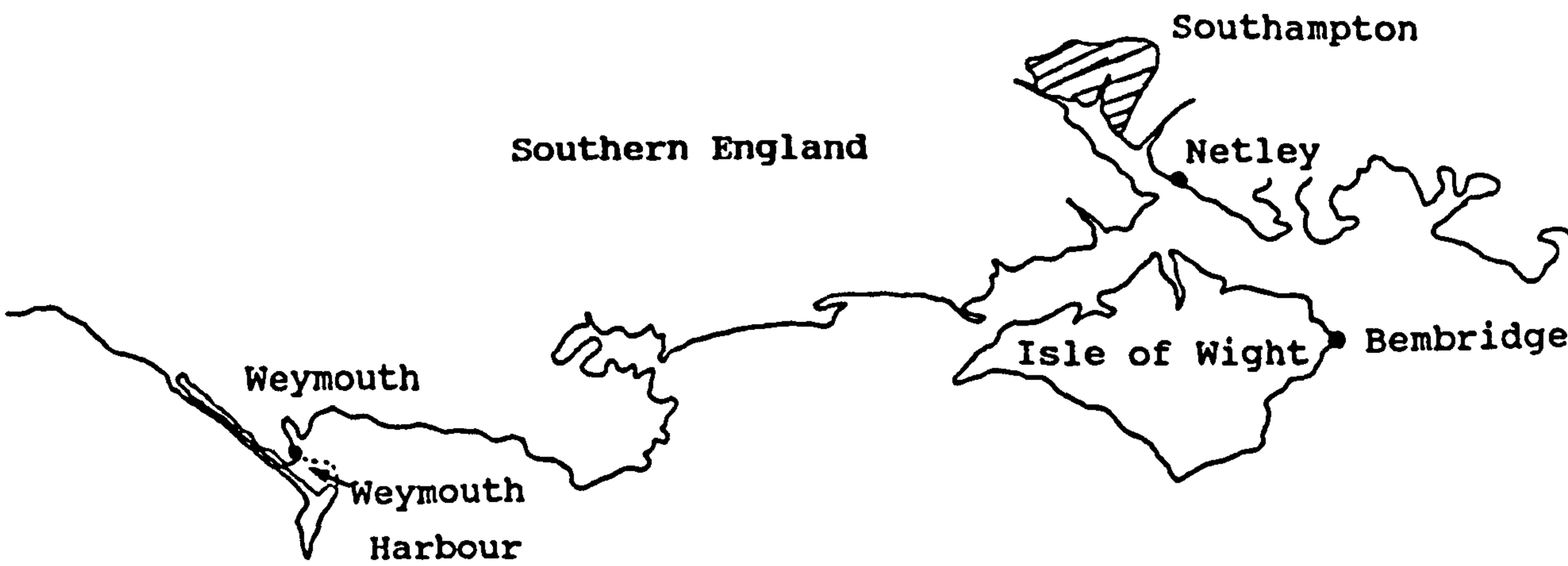


Figure 2.1.5 'D': Location of Bembridge, Netley and Weymouth Harbour

Abbreviations of Anthozoa collected

Av = A. viridis

Ab = A. ballii

Cp = C. pedunculatus

Is = I. sulcatus

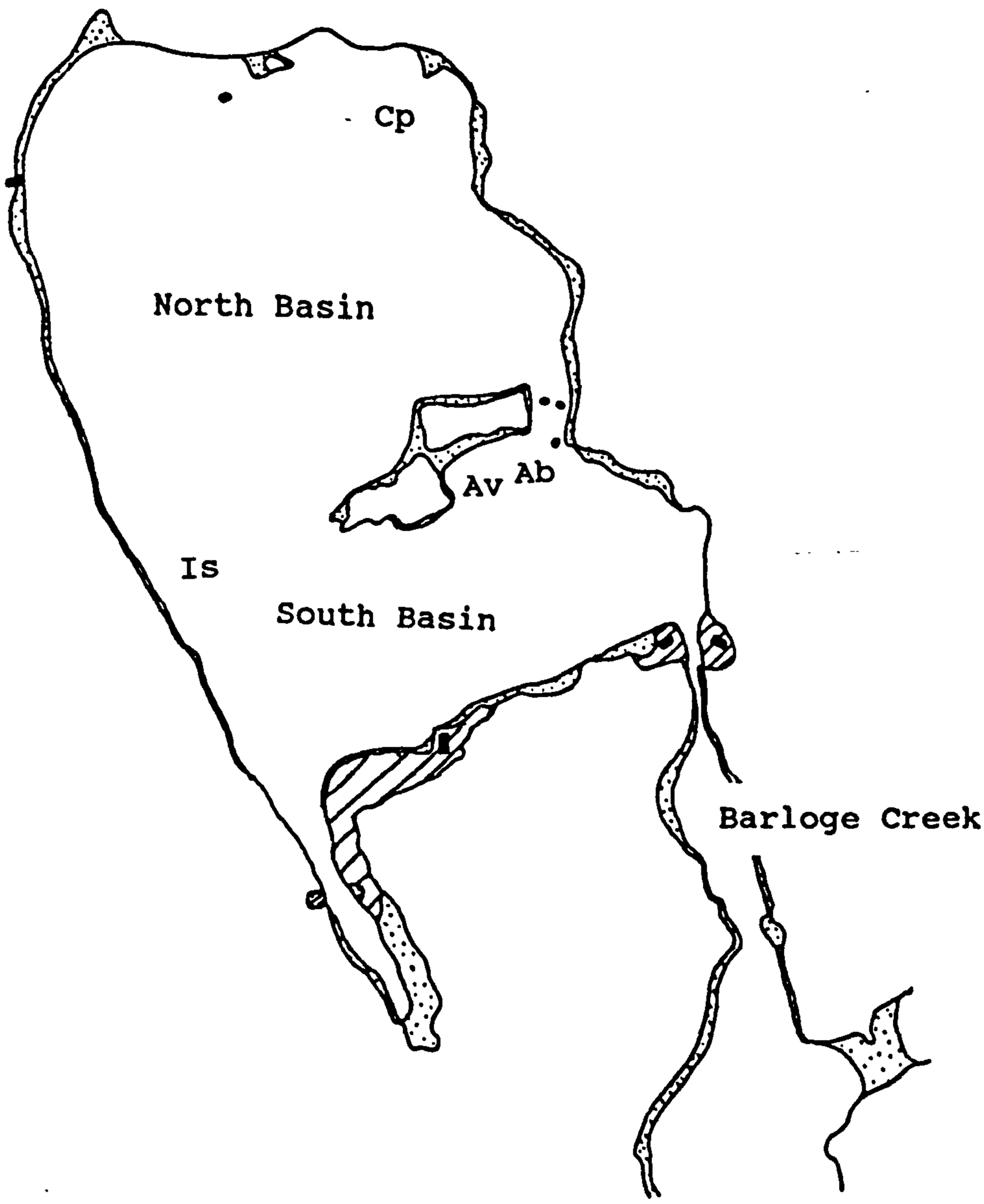


Figure 2.1.6 Map of Lough Hyne Collection Sites

2.2.3 LABORATORY MAINTENANCE OF ANTHOZOA

2.2.3.1 Introduction

A laboratory system was used which would maintain healthy symbioses for experimental use. The health of the symbioses, as reflected by symbiosis stability, may be affected by the following environmental parameters: Temperature; salinity; light; available sources of heterotrophic nutrition; and availability of dissolved inorganic nutrients. Extremes of these parameters may result in "bleaching" (a decrease in the symbiont population density) or overgrowth (caused by an increase in the symbiont population density).

(a)Temperature

Increased temperature is believed to be the predominant factor involved in coral "bleaching" (Jokiel and Coles 1977; Fankboner and Reid 1981; Brown and Howard 1985; Gates et al. 1992; Suharsono and Brown 1992; Suharsono et al. 1993)) and has also been shown to result in the expulsion of symbionts from the temperate anemone Anthopleura xanthogrammica (O'Brien and Wyttenbach 1980). In addition, decreased temperatures have been demonstrated to lead to symbiont expulsion in subtropical A. pallida and A. pulchella (Steen and Muscatine 1987; Muscatine et al. 1991). Temperature shock causes release of intact host endodermal cells containing symbionts, possibly as a result of host cell adhesion dysfunction, as has been demonstrated in A. pulchella and the coral Pocillopora damicornis (Gates et al.

1992). Temperature also influences the population dynamics of the symbionts, which are less thermally adaptable than the hosts (Clark and Jensen 1982). For example, O'Brien and Wyttenbach (1980) demonstrated a reversible decline in the mitotic index (MI) (the percentage of cells dividing) of 'zoochlorellae' in the temperate anemone Anthopleura xanthogrammica from 19 to less than 1 % when the anemones were maintained at 20 ^{o}C. Similarly, Suharsono and Brown (1992) showed a decrease in the MI of the symbiont population of A. viridis from approximately 4.5 to 2.5 % when the maintenance temperature was raised from 16 to 26 ^{o}C. These observations are consistent with the substantial decrease (up to 80 %) in photosynthetic carbon fixation by heat-stressed Symbiodinium sp. (Clark and Jensen 1982). Prolonged temperature shocks have resulted in the mortality of corals or a much reduced calcification rate. Jokiel and Coles (1977) reported that the coral Pocillopora damicornis suffered 95 % and 100 % mortality when maintained at 31 and 18.3 ^{o}C respectively. In contrast, mortality of this species was 0 % between 23.3 and 29.6 ^{o}C. Jokiel and Coles (1977) also showed that the peak calcification rate of P. damicornis of 2.5 mg $CaCO_3$/g/day occurred at 25 ^{o}C, but that this rate decreased to -0.5 and 0.1 mg $CaCO_3$/g/day at 31 and 18.3 ^{o}C respectively.

(b)Salinity

Increased and decreased salinity also result in the expulsion of symbionts (Goreau, T.F. 1964), where decreased

salinity has been suggested to cause necrosis by hypoosmotic shock (Gates et al. 1992). Although, using the coral Siderastrea siderea, Muthiga and Szmant (1987) demonstrated that a change in salinity of more than 10 $^{o}/oo$ may be required before marked changes in cell chlorophyll content and symbiosis photosynthesis and respiration are observed.

(c)Irradiance

Irradiance and light quality may affect the stability of the symbiosis or cause photoadaptation of the symbionts. Firstly, high irradiance (Coles and Jokiel 1978) and ultraviolet radiation (UV) (Jokiel and York 1982; Harriot 1985) may enhance "bleaching", synergistically acting with photosynthetically produced oxygen causing proteins and nucleic acids to denature. Protection against UV is produced in the form of the enzymes superoxide dismutase (SOD) and catalase, which inactivate superoxide radicals ($O2^{-}$) and hydrogen peroxide (H_2O_2) respectively. These enzymes increase in concentration within the symbionts in response to a direct increase in UV (Lesser and Shick 1989) or proportionately to the chlorophyll content (Dykens 1984). Such photoacclimatization has been shown to be seasonal (Dykens and Shick 1984), limiting oxygen toxicity. In addition, UV protection is provided by the animal's tissues or mucus, which may contain UV absorbing mycosporine-like amino acids (Dunlap et al. 1986; Drollet et al. 1993; Stochaj et al. 1994), or through behavioural modifications (Dykens and Shick 1984). Dykens and Shick (1984) described

the adhesion of debris to the column by A. elegantissima in response to elevated irradiance levels. Secondly, irradiance influences photosynthetic photoadaptation, which may include changes in symbiont density (Steele 1976; McCloskey and Muscatine 1984). Light quality also results in photoadaptation, with white and blue light inducing increased symbiont growth in M. verrucosa (Kinzie et al. 1984).

(d)Heterotrophic nutrition

The requirement of heterotrophic nutrition (zooplankton, bacteria and dissolved organic matter (DOM)) in the diet of symbiotic anthozoans depends on both the host species and the environment in which it is living (eg. McCloskey and Muscatine 1984; Muller-Parker 1985; Cook et al. 1988; Muscatine et al. 1989b). Starvation has been shown to lead to a decrease in symbiont density (Clayton and Lasker 1984), symbiont division rates and cellular chlorophyll-a contents (Cook et al. 1988). Particulate nutrition provided in the form of Artemia nauplii was not found to result in an increased symbiont density when provided for freshly collected corals because the increase in symbiont numbers was parallelled by an increase in host cell numbers (Muscatine et al. 1989a).

(e)Inorganic nutrients

Low ambient nitrogen levels, rather than phosphorus levels, have been shown to limit symbiont growth, with ammonium spikes causing an increase in the growth rate of coral

symbionts and a resultant increase in the symbiont density (Muscatine et al. 1989a; Stimson and Kinzie 1991). Also, using computer simulation, C.E. Taylor et al. (1989) predicted that the addition of inorganic nutrients could lead to the overgrowth of hydra polyps by their algal symbionts.

These factors may all result in a loss of symbiosis stability. Therefore the following maintenance regime was employed to provide a stable, non-stressful environment.

2.2.3.2 Materials and methods

(a)Stock tank set-up

All the species of temperate symbiotic Anthozoa were maintained in stock tanks positioned on a wet bench. The wet bench was beneath a south-east facing window and the anthozoans were illuminated by the natural light cycle. The tanks consisted of plastic crates into which an undergravel air-filter covered with approximately 7 cm of shell gravel was placed. Water pumped from the Menai Strait and passed through a sedimentation gravity tank was run through the tanks at all times, thereby maintaining the salinity (32 - 34 $^{o}/oo$), water nutrient levels and temperature at naturally prevailing levels. The tanks were strongly aerated at all times, preventing oxygen stress. The tanks were regularly cleared of algal growth and any dead animals, and were completely cleaned once every few months. Each tank was devoted to only one host species from one location, with no

water flow between tanks. Thus aggressive behaviour between species was prevented, as was the possibility of any released symbionts establishing a symbiosis with a different host species. The environment in each tank was set up in accordance with the habitat from which the anemones were collected. Therefore, large rocks were supplied for settlement of A. viridis and I. sulcatus, rocks possessing C. pedunculatus were buried in the gravel and A. ballii were permitted to bury directly into gravel.

A. pallida were maintained in a 5 l glass aquarium fitted with an undergravel filter which was covered by 5 cm of shell gravel. The tank was strongly aerated. The top of the aquarium was covered by clear perspex to prevent evaporation, but the salinity was still measured weekly and adjusted to 33 ^{o}/oo, the approximate salinity of water from the Menai Strait, using fresh water. 50 % of the sea water was exchanged with fresh sea water monthly. The tank was heated to 25 ^{o}C with a thermostatic heater and illuminated on a 12 hour light : 12 hour dark cycle with a 60 W white bulb. The method of maintenance recommended by Walters (pers. comm.), employing a clean glass tank and filtered sea water (FSW), was found to cause "bleaching" of the anemones indicating unidentified stressful conditions.

(b)Maintenance of Anthozoa prior to experimental use

Due to both subtropical and temperate anthozoans being in use and the intended inclusion of tropical 'strains' and species Symbiodinium, a compromise temperature was required

at which all experiments would be run. Tropical and subtropical symbionts do not survive below approximately 20 ^{o}C (Prof. R.K. Trench, University of California, Santa Barbara, pers. comm.). However, within their south-western European range the temperate Anthozoa in this study experience average temperatures greater than those in British waters. For example, Mediterranean A. viridis used by Stambler and Dubinsky (1987) were found and maintained at 22 ^{o}C. Thus, the possibility of maintaining stable symbioses at 21 ^{o}C was investigated. Appendix 1 (pp.463-466) gives experimental data showing that none of the species of anthozoan investigated here appeared to be under stress, as reflected by the stability of their symbiont densities (symbiont number/mm^2 of tentacle or polyp), when maintained for 1 month at 21 ^{o}C. Consequently, prior to experimental use all animals were acclimatized to 21 ^{o}C for at least 4 weeks. A. viridis, A. ballii and C. pedunculatus were acclimatized in 30 l aquaria set up similarly to the A. pallida stock tank and A. pallida and I. sulcatus were maintained in 50 ml transparent polystyrene pots (BDH). Because of the small volumes of these pots, the water was changed every 2 days or 4-5 hours after feeding. All the tanks and containers were positioned in a temperature controlled room and illuminated by warm white fluorescent light at 80 μE/m^2/s. The light intensity was measured using a scalar irradiance meter (Biospherical Instruments Inc. model QSL-100). The meter scale in quanta/cm^2/s was

converted into microeinsteins (μE) using the conversion of 6.022×10^{17} quanta/cm^2/s = 1 μE/cm^2/s.

(c)Control of diatom growth

The conditions under which the anthozoans were kept were also favourable to diatom growth, resulting in extensive diatom mats across all surfaces of the aquaria and the smothering of the animals. Germanium dioxide (GeO_2), which is non-toxic to Anthozoa in low concentrations (Turner 1988), was employed to control this growth. Germanium is taken up by diatoms in preference to the silica with which they manufacture their frustules, hence preventing cell division. A stock solution of 0.25g/l GeO_2 (Sigma Chemical Co.) in FSW was made up, allowing the GeO_2 to dissolve overnight. This stock solution was then stored in a refrigerator. 2 ml/l of the stock solution was added to the aquaria once every 6-8 weeks to control diatom growth.

(d)Feeding of the anthozoans

All the animals were fed newly hatched Artemia nauplii twice weekly. Artemia cysts were hatched in a glass flask containing FSW and bubbled through with air. Hatching occurred after 2 days and the nauplii were settled out by switching off the air supply. The Artemia were then pipetted onto the oral discs of the anthozoans in a heavy suspension and the animals fed to repletion.

2.2.3.3 Results

The laboratory system for the maintenance of the symbiotic Anthozoa was successful in providing healthy symbioses for experimental use. No evidence of "bleaching" (ie. a noticeable paling in the colour of the anthozoan or excessive symbiont containing mucus production) was observed and the animals were observed to reproduce successfully. A. viridis spawned annually in May/June and reproduced asexually by longitudinal fission at least annually. C. pedunculatus produced viviparous young, although the frequency of this production decreased with time in the laboratory. A. ballii was not observed to reproduce asexually but spawned annually under constant conditions or could be induced to do so in response to the movement from the stock tanks to the warmer 'preparation' tanks. A. ballii planula metamorphosis was only observed on one occasion and the juvenile polyps only survived for 2 weeks. I. sulcatus was observed to reproduce asexually resulting in a greater percentage cover of the surface on which it was growing. A. pallida reproduced very rapidly by pedal laceration resulting in a 5 fold increase in numbers within 1 month of this species being obtained; Aiptasia was not observed to reproduce sexually.

2.2.3.4 Discussion

The absence of either symbiont "bleaching" or overgrowth indicated that the conditions under which the symbioses were

maintained provided environmental parameters within the tolerated range of the hosts and their symbionts. This was borne out by the successful reproduction of all the host animals. However, the reproductive rate of these anthozoans in the field is unknown and awaits investigation. Therefore any change in the 'fitness' of these animals with respect to reproductive success whilst in the laboratory could not be ascertained.

2.3 ISOLATION AND MAINTENANCE OF THE ALGAL SYMBIONTS

2.3.1 Introduction

Cultured symbionts were required in order to characterize the cells when not under the influence of the host cell environment. The ambient environment inhabited by hosts and the intracellular environment occupied by the algae may vary greatly, hence it is recommended that cultured algae are used where possible when investigating genetic characteristics (Schoenberg and Trench 1980a). The earliest reported cultures of *Symbiodinium* cells are by Kawaguti (1944) and McLaughlin and Zahl (1957), who cultured algae axenically from the jellyfish *Cassiopeia* sp. and the anemone *Condylactis* sp.. Mclaughlin and Zahl (1957) used the artificial sea water medium B+, later adapting this to the M9 medium (Mclaughlin and Zahl 1959). Using M9 medium, Freudenthal (1962) cultured and classified *S.*

microadriaticum from Cassiopeia sp.. Another artificial sea water medium, SM, has also been used successfully to culture Symbiodinium sp. (Loeblich and Sherley 1979), as have the enriched sea water media GLM (Loeblich and Sherley 1979) and silica free F/2 (Bigelow laboratory, Maine, pers. comm.). But it is the artificial sea water medium ASP8-a which has been the most extensively used culture medium for Symbiodinium spp.(eg. Fitt et al. 1981; Fitt and Trench 1983a; Wilkerson and Trench 1985; Blank 1987). ASP8-a was originally developed by Ahles in 1967 who adapted the B+ and M9 media, adding a more extensive mix of nutrients and vitamins (cited in Guillard and Keller 1984). The only vitamins which most dinoflagellates are known to require are B_{12}, thiamin and biotin, although biotin is not thought to be essential to Symbiodinium spp. (Taylor, D.L. 1975). However, ASP8-a's success indicates that the presence of other vitamins can be advantageous (Guillard and Keller 1984).

As in the case of the intact symbioses, cultured symbionts require stable, favourable environmental conditions in which to grow. As discussed earlier (chapter 2, section 2.2.3.1 (pp.33-34)), the algal symbionts can be temperature stressed. In addition, the pH and salinity of the medium and the light quality should approximate that experienced by the symbionts when in the host. Under such conditions, Symbiodinium sp. from many host species, including all the British symbiotic Anthozoa with the exception of A. viridis

(Taylor, D.L. 1969a), have never been cultured successfully. The following describes the procedures used in the attempted culture of the symbionts of all the host species under investigation.

2.3.2 Materials and Methods

(a) Isolation of the algal symbionts

In order to take account of any intraspecific variation, 3 tentacles were removed from 5 different specimens of *A. viridis* using a pair of scissors. *A. ballii* was treated similarly. Alternatively, because of the small size of the tentacles of the other host species, either 5 complete oral discs were removed using a razor blade, as in the case of *C. pedunculatus*, or 5 complete polyps were collected, as in the case of *I. sulcatus* and *A. pallida*. The excised material was then homogenized in 10 ml of autoclaved, UV irradiated, 0.2 µm filtered sea water (FSW) in a hand-held glass tissue grinder. The resultant homogenate was centrifuged at x1200 rpm for 10 minutes. The supernatant containing the animal tissue fragments was discarded and the algal pellet was resuspended in 10 ml autoclaved FSW using a sterile hypodermic needle and 10 ml syringe. The homogenate was shaken vigorously and centrifuged at x1200 rpm for 5 minutes. This procedure was repeated until the supernatant appeared clear of host debris. During the preparations for initial culturing attempts the second wash included 0.05% sodium dodecyl sulphate (SDS) (Sigma Chemical Co.). 0.05%

SDS has been shown to enable the isolation of Chlorella cells "essentially free" of host hydra debris without affecting algal viability (McAuley 1986). Following the final centrifugation, the symbiont pellet was resuspended in 1 ml autoclaved FSW.

(b)Obtaining Symbiodinium from other host species

It was intended to compare the characteristics and specificity of Symbiodinium spp. from a wide geographical and climatic range. Therefore, subcultures of S. pilosum, S. microadriaticum and Symbiodinium sp. from A. elegantissima were obtained from Professor R. K. Trench of the University of California, Santa Barbara. Subcultures of Symbiodinium sp. from A. pulchella were obtained from Professor L. Muscatine of the University of California, Los Angeles. All of these subcultures were received in December 1990.

(c)Preparation of culture media

Initially 3 different culture media were used in an attempt to find a successful medium for the culture of temperate dinoflagellate symbionts: ASP8-a (Blank 1987), M9 and silica-free F/2 (Guillard and Keller 1984). The subcultures obtained from the University of California had been grown originally in ASP8-a and were therefore continued to be cultured in this medium only. These media were made up using the constituents given in table 2.3 (pp.46-47), which were all weighed to 4 decimal places using an Oertling pan balance. 1 l distilled water was first autoclaved at 121 ^{o}C

TABLE 2.3 THE COMPOSITION OF CULTURE MEDIA (F/2, M9 AND ASP8-A) USED FOR THE CULTURE OF SYMBIOTIC DINOFLAGELLATES.

Compound	F/2 silica-free enriched sea water (Guillard and Keller 1984)	M9 artificial sea water (Guillard and Keller 1984)	ASP8-a artificial sea water (Blank 1987)
Group 1: Major nutrients, elements and buffer (Mol./l)			
$NaNo_3$	8.83 E-4	-	5.9 E-4
NH_4Cl	1 E-4	-	-
$NaH_2PO_4.H_2O$	3.63 E-5	-	-
NaCl	-	4.45 E-1	4.3 E-1
$MgSO_4.7H_2O$	-	3.65 E-2	3.7 E-2
KCl	-	8.05 E-3	9.4 E-3
$CaCl_2$	-	7.49 E-3	7.5 E-3
$Na_2CO_3.H_2O$	-	2.42 E-6	-
$NaNO_3$	-	5.88 E-4	5.9 E-4
$(NH_4)_2SO_4$	-	7.57 E-6	-
$Na_2glyceroPO_4$	-	6.35 E-5	-
$Na_2SiO_3.9H_2O$	-	3.52 E-4	-
Tris	-	3.17 E-3	8.3 E-3
Na_3NTA	-	-	1.6 E-4
KH_2PO_4	-	-	7.4 E-5
NH_4NO_3	-	-	1.5 E-5
Group 2: Metals, minor elements and chelators (Mol./l)			
$FeCl_3.6H_2O$	1.17 E-5	2.36 E-5	1.8 E-6
$Na_2.EDTA$	1.17 E-5	8.06 E-5	2.7 E-5
$MnCl_2.4H_2O$	9 E-7	2.18 E-5	-
$ZnSO_4.7H_2O$	8 E-8	-	8.3 E-7

Table 2.3, group 2, contd.	F/2 silica-free enriched sea water (Guillard and Keller 1984)	M9 artificial sea water (Guillard and Keller 1984)	ASP8-a artificial sea water (Blank 1987)
$CoCl_2.6H_2O$	5 E-8	5.09 E-7	-
$CuSO_4.5H_2O$	4 E-8	-	-
$ZnCl_2$	-	2.29 E-6	-
H_3BO_3	-	5.55 E-4	-
NTA	-	1.05 E-3	-
$MnSO_4$	-	-	1.1 E-5
$CoSO_4.7H_2O$	-	-	2.6 E-7
Group 3: Vitamins (Mol./l)			
Vitamin B_{12}	3.7 E-10	7.42 E-10	1 E-9
Thiamin (HCl)	3 E-7	1.54 E-6	3 E-7
Biotin	2.1 E-9	4.3 E-9	8 E-9
Niacin	-	8.12 E-8	4.1 E-7
Putrescine $(HCl)_2$	-	4.54 E-8	1.2 E-7
Ca d-Pantothenate	-	4.2 E-8	2.1 E-7
Riboflavin	-	1.33 E-8	5 E-9
Pyridoxine (HCl)	-	1.95 E-8	9.7 E-8
Pyridoxamine $(HCl)_2$	-	8.3 E-9	4.1 E-8
PABA	-	7.29 E-9	3.7 E-8
Choline-H_2.Citrate	-	1.69 E-7	8.5 E-7
Myo-Inositol	-	5.55 E-7	2.8 E-6
Thymine	-	6.34 E-7	3.2 E-6
Orotic acid	-	1.28 E-8	5.6 E-8
Folic acid	-	5.67 E-10	4 E-9
Folinic acid	-	3.91 E-11	3.9 E-10
Urea	-	1.67 E-5	-

for 20 minutes in a 5 l conical flask bunged with non absorbent cotton wool. Once the water was cooled to room temperature, the basic solution was made using the compounds in group 1. Having ensured that the basic solution was completely dissolved the 'supplementary' compounds in group 2 were added. The final solution was then autoclaved as previously described. Due to the very low concentrations of vitamins required, it was necessary to make a stock solution of the vitamin supplement of x 10,000 in 1 l autoclaved distilled water. This stock was stored in 5 ml plastic vials in a freezer over the duration of the culturing period. 0.1 ml of the vitamin stock solution was added to the autoclaved solution of the group 1 and 2 compounds after cooling. The vitamins were not autoclaved to prevent denaturation. Thus, to reduce bacterial contamination, the vitamin stock was added to the medium using a sterile 1 ml syringe and a 0.2 µm filter. The pH of the medium was then adjusted to 8.2 by titration using 1N HCL and a pH meter. This was the standard procedure used throughout the culturing of the symbionts, although when cultures were being initiated antibiotics and GeO_2 were employed to control bacterial and diatom growth respectively. The antibiotic used was penicillin-streptomycin-neomycin (Sigma Chemical Co.), which was added to give a final concentration of 0.004% (Guillard and Keller 1984). The cultures were treated with antibiotics for 36 hours and the medium then replaced with antibiotic-free medium (Stein 1973). A GeO_2 stock solution was prepared in

fresh culture medium and added, filtering through a 0.2 µm sterile filter, to give the concentrations described in chapter 2, section 2.2.3.2 (c) (p.40). GeO_2 was used throughout the first month of culturing. Subsequent use of antibiotics and GeO_2 only occurred when cultures required cleaning. Trench (1981) described the use of agar plating in obtaining clonal symbionts for culture in liquid ASP8-a, so a further adaptation of the media was the addition of agar to produce a semi-solid medium. 1 g of agar (Sigma Chemical Co.) was autoclaved in 10 ml distilled water at the same time as the medium was being prepared. The conical flask used for autoclaving the agar was itself placed in a beaker of water to prevent the rapid cooling and solidification of the agar following autoclaving. Immediately after autoclaving the agar and medium were mixed vigorously. The solution was then permitted to cool. Before the agar had set the vitamins and GeO_2 were added; antibiotic treatment was performed prior to plating. A low agar concentration and separate autoclaving of agar and medium have been stressed as being important in the success of plating methods of dinoflagellate culture (Guillard and Keller 1984).

(d)Inoculation of the culture medium

3 forms of culture vessel were used for culturing in liquid media: 50 ml clear glass conical flasks bunged with non-adsorbent cotton wool, 10 ml screw top clear glass test tubes and sterile plastic petri dishes. The flasks and test tubes were sterilized by autoclaving before use. 50 ml of

culture medium was then decanted into the conical flasks and the test tubes were half filled, holding the opening of the vessels near a Bunsen burner flame at all times to limit bacterial contamination. Using a sterile 1 ml syringe and a hyperdermic needle, each culture vessel was inoculated with 0.1 ml of the prepared algal suspension. A different syringe and needle was used for the symbionts of each host species to prevent the same vessel being inoculated with more than one 'strain'. Whilst still warm, the semi-solid agar medium was poured into sterile petri dishes and allowed to set. Once the agar plates were cool, algal suspension was streaked over the surface using a sterile syringe and needle. This algal suspension had been treated with the antibiotic mixture in culture medium for 36 hours prior to plating. All work done using petri dishes ensured that the top of the dish was lifted only slightly to prevent bacterial and fungal contamination.

All the flasks, tubes and petri dishes were clearly labelled with the host species and date of isolation to prevent confusion.

(e)Culture of the algal symbionts

The flasks and test tubes were placed in an enclosed box and illuminated from below by warm white fluorescent lighting. The tops of the test tubes were left loose to allow gas exchange and hence prevent culture collapse. Because Symbiodinium spp. grow attached to surfaces (Deane and O'Brien 1978; Dr. N. Stambler, Tel-Aviv University, pers.

comm.) the cultures were not disturbed until subculturing. The petri dishes were inverted to prevent the collection of condensation on the agar surface and were placed on a metal grid over a warm white fluorescent light. The irradiance was manipulated using layers of mesh to give an irradiance of 80 $\mu E/m^2/s$. An irradiance of no more than 170 $\mu E/m^2/s$, with all visible wavelengths represented, is recommended for successful dinoflagellate culture (Guillard and Keller 1984) and the value of 80 $\mu E/m^2/s$ has proved widely successful for the culture of Symbiodinium spp. (eg. Schoenberg and Trench 1980a; Fitt and Trench 1983a). All the cultures were maintained on a 12 hour light : 12 hour dark cycle at 21 ^oC in a temperature controlled room.

(f)Subculturing and checks on culture health and axenicity

Subculturing occurred every 4 weeks. Firstly, the health and axenicity of the cultures was investigated by scraping a sample of the culture from the bottom of the flask or tube using a flame sterilized pasteur pipette. The vessel opening was held over a flame at all times. The sample was mounted on a slide and its axenicity determined using phase contrast. Epifluorescence was utilized to estimate the health of the culture. Healthy cells were expected to fluoresce deep red due to the emittance of wavelengths greater than 600nm when excited by wavelengths in the range 400-440 nm. This fluorescence is proportional to the concentration of chlorophyll in the alga, a variable which was expected to decline in degenerating cells as a result of

a reduction in chloroplast size and an increase in the number and size of cytoplasmic inclusions (Steele 1975). The percentage of cells fluorescing was not quantified, but a culture in which the majority of cells fluoresced brightly was continued. Scrapings of the cultures were placed in sterile petri dishes and observed under a binocular microscope using the x 10 objective. Using a glass pasteur capillary pipette made by drawing out a standard pipette tip over a Bunsen burner flame, healthy looking cells (dark green/brown in colour without large "accumulation bodies") free of contaminants were pipetted out and inoculated into freshly prepared sterile culture medium. Attempts were also made to obtain clonal cultures by inoculating each flask with just 1 cell. But most of the culture was left undisturbed and approximately 90 % of the medium replaced with fresh medium. If bacterial and diatom contamination appeared substantial the culture was treated accordingly.

2.3.3 Results

Growth of the symbionts was only observed when the ASP8-a liquid medium was used without the involvement of SDS in the isolation procedure and was not evident on any of the agar plate media. Growth of the cultures was very slow, only being evident by eye over a period of several weeks. The symbionts were only found attached to the bottom and sides of the culture vessels in the case of the temperate symbionts, although motile stages of the tropical and

subtropical symbionts, as well as the symbionts of A. elegantissima, were observed swimming in the medium.

Subculturing of the British symbionts by the inoculation of cells removed from the vessel surfaces never resulted in a successful culture but the addition of fresh medium to existing cultures did enable the continued growth of these cultures. The use of this method necessitated the clearing of some of the available growing surfaces to enable fresh algal growth. In addition, antibiotics needed to be added monthly to the medium because of the inability to thoroughly sterilize the culture vessels during the culture period. Cultures were obtained for all the symbionts of the British Anthozoa and A. pallida, but with varying degrees of success. Symbionts isolated from A. ballii, A. viridis (from Shell Island, Bembridge and Weymouth) and A. pallida were cultured on most occasions and were maintained in culture for up to 8 months. Symbionts of C. pedunculatus (Lough Hyne and Netley), I. sulcatus and A. viridis (Trearddur Bay and Loch Sween) were cultured after several attempts and were maintained in culture for up to 6 months. Less successful were Symbiodinium sp. from A. viridis (Lough Hyne), which were only cultured on one occasion. This culture was unfortunately lost because of experimental error. The cultures obtained from the University of California were maintained for several months, but, perhaps due to variations between the conditions in which they were originally cultured and those used here, the health of these

cultures was observed to slowly deteriorate leading ultimately to their collapse.

2.3.4 Discussion

For the first time the symbionts of A. ballii, C. pedunculatus and I. sulcatus were cultured, as were symbionts from A. viridis and A. pallida. However, their requirement of a surface upon which to grow on and their intolerance to disturbance resulted in problems when trying to maintain the cultures over many generations. The concept of axenicity is rarely achievable in practice, with a residual number of bacteria always being present even after treatment with antibiotics (Guillard and Keller 1984). Diatoms were controlled successfully by the use of GeO_2 throughout the life of the cultures. Despite antibiotic washes, bacterial growth was a problem and probably the cause of the eventual culture collapse. But a possible contribution towards this collapse by the antibiotic itself cannot be discounted; the failure of some 'strains' or species of Symbiodinium to grow has been attributed to the presence of antibiotics (Polne-Fuller 1991). In order to overcome this problem successful subculturing of the algae is required, enabling growth to continue in sterile glassware. The breakdown of residual animal material after symbiont isolation may also create a favourable environment for bacteria. The lack of culture growth when SDS was used may indicate an effect on cell viability or the need for

some compound present in the animal debris during the early stages of the culture's life. If the former is true, then a non-chemical method of host debris removal may prove useful. For example, the amoeba Trichosphaerium sp. was shown to cleanse Symbiodinium sp. of contaminating debris and microorganisms, and release the indigestible Symbiodinium cells which could then be cultured (Polne-Fuller 1991). But axenicity is only required where aspects of the contaminants may be confused with those of the cultured organisms, as would occur in DNA profiling or isoenzyme analysis. Therefore, the acquisition of axenicity was not essential for this research other than to prolong culture life.

The inability of the symbionts to grow in either the F/2 or M9 media indicated that their nutrient or vitamin requirements were not met by the media in this investigation. The varying success rates of the cultures of algae isolated from different host species when in ASP8-a also suggests that the symbionts of different host species may have different vitamin requirements. Unfortunately, very little is known concerning the specific vitamin requirements of symbiotic dinoflagellates; there is no evidence for the necessity of any of the organic additions to the culture media but vitamin-requiring 'strains' may exist, as in the Bacillariophyceae (diatoms) (Guillard and Keller 1984).

The slow growth of the cultures and the inability to subculture them resulted in difficulty obtaining large volumes of cells. In this research this problem was only

apparent during pigment analysis, where freshly isolated symbionts eventually had to be used. Symbiotic algae have been shown to change with time after isolation from the host, with respect to their morphology and physiology. For example, Freudenthal (1962) noted that the "assimilation product" of S. microadriaticum occupied less than half the symbiont cell in new cultures but occupied up to 80 % of the cell in old cultures. Muscatine et al. (1972) demonstrated that photosynthate release by freshly isolated symbionts of the coral Agaricia agaricites in sea water decreased by 50 % over a period of 6 hours. A further physiological difference between freshly isolated and cultured symbionts was shown by Domotor and D'Elia (1984), who demonstrated that the inhibition of nitrate uptake by ammonium occurred only when the cells were in culture. Consequently, cultured Symbiodinium cells cannot be considered as being the same as the cells in situ. Therefore, the use of freshly isolated symbionts was preferable to cultured cells in this work, except for the investigation of genetic differences in morphology, cell division behaviour and pigmentation.

2.4 SUMMARY

(i)Of the 74 species of British Anthozoa only 5 contain endosymbiotic dinoflagellates: A. mutabilis, A. viridis, A. ballii, C. pedunculatus and I. sulcatus.

(ii)With the exception of A. mutabilis, these Anthozoa were collected from south and westerly locations around the British Isles. In addition, the subtropical A. pallida was obtained from Bermuda.

(iii)The Anthozoa were maintained successfully in the laboratory in stock tanks, under the influence of natural environmental conditions, and in experimental preparatory tanks at an elevated temperature of 21 ^{o}C. No evidence of stress to the symbioses was observed, as expressed by the destabilizing of the symbiont population.

(iv)The algal symbionts of the British Anthozoa, with the exception of A. viridis, had not been cultured before.

(v)The algal symbionts of these host species were cultured for up to 8 months in ASP8-a artificial sea water medium but not on ASP8-a agar plates. The algae were cultured with varying degrees of success, suggesting differences in the environmental requirements (possibly vitamins or nutrients) of the symbionts from different host species.

(vi)The cultures grew slowly and the cells would not tolerate disturbance. Consequently, the quantity of material available for practical purposes was limited.

(vii)The symbionts were not cultured in M9 or silica-free F/2 media, indicating that the vitamin or nutrient requirements of the algae were not met.

CHAPTER 3

CHARACTERIZATION OF THE ALGAL SYMBIONTS

3.1 GENERAL INTRODUCTION

The aim of this chapter was to characterize, at a preliminary level, the symbionts of A. viridis, A. ballii, C. pedunculatus, I. sulcatus and A. pallida with respect to: (a) Morphology; (b) pigment composition; and (c) diel division behaviour.

Many investigations have indicated the presence of several genetic entities within the genus Symbiodinium (table 3.1, p.60) and have ultimately led to the identification of the species named in chapter 1, section 1.2.2 (p.6).

The precise identity of the symbionts of the British Anthozoa has not been determined, although the symbionts of A. viridis have been investigated with respect to their pigment composition (Taylor, D.L. 1967), ultrastructure (Taylor, D.L. 1968b; Dodge 1973; LeVay 1989) and behaviour in vitro (Taylor, D.L. 1969a), and those of A. ballii, C. pedunculatus and I. sulcatus have been investigated briefly with regards to ultrastructure and isoenzyme patterns (LeVay 1989). In contrast, the cultured symbionts of A. pallida have been widely included in studies of genetic variation in the genus Symbiodinium (Steele 1975; Schoenberg and Trench 1980a, b and c; Fitt et al. 1981; Fitt and Trench 1983a; Rowan and Powers 1991a; Markell and Trench 1993). The following therefore describes the comparison of several of

TABLE 3.1 PREVIOUSLY IDENTIFIED CHARACTERISTICS SUGGESTING THE EXISTENCE OF DIFFERENT STRAINS OR SPECIES OF SYMBIODINIUM IN DIFFERENT HOST SPECIES.

Characteristic(s) differing between *Symbiodinium* from different host species	Author and date
Morphology	Schoenberg and Trench (1980b); Blank and Trench (1985); Trench and Blank (1987); Banaszak *et al.* (1993)
Photoadaptive mechanisms and concentrations of different pigments	Chang *et al.* (1983)
Isoenzyme and soluble protein patterns	Schoenberg and Trench (1980a)
Isoelectric forms and apoprotein compositions of peridinin-chlorophyll-*a* proteins	Chang and Trench (1982); Iglesias-Prieto *et al.* (1991)
Behaviour in culture	Fitt *et al.* (1981); Fitt and Trench (1983a)
Ability to reinfect 'homologous' and 'heterologous' hosts	Kinzie and Chee (1979); Schoenberg and Trench (1980c)
Components of the macromolecules released in culture	Markell and Trench (1993)
Fatty acid composition	Bishop and Kenrick (1981)
Sterol composition	Withers (1987)
DNA base compositions and homologies	Blank *et al.* (1988); Blank and Huss (1989)
Small ribosomal subunit RNA (ssRNA) sequences	Rowan (1991); Rowan and Powers (1991a); Rowan and Powers (1991b)

the characteristics of the symbionts of the British Anthozoa and of A. pallida, and the comparison of these symbionts with previously described Symbiodinium spp. isolated from different host species.

3.2 SYMBIONT MORPHOLOGY

3.2.1 Introduction

The aims of this investigation were: (a) To assess the degree of morphological homogeneity between the symbionts of the different host species and also between the symbionts of the same host species but from different geographical locations and (b) to determine whether the symbionts of the British Anthozoa satisfy the morphological criteria of the genus Symbiodinium.

The taxonomy of dinoflagellate symbionts has been based largely upon morphological studies. The type species S. microadriaticum (Freudenthal 1962) from Cassiopeia sp. was described using morphological and behavioural characteristics observed by light microscopy (LM). Freudenthal's assignment of S. microadriaticum to the dinoflagellates was a result of the two dissimilar flagella and the transverse girdle of the motile stage, and the large granular nucleus. But this author's placement of S. microadriaticum in the family Blastodiniaceae and genus Symbiodinium was a reflection of its symbiotic nature rather than its morphology. Freudenthal's taxonomic description was

revised later using details obtained from electron microscopical investigations of the symbionts of Cassiopeia sp. and Condylactis sp. (Kevin, M.J. et al. 1969), and a list of diagnostic ultrastructural characteristics was compiled from electron microscopical examinations of symbionts from 6 species of Pacific giant clam (Tridacnidae) (Taylor, D.L. 1969a). As a result of these early investigations, D.L. Taylor (1974) concluded that all symbiotic gymnodinioid dinoflagellates were the same species, having already suggested that any morphological variations observed between symbionts from different host species reflected different stages in the symbiont life history (Taylor, D.L. 1973). In addition, D.L. Taylor (1974) suggested placing S. microadriaticum in the free-living genus Gymnodinium because of observations of flagellated symbionts in situ. Loeblich and Sherley (1979) later proposed the replacement of Gymnodinium (=Symbiodinium) microadriaticum with the name Zooxanthella microadriatica. This change was on account of the identical nature of the amphiesmal plates of symbionts from Cassiopeia sp. and a free-living dinoflagellate, and the morphological similarity of this free-living dinoflagellate to the type species Zooxanthella nutricula (Brandt). However, the less confusing genus name Symbiodinium has been accepted in recent years, and the inclusion of these symbionts in the genus Gymnodinium has been dismissed due to the daily transformation from the motile to coccoid stage and mitotic

division only occurring in the coccoid stage (Blank and Trench 1986).

Dispelling earlier conclusions, morphological studies using both LM and electron microscopy (EM) have been instrumental in indicating the presence of different strains or species of Symbiodinium. Steele (1975) noted that motile symbiont cells from A. pallida possessed "accumulation bodies" not observed at this stage in the descriptions of Freudenthal (1962). Schoenberg and Trench (1980b) observed differences in both the dimensions at the "two-cell stage" and the ultrastructure of symbionts isolated from 17 host species and cultured under identical conditions. Blank (1986) also reported ultrastructural differences between symbionts from A. viridis, A. elegantissima and Z. sociatus, and the type species S. microadriaticum, particularly with respect to chloroplast structure. The most conclusive morphological evidence for different strains or species of Symbiodinium was produced by 3-D ultrastructural comparisons. 3-D reconstructions of symbiont nuclei from Cassiopeia xamachana, Heteractis lucida, A. elegantissima and M. verrucosa revealed differences in chromosome numbers and volumes, which were stated as not being consistent with different ploidy states within the same symbiont species (Blank and Trench 1985). Further 3-D reconstructions of the symbionts of R. lucida, M. verrucosa and Z. sociatus also showed differences in the number and volume of chloroplasts, organisation of the thylakoids, mitochondrial

number and volume, the number of pyrenoid stalks and the nuclear volume. These differences resulted in emendations to the descriptions of the genus Symbiodinium and the species S. microadriaticum, and the naming of 3 new species of Symbiodinium (Trench and Blank 1987). Blank (1987) also investigated the microarchitecture of the symbionts of M. verrucosa. This author constructed styrofoam models of cells and organelles from electron micrographs of serially sectioned symbionts. The observed variations in chromosome numbers and volumes, and nuclear volumes led to the view that the genus Symbiodinium contained separate species (Blank 1987; Trench and Blank 1987).

The morphologies of the symbionts of the British Anthozoa, with the exception of the symbionts of A. viridis, have not been investigated in detail before. Therefore, light and electron microscopy were employed to describe and compare the morphologies of these symbionts and the symbionts of A. pallida when in culture and in vivo. Thus, any evidence suggesting the presence of genetically distinct populations of Symbiodinium within these hosts could be identified.

3.2.2 Materials and methods

(a)Light microscopy (LM)

(i)Location of symbionts in host tissues

Depending on the size of the host animal, either tentacles or whole polyps were relaxed in 7.5 % magnesium chloride ($MgCl_2$) in filtered sea water (FSW) for approximately 10 minutes. The polyp or tentacle was then placed on a slide and squashed lightly under a coverslip. The endoderm, mesogloea and epidermis were viewed under a Leitz Orthoplan light microscope and the location of the symbionts within these tissues noted.

(ii)Observations of symbiont morphology

Symbionts were isolated from 5 different individuals of each host species from each location, as described in chapter 2, section 2.3.2 (a) (pp.44-45). In addition to the cultures of S. microadriaticum, S. pilosum and the symbionts of A. pulchella and A. elegantissima, cultured algae which had been in culture for approximately 3 months were scraped from the glass surfaces on which they were growing two weeks after subculturing. A sample of the culture medium above the growth surface was also taken to investigate the presence of motile cells. The algae were then mounted live in FSW or in culture medium on a glass slide under a coverslip and viewed on a light microscope. The cells were observed under the x 10, x 25 and x 40 objectives, and under oil immersion using the x 100 objective, and their morphological characteristics were noted. In order to investigate the possibility of

making chromosome counts, the chromosomes were fixed in Carnoy's fixative for 30 seconds followed by 3 further changes of Carnoy's of 20 minutes duration. The chromosomes were then stained with aceto-orcein and observed with the light microscope under the x 40 and x 100 objectives.

(iii)Measurement of symbiont dimensions

The dimensions of the "2-cell stage", which was still surrounded by the parent amphiesma, of 40 cells in each sample was measured both parallel and perpendicular to the cell division plane giving the cell width and length respectively. Only the dimensions of living cells were measured, using a light microscope and a previously calibrated ocular micrometer. The dimensions of the symbionts were compared between cultured and freshly isolated cells using the statistics discussed below.

(iv)Statistical tests used

Normality of samples was tested using 'nscore' followed by correlation. Variance homogeneity within the relevant samples was tested using Bartlett's statistic, the most robust of the available tests of variance homogeneity. Statistical comparisons between two samples were performed using Student's t-test when the data was normal or the Mann-Whitney test when the data was not normally distributed. Significant differences between more than two samples were identified using one-way analysis of variance (ANOVA) followed by Tukey's test or Scheffe's test, to identify where the significant differences lay, when data

was normally distributed and the variances were homogenous. Tukey's test and Scheffe's test were used when the sample sizes were equal and unequal respectively. When the data was not normally distributed or the variances were not homogenous, more than two samples were tested for significant differences using the Kruskal-Wallis non-parametric test, and subsequently by 'multiple comparisons' to identify between which samples the significant differences occurred. All statistics were performed using the 'MINITAB' statistical package and significant differences were tested for at the 5 % (ie. $P<0.05$) level. These same significance tests were used throughout this work. **All significant differences referred to in this thesis were significant when $P<0.05$, except where stated.**

(b)Electron microscopy

Cultured and freshly isolated symbionts from the British Anthozoa (except cultured symbionts of A. viridis from Lough Hyne, which were not available) and A. pallida were obtained as above. However, cultures of S. microadriaticum, S. pilosum and the symbionts of A. pulchella and A. elegantissima were no longer available for any of the characterization work. These isolated symbionts were investigated using both SEM and TEM. Also, tentacles or polyps of the anthozoans, depending on the size of the animals, were excised with the aim of investigating the position of the symbionts within the endoderm using TEM

only. The preparation of samples for the scanning (SEM) and transmission (TEM) electron microscopes was as follows:

(i)Fixation

Fixation of samples is required to preserve cell structure by cross-linking structural components, thereby protecting the samples from further preparatory steps.

The algae, tentacles or polyps were placed in labelled Eppendorf centrifuge tubes. The suspended isolated algae were centrifuged in an Eppendorf centrifuge for 2 minutes to obtain an algal pellet, the supernatant having been pipetted off. This procedure was repeated after each of the following steps, in which the pellet was resuspended, until the sample was embedded.

To each sample was added 1 ml 50 % 0.1 M phosphate buffer (to maintain pH) : 50 % 0.6 M sucrose (to maintain osmotic balance) at pH 7.4 with 3 % glutaraldehyde (the fixative). The samples were then fixed at 4 ^{o}C for 30 minutes. All work where glutaraldehyde was being pipetted was performed in a fume hood.

The buffer was made by preparing 2 separate 0.2 M stock solutions of NaH_2PO_4 and Na_2HPO_4. 27.8 g of NaH_2PO_4 and 53.65 g of Na_2HPO_4 were dissolved in 1 l distilled water. 19 ml NaH_2PO_4 solution and 81 ml Na_2HPO_4 solution were then added together, resulting in phosphate buffer of pH 7.3. A 1.2 M sucrose solution was prepared by dissolving 410.76 g sucrose in 1 l distilled water. 1.2 ml of 25 % glutaraldehyde was then added to 8.8 ml 50 % phosphate

buffer : 50 % sucrose to give a final concentration of 3 % glutaraldehyde.

(ii)Washing

Following fixation the supernatant was pipetted off and the samples washed to remove the fixative and sucrose.

The samples were resuspended and then centrifuged in 3 different washes: Wash I: 1 minute in 50 % phosphate buffer (0.1 M) : 25 % sucrose (0.6 M) : 25 % distilled water; Wash II: 1 minute in 50 % phosphate buffer (0.1 M) : 12.5 % sucrose (0.6 M) : 37.5 % distilled water; Wash III: 3 x 1 minute in 50 % phosphate buffer (0.1 M) : 50 % distilled water.

(iii)Post-fixation

The samples were placed in 1 % osmium tetroxide (OsO_4) in phosphate buffer and post-fixed at 4 ^{o}C for 30 minutes. A 10 % OsO_4 stock solution was made by dissolving 0.2 g OsO_4 in 2 ml distilled water overnight, the day before use. The stock solution was then added to 0.1 M phosphate buffer in the ratio 1 : 9. All preparation of the OsO_4 stock solution and the pipetting of this solution was performed in a fume hood.

(iv)Washing 2

To wash the samples free of OsO_4, the OsO_4 solution was pipetted off and the samples were shaken in distilled water for 1 minute. This procedure was then repeated once.

(v)Dehydration

In order to remove water from the specimens before use in SEM or TEM it was necessary to dehydrate them. Resins used in TEM are not miscible with water and hence water would hinder a successful embedding of the sample. To dehydrate the samples a series of alcohol washes followed by centrifugation were employed: 1 minute in 30 % acetone; 1 minute in 50 % acetone; 1 minute in 70 % acetone; 5 minutes in 100 % acetone; 5 minutes in 100 % propylene oxide .

At this point the preparatory procedure differed depending on whether the TEM or SEM was being used.

(vi)SEM preparation

The dehydrated algal pellets were pipetted out of the Eppendorf tubes and into labelled plastic capsules which had plankton mesh of 2 μm mesh size placed over the bottom. This mesh size permitted the movement of the relevant liquids into and from the capsule, but prevented the loss of the symbiont cells. These capsules were stood, bottom down, in a glass well containing acetone to ensure that the samples were in alcohol at all times. Once the samples were in place, the top of the capsules was sealed with a second piece of 2 μm plankton mesh. The capsules were then placed in an acetone filled holder and placed into a critical point drier (CPD). The sealed CPD chamber was then filled with liquid CO_2 and excess air bled out. The CO_2 was vented off

and the chamber 3/4 filled with CO_2 again. This latter procedure was repeated a further 3 times to vent off most of the acetone. Following this, the chamber was 3/4 filled with CO_2 and allowed to stand for 15 minutes. The chamber was then vented repeatedly until all traces of acetone, as detected with tissue from the exhaust pipe, had disappeared. The chamber was 3/4 filled again and hot tap water poured into the jacket surrounding the chamber until a chamber temperature of 32 ^{o}C was achieved, causing the liquid level in the chamber to disappear. Subsequently, the chamber was returned to atmospheric pressure and the dried samples removed.

Next it was necessary to mount the samples. Aluminium 'stubs' were covered in double sided sticky tape and the dried samples were adhered to this surface. The samples were then coated in gold using a Polaron splutter coater. The 'stubs' were pumped down to a pressure of 0.1 mbar in the splutter coater chamber and the chamber was flushed with argon for 10 seconds. The chamber was then pumped to 0.07 mbar and again argon was leaked in. Following this the chamber was pumped down to 0.03 mbar and thermally equilibrated at 5 ^{o}C for 5 minutes. Finally the voltage was switched to 2.5 KV, argon flushed into the chamber to give a current reading of 18 mA and the samples coated for 5 minutes to give a coat thickness of 675 Angstroms.

The prepared samples were mounted in and observed under a

scanning electron microscope (Cambridge Instruments Stereoscan S-120).

(vii)TEM preparation

Following dehydration, the algal pellets, tentacles and polyps were infiltrated with resin and embedded.

Spurr's resin, which possesses good cutting and staining properties and can be stored frozen for several months, was used for infiltration and embedding. Spurr's resin was made by adding the following in the order given:

11.5 g vinylcyclohexene dioxide (VCD), 7 g diglycidyl ether of polypropylene glycol (DER) 736 (to control hardness), 31 g nonenyl succinic anhydride (NSA) and 0.5 g dimethylaminoethanol (DMAE) (the catalyst). The resin was stored in sealed containers in a deep freeze and was defrosted when required.

The samples were put into plastic caps which provide a large surface area over which alcohol evaporation can occur. The samples were infiltrated by adding 1 : 1 100 % propylene oxide : Spurr's resin for 15 minutes (propylene oxide was used as it is more miscible with resin and more readily evaporates than acetone). Subsequently, the samples were infiltrated with 100 % resin for 30 minutes, embedded in fresh 100 % resin and polymerized at 65 ^{o}C overnight.

The resin blocks were trimmed, leaving the sample surrounded by a small amount of resin, using a saw. The block was then clamped into a chuck and a razor blade used to trim this

block into a pyramid. The top of this pyramid was cut to obtain a flat, smooth cutting face with the sample near the surface in the centre of this face. The chuck was loaded into a LKB ultratome and 1 µm sections cut using a glass knife. The sections were collected in a distilled water filled trough, removed and placed on a slide. Subsequently, the sections were dried on a hot plate and stained with toluidine blue in borax (1 g toluidine blue and 1 g borax in 100 ml distilled water). The sections were examined under a light microscope to ensure that further thin sections would contain the desired material. Thin grey sections (60 - 90 nm) were then cut, mounted on either '200' copper grids or slot grids and double stained with uranyl acetate (1 1/4 hours) and lead citrate (4 minutes) using a Hiraoka grid staining kit (Polaron Instruments Inc.). Slot grids were employed in an attempt to obtain serial sections with the aim of constructing a 3-D image of the cells. The prepared samples were finally observed using either a Corinth or Philips-200 transmission electron microscope. The observed structures were identified by comparison with published electron micrographs (eg. Taylor, D.L. 1969a; Taylor, D.L. 1969b; Loeblich and Sherley 1979; Schoenberg and Trench 1980b; Blank 1987; Trench 1987; Trench and Blank 1987).

3.2.3 Results

(a)Light microscopy

(i)Location of the symbionts

The symbionts of all the host species under investigation were located within the endoderm (plate 3.1 A (p.77) and appendix 2, plate 1 (p.468)). But the very high concentrations of the algae rendered the endodermal cells themselves indistinguishable. No symbionts were observed within the epidermis or mesogloea. The symbionts were distributed throughout the tentacles and oral disc of all the host species, and throughout the polyps of I. sulcatus and A. pallida.; the distribution of symbionts within C. pedunculatus, A. viridis and A. ballii was not investigated further.

(ii)Morphological features of freshly isolated symbionts

Light microscopy revealed few cellular structures (plate 3.1 B (p.77) and appendix 2, plate 2 (p.469)). Freshly isolated cells were all coccoid in nature and were regularly observed to be in a state of mitosis with a distinct cell plate dividing two daughter cells and a thin, unornamented amphiesma surrounding the cells. Triads of daughter cells were observed on one occasion, in a preparation of symbionts from A. ballii. The only obvious cellular inclusions visible were a central pyrenoid surrounded by a paler starch sheath, a large peripheral chloroplast(s) with a distinct yellow/brown colouration, several vacuoles and occasionally a dark, spherical "accumulation body" which was more

prominent in degenerating cells. The "accumulation body" was observed to be present in either one or both of the daughter cells during division. No flagellated cells were evident. The nuclei could be observed as a pink/red spot when stained with aceto-orcein and occasionally some chromosomes were evident. However, the nucleus was frequently obscured by cellular inclusions and the chromosomes could not be viewed clearly enough to perform accurate counts.

(iii)Morphological features of cultured symbionts

The cells were again coccoid in nature and a "2-cell dividing stage" was regularly observed, with no triads or tetrads of dividing cells being evident (plate 3.1 C (p.77)). The only cellular inclusions visible were those seen in the freshly isolated symbionts and whether the amphiesma was thicker in culture than in situ could not be determined at this magnification. However, many of the cells appeared less pigmented and possessed much larger "accumulation bodies" than the freshly isolated cells. There were two obvious differences between the cultured algae and the freshly isolated cells. Firstly, motile cells were present in the cultures of S. microadriaticum, S. pilosum and the symbionts of A. elegantissima, A. pulchella and A. pallida. Motile cells of A. pallida symbionts were observed within a month after the culture was initiated. These motile cells were obviously gymnodinioid dinoflagellates, with the longitudinal and transverse flagella being visible. The cells were divided into hypocone and epicone regions, the

epicone being larger than the hypocone. Once again, the only structures visible in these cells were a pyrenoid and chloroplast(s). The cells moved either rapidly forwards whilst gyrating transversely or by gyrating 'on the spot' with the cell 'body' at right angles to the flagella. No clear photographs are available of these motile cells, however light micrographs are given by Steele (1975) and Schoenberg and Trench (1980b). Notably, no motile stage was observed in the cultures of any of the symbionts of the British Anthozoa.

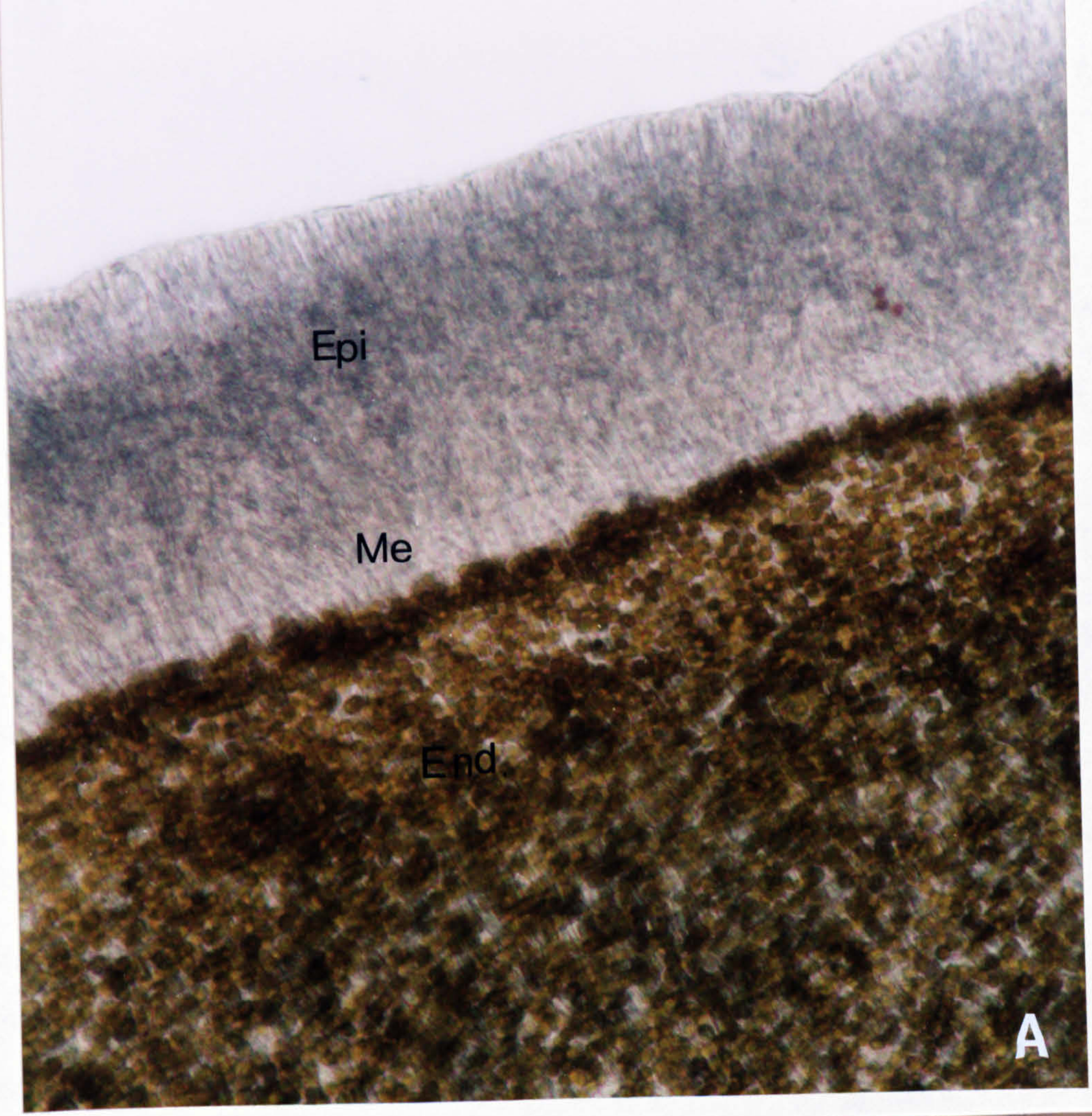
The second difference was the 'matrix' of clear material surrounding the coccoid cells, causing cells that were disturbed from the growth surface to clump. This 'matrix' was believed to be compounds excreted by the cells (Trench, pers. comm.), but the cementing material was more prominent in collapsing cultures. No such 'matrix' was observed around freshly isolated cells, although any isolation procedure would have disrupted a matrix even if it were originally present.

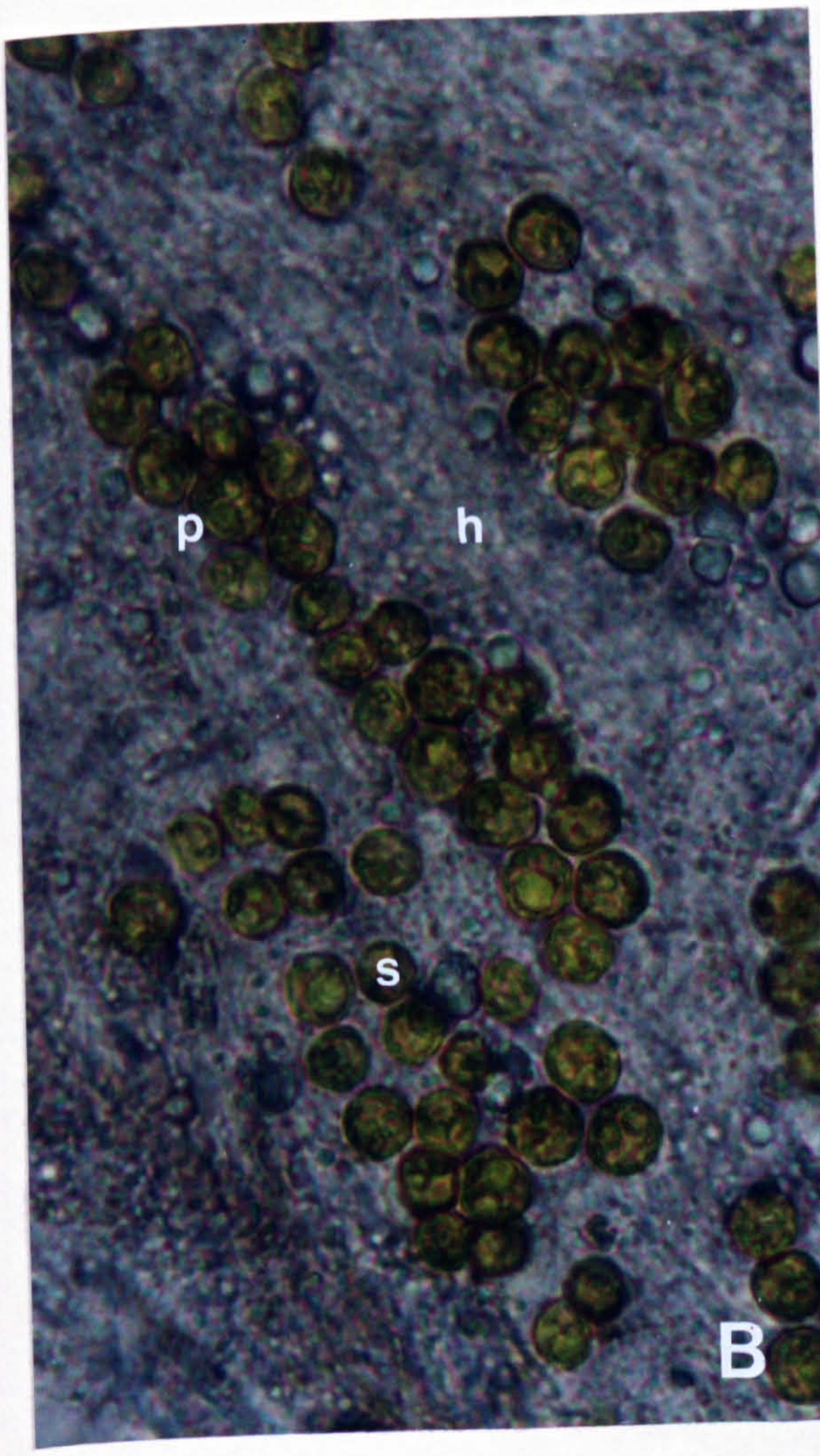
Plate 3.1: Light micrographs showing general features.

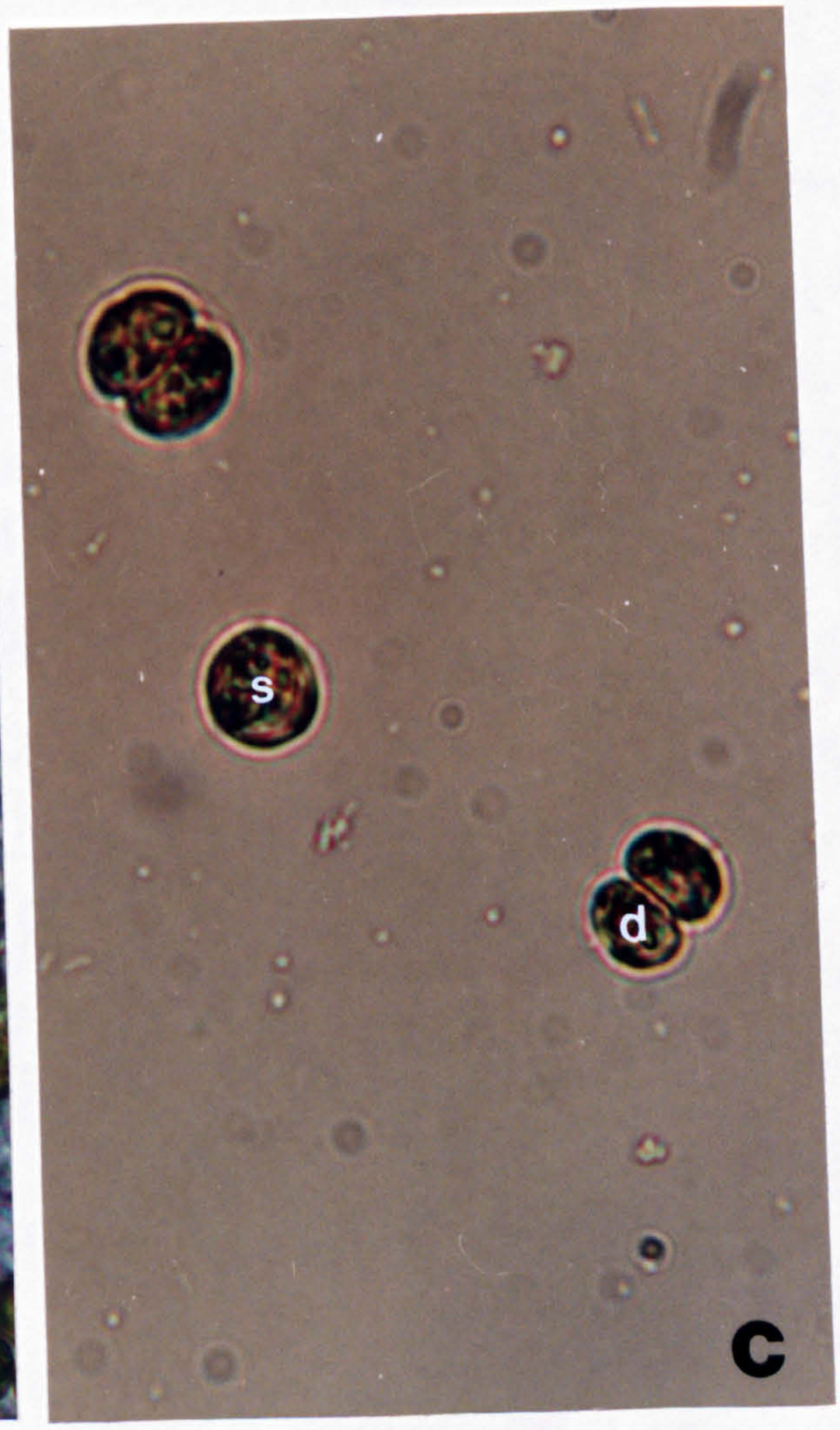
(A) A tentacle squash of A. viridis (Lough Hyne) showing the location of the brown symbionts within the endoderm (magnification approx. x200).
Epi = epidermis; Me = mesogloea; End = endoderm.

(B) Symbiont cells freshly isolated from C. pedunculatus (Lough Hyne) (magnification approx. x750).
s = symbiont cell; h = host homogenate; p = symbiont cell with a prominent pyrenoid.

(C) Cultured symbiont cells originally isolated from A. viridis (Shell Island) (magnification approx. x1000).
s = symbiont cell; d = symbiont cell in "2-cell dividing stage".


Epi
Me
End.
A


p
h
s
B


s
d
C

(iv)Dimensions of freshly isolated and cultured symbionts

The lengths and widths of the "2-cell stage" of the live cultured and freshly isolated symbionts are shown in table 3.2 (p.82), and the lengths and widths of the cultured algae from different host species and from A. viridis from different locations are illustrated in graphs 3.1 (p.83) and 3.2 (p.84) respectively. The results of t-tests comparing the dimensions of cultured and freshly isolated cells are given in appendix 3, table 1 (p.471). Symbionts from A. pallida were significantly larger and symbionts of A. ballii and C. pedunculatus (Lough Hyne) were significantly smaller in culture than when freshly isolated from the host. The dimensions of all other cultured cells were consistent with their size when freshly isolated.

Using the Kruskal-Wallis test, the dimensions of the symbionts of A. ballii, C. pedunculatus (Lough Hyne), A. viridis (Lough Hyne), A. viridis (Shell Island), I. sulcatus, A. pallida, A. pulchella, Cassiopeia sp., A. elegantissima and Z. sociatus were compared. The cell lengths ($H=254.77$, $P<0.0001$) and widths ($H=258.78$, $P<0.0001$) of the cultured symbionts were significantly different, as were the lengths ($H=166.57$, $P<0.0001$) and widths ($H=172.54$, $P<0.0001$) of the freshly isolated cells. The results of more detailed multiple comparison tests between the cultured and freshly isolated samples are shown in appendix 3, tables 2-5 (pp.472-474). From these tables and graphs 3.1 and 3.2 the presence of 2 size classes of cultured symbionts can be

seen. Group I included symbionts of A. pulchella, Cassiopeia sp., A. pallida, A. viridis (Loch Sween), C. pedunculatus (Lough Hyne) and C. pedunculatus (Netley). Group II included symbionts of A. viridis (Shell Island), A. viridis (Weymouth), A. viridis (Bembridge), A. viridis (Trearddur Bay), A. ballii, I. sulcatus, A. elegantissima and Z. sociatus. Symbionts of A. viridis (Lough Hyne) overlapped the two groups.

Tables of freshly isolated symbiont dimensions revealed 4 less distinct size classes. Group I contained Symbiodinium sp. from A. pallida and A. viridis (Loch Sween). Group II included symbionts from A. viridis (Loch Sween), C. pedunculatus (Netley), C. pedunculatus (Lough Hyne) and A. viridis (Lough Hyne). Group III contained symbionts of C. pedunculatus (Lough Hyne), A. viridis (Lough Hyne), A. viridis (Shell Island), A. viridis (Weymouth Harbour), A. viridis (Bembridge), A. viridis (Trearddur Bay) and I. sulcatus. Group IV included symbionts of I. sulcatus and A. ballii. As is apparent from the degree of overlap between these size classes, the sizes of the freshly isolated symbionts could be considered as being a continuum.

In addition to their inclusion in the analyses above, the dimensions of the symbionts of C. pedunculatus and A. viridis from different geographical origins were compared on an intra-host specific basis. The dimensions of the symbionts of C. pedunculatus from Lough Hyne and Netley were compared using Student's t-test, revealing the cultured cell

lengths not to be significantly different (T=1.20, P=0.23), in contrast to the cell widths (T=3.12, P=0.0027). The lengths (T=1.49, P=0.14) and widths (T=0.70, P=0.49) of the freshly isolated symbionts of C. pedunculatus from Lough Hyne and Netley were not significantly different. The dimensions of the symbionts of A. viridis from different geographical locations also showed significant differences: The lengths of the cultured algae were significantly different (H=49.27, P<0.0001), as were the widths (F=24.83, P<0.0001), and the freshly isolated symbionts also exhibited significantly different lengths (H=61.0, P<0.0001) and widths (H=72.38, P<0.0001). Appendix 3, tables 6-9 (pp.475-476), show the results of multiple comparisons of these measurements. 2 size classes of the symbionts of A. viridis were evident, with symbionts of A. viridis (Loch Sween) being significantly smaller than those of A. viridis from all other locations.

Key of abbreviations used in tables and graphs throughout this thesis:

Cp = C. pedunculatus (Lough Hyne)

CpNt = C. pedunculatus (Netley)

Ab = A. ballii

Is = I. sulcatus

AvLH = A. viridis (Lough Hyne)

AvSI = A. viridis (Shell Island)

AvTB = A. viridis (Trearddur Bay)

AvLS = A. viridis (Loch Sween)

AvWH = A. viridis (Weymouth Harbour)

AvBm = A. viridis (Bembridge)

Ap = A. pallida

Apul = A. pulchella

Ca = Cassiopeia sp.

Zs = Z. sociatus

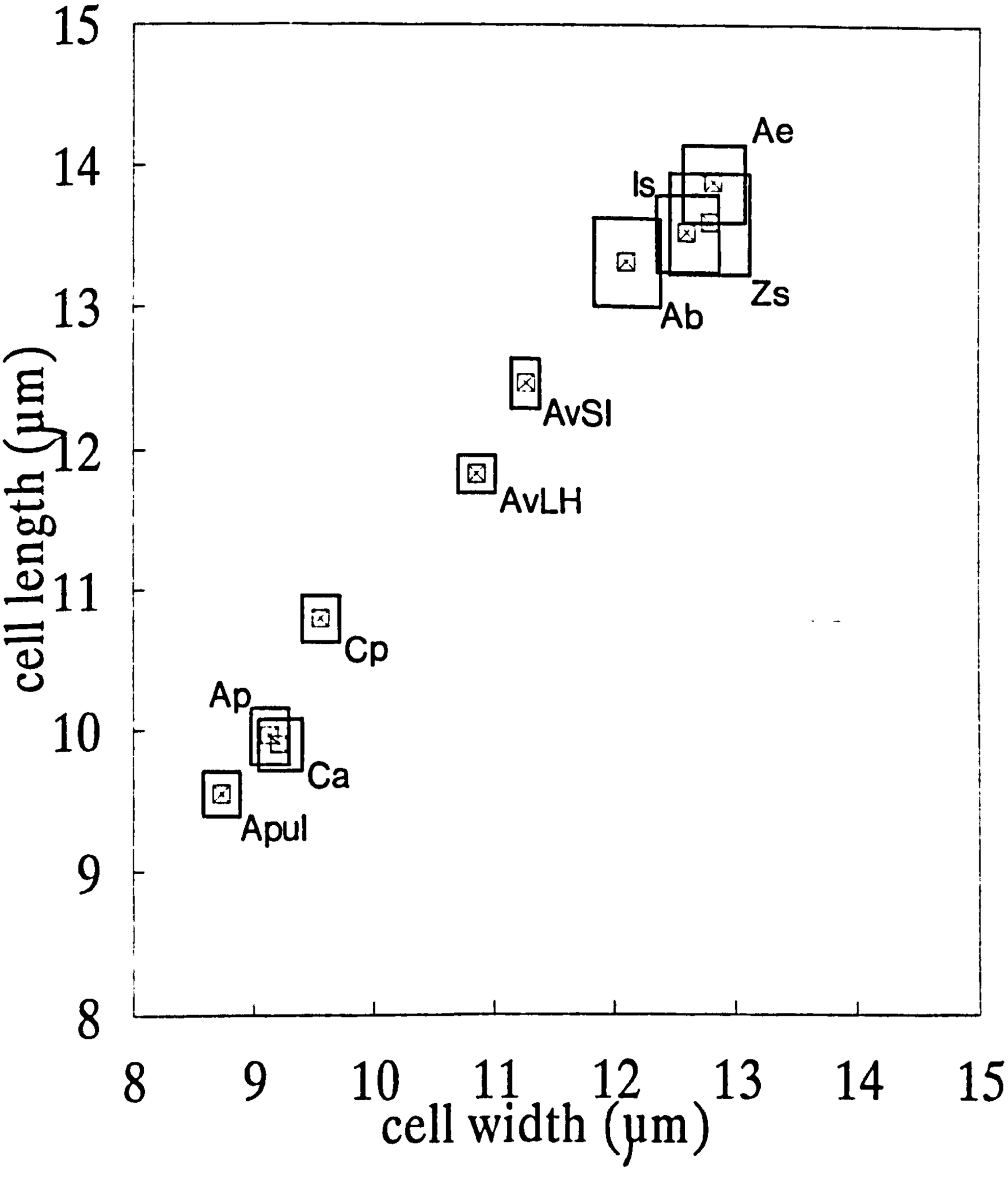
Ae = A. elegantissima

TABLE 3.2 DIMENSIONS OF "2-CELL STAGE" OF CULTURED AND FRESHLY ISOLATED SYMBIONTS

HOST SPECIES	CULTURED CELL LENGTH +/- S.E. (µm) (N=40)	CULTURED CELL WIDTH +/- S.E. (µm) (N=40)	FRESHLY ISOLATED CELL LENGTH +/S.E. (µm) (N=40)	FRESHLY ISOLATED CELL WIDTH +/- S.E. (µm) (N=40)
Apul	9.55 +/- 0.18	8.73 +/- 0.16	-	-
Ca	9.90 +/- 0.19	9.21 +/- 0.18	-	-
Ap	9.96 +/- 0.21	9.13 +/- 0.18	9.14 +/- 0.11	7.84 +/- 0.10
Cp	10.80 +/- 0.17	9.56 +/- 0.16	11.39 +/- 0.16	10.11 +/- 0.18
AvLS	10.97 +/- 0.16	9.76 +/- 0.15	10.81 +/- 0.16	9.75 +/- 0.15
CpNt	11.04 +/- 0.11	10.15 +/- 0.11	11.09 +/- 0.12	10.26 +/- 0.12
AvLH	11.83 +/- 0.14	10.86 +/- 0.14	11.61 +/- 0.18	10.45 +/- 0.17
AvWH	12.16 +/- 0.12	11.38 +/- 0.14	12.21 +/- 0.11	11.26 +/- 0.12
AvBm	12.21 +/- 0.12	11.22 +/- 0.14	12.30 +/- 0.14	11.34 +/- 0.11
AvTB	12.38 +/- 0.14	11.75 +/- 0.16	12.38 +/- 0.10	11.50 +/- 0.15
AvSI	12.47 +/- 0.19	11.27 +/- 0.11	12.14 +/- 0.10	11.24 +/- 0.10
Ab	13.32 +/- 0.31	12.10 +/- 0.27	13.96 +/- 0.16	12.79 +/- 0.14
Is	13.53 +/- 0.28	12.60 +/- 0.26	13.43 +/- 0.21	12.22 +/- 0.16
Ae	13.60 +/- 0.28	12.79 +/- 0.25	-	-
Zs	13.88 +/- 0.35	12.82 +/- 0.34	-	-

Graph 3.1: Cultured Symbiodinium cell length vs. cell width.

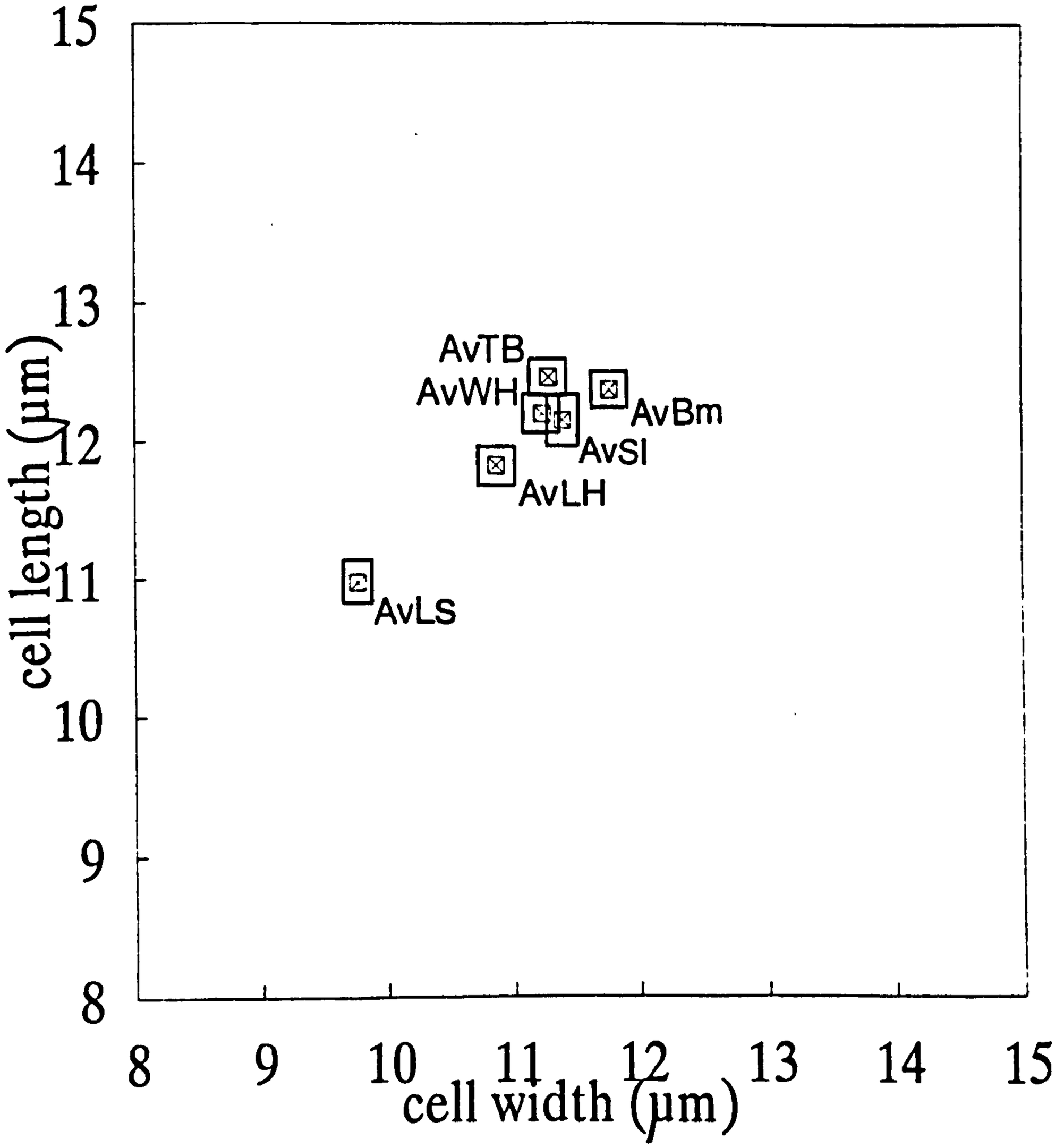
(N=40) (+/- S.E.).



Key: Ap = A. pallida; Apul = A. pulchella; Ca = Cassiopeia sp.;
Cp = C. pedunculatus (Lough Hyne); AvLH = A. viridis (Lough Hyne)
AvSI = A. viridis (Shell Island); Ab = A. ballii; Is = I. sulcatus
Ae = A. elegantissima; Zs = Z. sociatus

Graph 3.2: Cell length vs cell width for cultured symbionts of A. viridis from different locations

(N=40) (+/- S.E.).



Key: AvLH = A. viridis (Lough Hyne); AvSI = A. viridis (Shell Is.)
AvLS = A. viridis (Loch Sween); AvTB = A. viridis (Trearddur Bay);
AvWH = A. viridis (Weymouth Harbour); AvBm = A. viridis (Bembridge)

TABLE 3.3 ULTRASTRUCTURAL FEATURES OBSERVED USING TEM COMMON TO ALL INVESTIGATED CULTURED AND FRESHLY ISOLATED SYMBIONTS.

Ultrastructural feature
(1)A large, pale 'mesokaryotic-type' nucleus containing tightly coiled chromosomes and a grainy nucleolus.
(2)A nuclear envelope consisting of 2 membranes.
(3)A peripheral multilobed chloroplast(s) containing thylakoids and a pale stroma and surrounded by an indeterminable number of envelope membranes.
(4)A large pyrenoid with a stalk(s) attached to the inner face of the chloroplast, the lamellae of which did not invade the pyrenoid.
(5)Starch granules.
(6)A membrane-bound dark"accumulation body"which was separate from the pyrenoid.
(7)Vacuoles containing calcium oxalate crystals.
(8)Mitochondria.
(9)A membrane enclosing the cell contents and overlaid by a variable (1-4) series of wavy membranes in an apparently amorphous layer and one tightly-fitting outer membrane.
(10)An unidentified 'coat' (possibly polysaccharide (Trench 1987)) on the outer surface of the outermost cell membrane.

(b)Electron microscopy

(i)S.E.M.

All the cultured algal cells were identical in form, being coccoid with a smooth unornamented amphiesma. No thecal plates were visible (plate 3.2, (p.89)). Freshly isolated cells often possessed a wrinkled surface, possibly due to being surrounded by the animal endodermal cell (Muscatine et al. 1991).

(ii)T.E.M.

From in situ observations it appeared that all the symbionts were intracellular within the host endoderm. The symbiont cells were enclosed singly by a host membrane, with the host cell being separated from the algal cell by a perialgal space (plate 3.3 (p.90)).

Several characteristics were common to the symbionts, both cultured and freshly isolated, from all the different host species. These features are listed in table 3.3 (p.85) and can be seen in plates 3.4, 3.5 and 3.6 (pp.91-93) and appendix 4, plates 1-5 (pp.478-482).

Some ultrastructural differences were observed between the symbionts of different host species. The most distinctive difference was with respect to the arrangement of the thylakoid lamellae. The thylakoid lamellae of all the symbionts ran parallel to one another, however the chloroplasts of symbionts from A. viridis (from all locations), A. ballii, I. sulcatus and C. pedunculatus (from both locations) also possessed peripheral thylakoid lamellae

(plates 3.4 (p.91), 3.7 B (p.94), and appendix 4, plates 1 and 2 (pp.478-479)). In comparison, the symbionts of A. pallida did not possess peripheral thylakoid lamellae (plate 3.7 A (p.94)). The thylakoids were always stacked in groups of 3 (plate 3.8 C (p.95)) except in the symbionts from I. sulcatus; these possessed thylakoid lamellae which predominantly were in groups of 2 although short stacks of 3 appeared sporadically (plate 3.8 A (p.95) and plate 3.8 B (p.95)). No grana were observed in any of the chloroplasts.

The second major difference observed was the presence or absence of a cell wall in the freshly isolated cells. The freshly isolated and cultured symbionts of A.viridis, I.sulcatus and A.ballii all possessed a cell wall (often referred to as the "pellicle") which overlaid the membrane series described in table 3.3 (p.85). A further single membrane surrounded the cell wall (plate 3.6 B (p.93) and appendix 4, plate 5 (p.482)). However, freshly isolated symbionts of C. pedunculatus and A. pallida did not possess such a structure. Instead, the symbionts of these two host species were only enveloped by the plasmalemma and a series of membranes, distinguished from residual host membranes by the closeness with which they were bound to the cell (plate 3.9 A (p.96)). But a "pellicle" was produced by both these symbiont 'strains' upon culturing (plate 3.9 B (p.96)). Thickening of the cell wall was not noted consistently in culture by the symbionts of A. viridis, I. sulcatus and A. ballii.

In addition to changes in amphiesma structure, the size and numbers of starch granules decreased, and the size and numbers of "accumulation bodies" and calcium oxalate containing vacuoles increased in culture. A further structure was only observed in one section of a cultured symbiont from A. pallida. This structure appeared as fibrous material surrounded by a membrane and resembled a flagellar hair-producing cavity, although any identification of this structure remains speculative (plate 3.9 C (p.96)). All other ultrastructural features appeared to be stable with culturing.

Attempts to obtain serial sections of cells for 3-D analysis of symbiont ultrastructure failed. Loss of a few sections in a series, variations in staining between sections and difficulties with locating the same cell from one section to another resulted in 3-D analysis not being viable within the time-scale available.

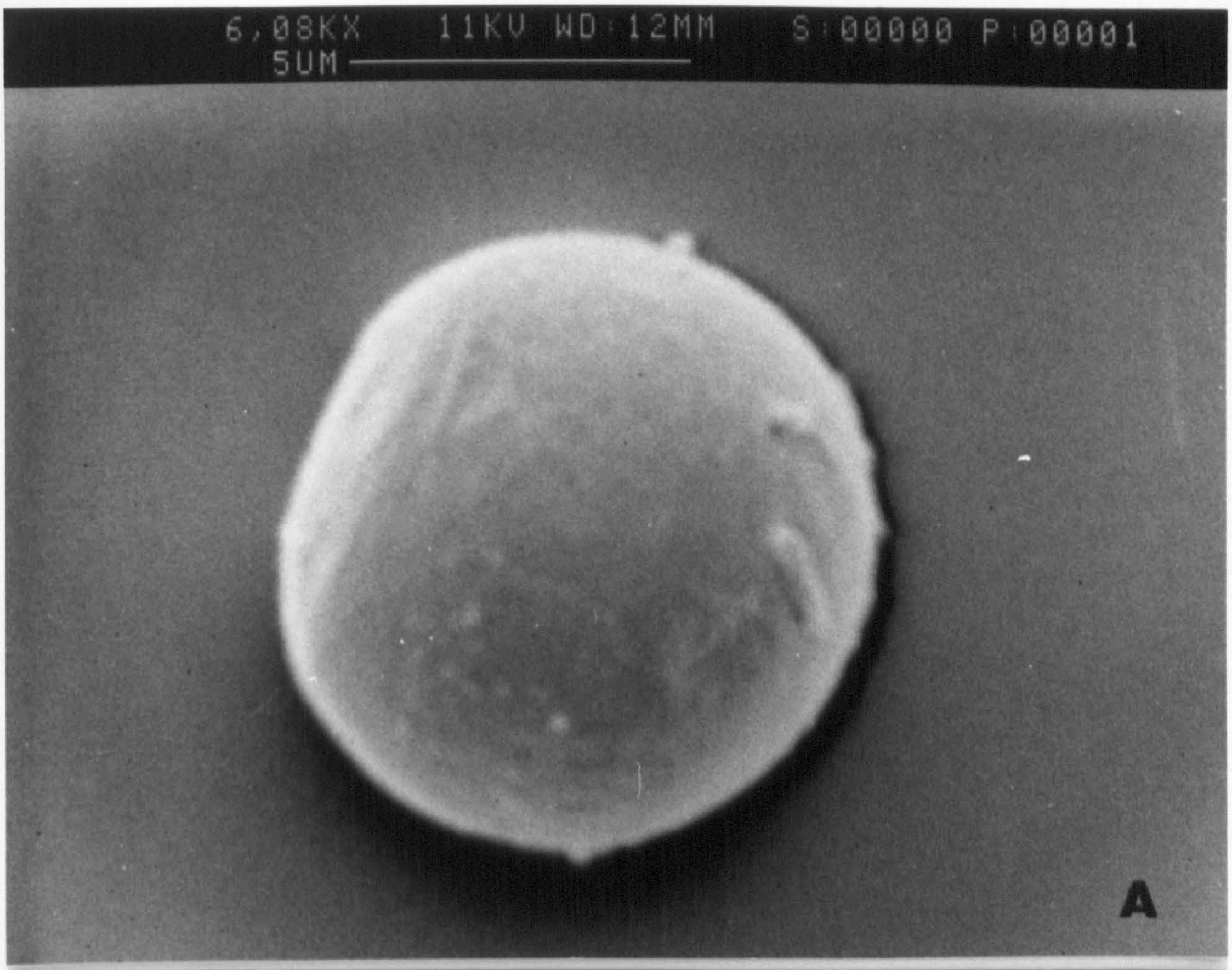
Plate 3.2: Scanning electron micrographs showing general features.

(A) A cultured symbiont cell originally isolated from *A. viridis* (Shell Island) (magnification approx. x9200).

Note the featureless cell surface.

(B) Cultured symbiont cells originally isolated from *A. pallida* (magnification approx. x2100).

Note the uniformity of the cells.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5UM
A

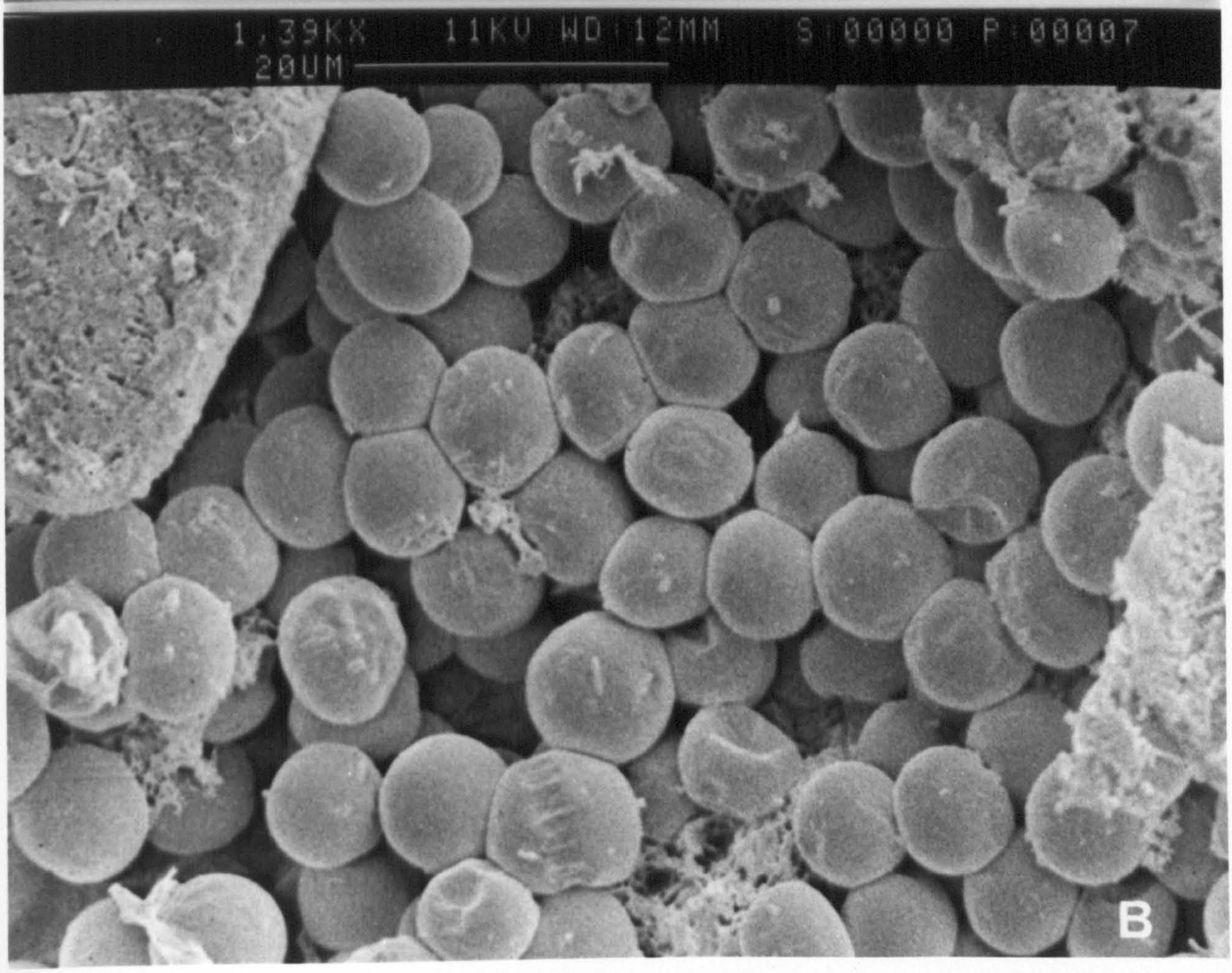

1,39KX 11KV WD:12MM S:00000 P:00007
20UM
B

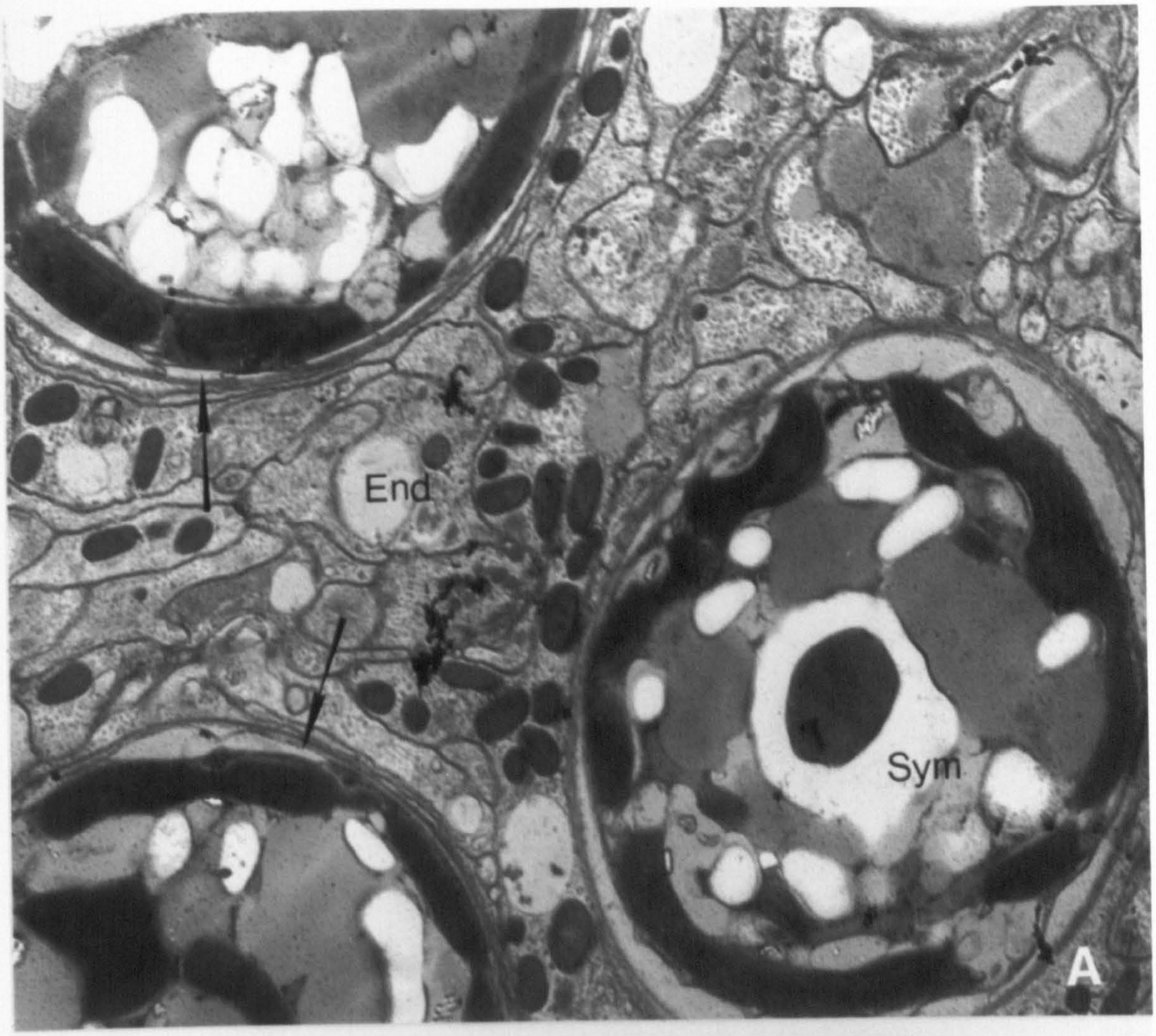
Plate 3.3: Transmission electron micrographs showing general features.

(A) Symbiont cells *in situ* in the endoderm of *I. sulcatus* (magnification approx. x9,650).

End = endoderm; Sym = symbiont cell; arrows indicate the position of the perialgal space.

(B) A symbiont cell *in situ* in the endoderm of *I. sulcatus* (magnification approx. x24,100).

End = endoderm; Sym = symbiont cell; cl = chloroplast; s = starch; cr = crystalline waste; ac = "accumulation body"; arrow indicates the position of the perialgal space.


End
Sym
A

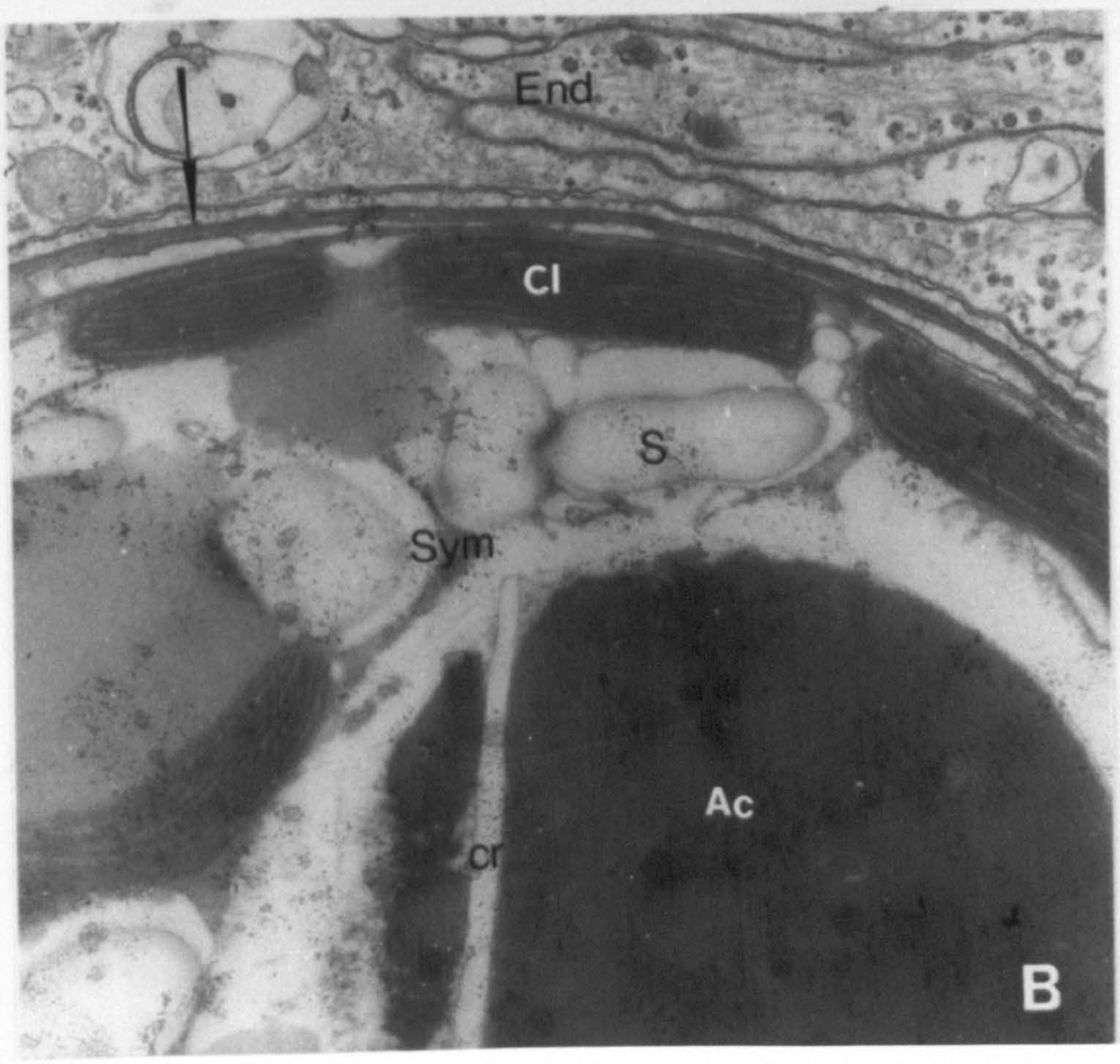

End
Cl
S
Sym
Ac
cr
B

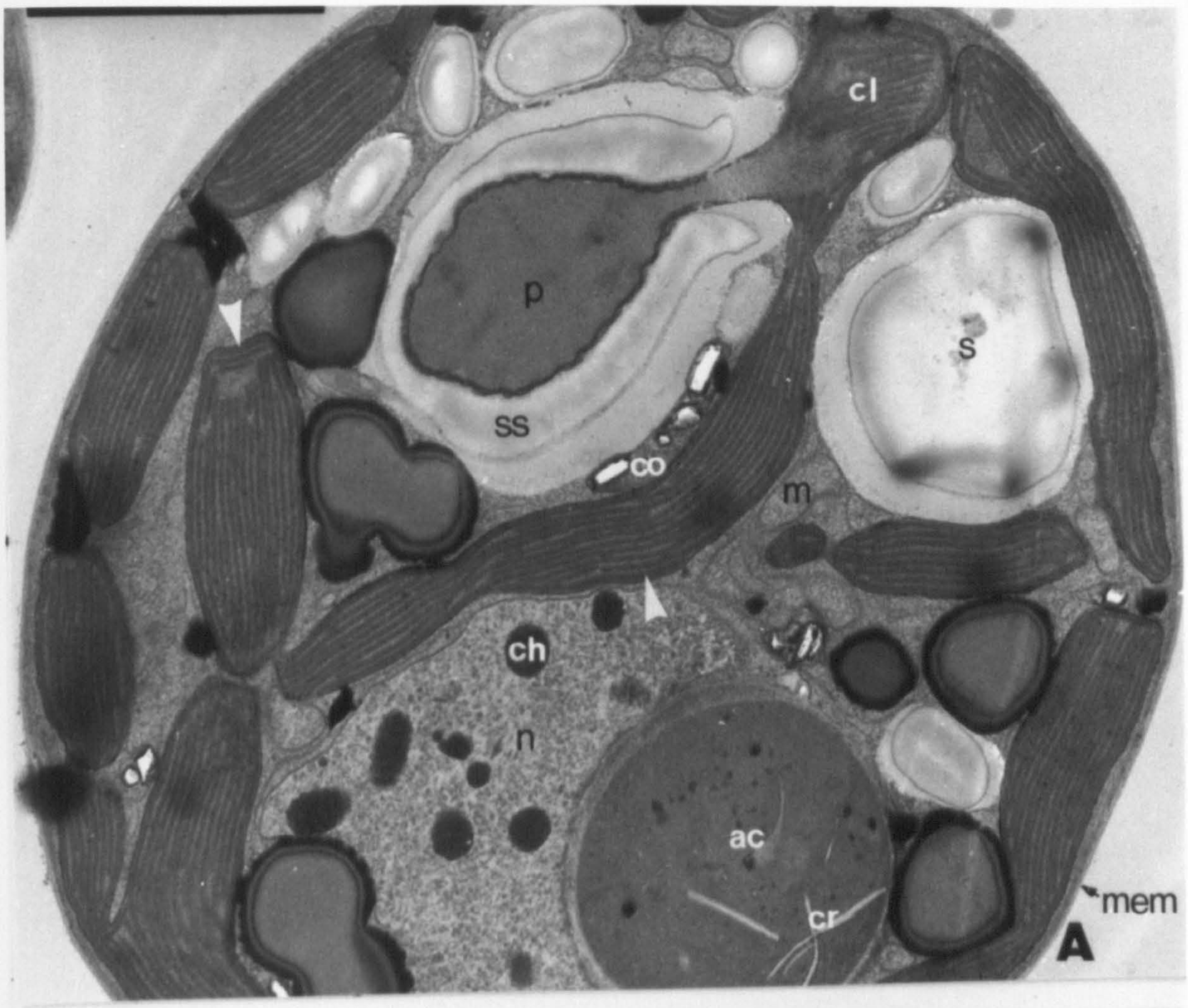
Plate 3.4: Transmission electron micrographs showing general features.

(A) A symbiont cell freshly isolated from C. pedunculatus (Netley) showing the general ultrastructural features of the genus Symbiodinium (magnification approx. x23,400).

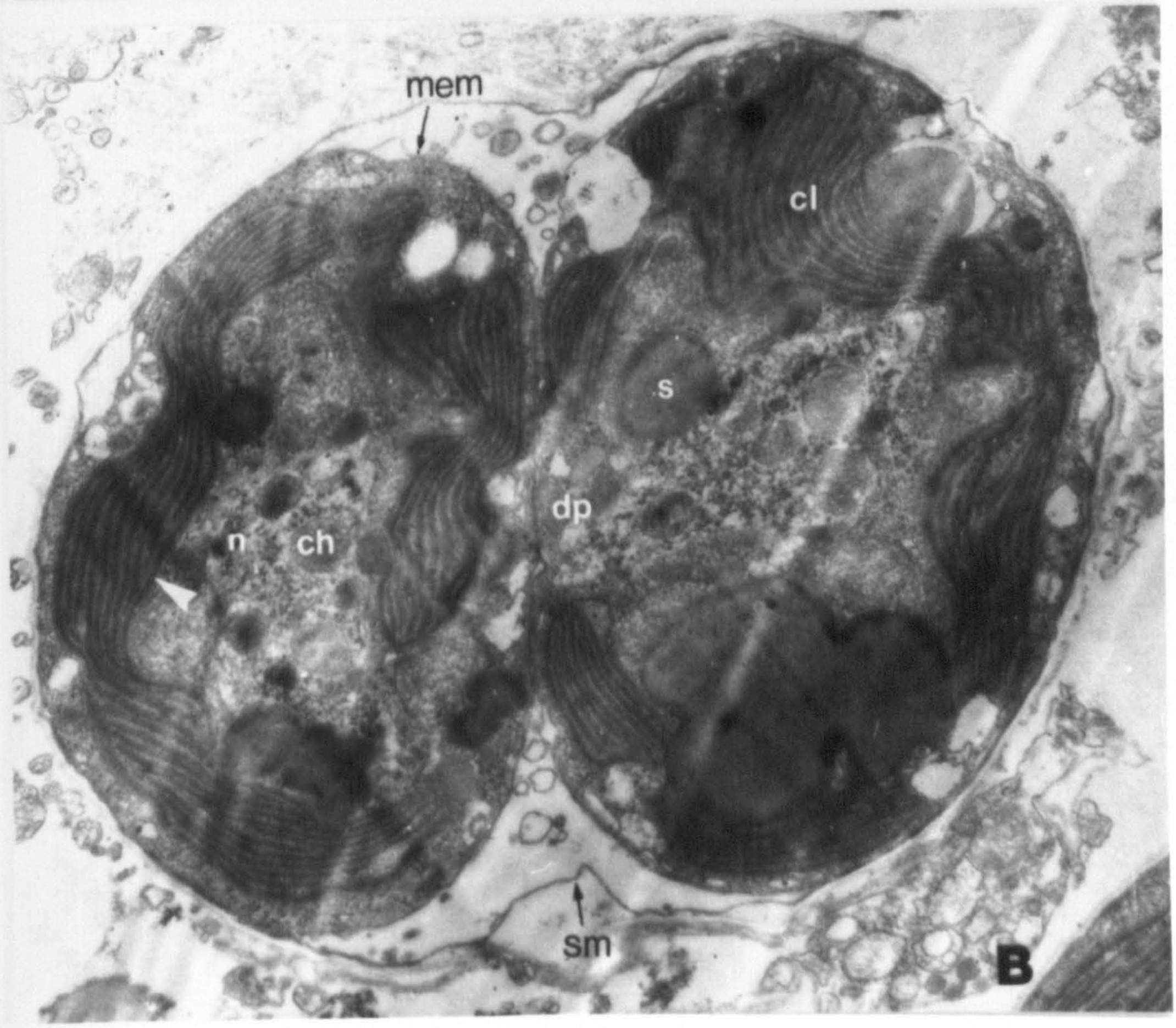
mem = membranes comprising the amphiesma; p = stalked pyrenoid with no invasive chloroplast lamellae; ss = starch sheath of pyrenoid; cl = chloroplast; ac = "accumulation body"; n = nucleus; ch = chromosome; m = mitochondrion; s = starch; co = calcium oxalate crystals; cr = crystalline waste; white arrows indicate the position of the peripheral thylakoid lamellae.

(B) A dividing symbiont freshly isolated from A. viridis (Lough Hyne) (magnification approx. x22,000).

mem = membranes/"pellicle" comprising the amphiesma of the daughter cells; sm = the shed membranes/"pellicle" of the amphiesma of the parental cell; dp = cell division 'plate'; cl = chloroplast; n = nucleus; ch = chromosome; s = starch; white arrow indicates the presence of a peripheral thylakoid lamella.



A



B

Plate 3.5: Transmission electron micrographs showing general features.

(A) Condensed, tightly coiled chromosomes in a symbiont cell freshly isolated from A. viridis (Lough Hyne) (magnification approx. x72,000).

N = nucleus; Ch = chromosome; nm = nuclear membranes; Cl = chloroplast; Ac = "accumulation body".

(B) The double nuclear envelope of a symbiont cell freshly isolated from A. viridis (Weymouth Harbour) (magnification approx. x236,250).

N = nucleus; Ch = chromosome; arrows 1 and 2 indicate the membranes of the nuclear envelope.

(C) The stalked pyrenoid, with non-invasive chloroplast lamellae, in a symbiont cell freshly isolated from C. pedunculatus (Netley) (magnification approx. x46,800).

P = stalked pyrenoid; SS = starch sheath of pyrenoid; S = starch; Cl = chloroplast; CO = calcium oxalate crystals.

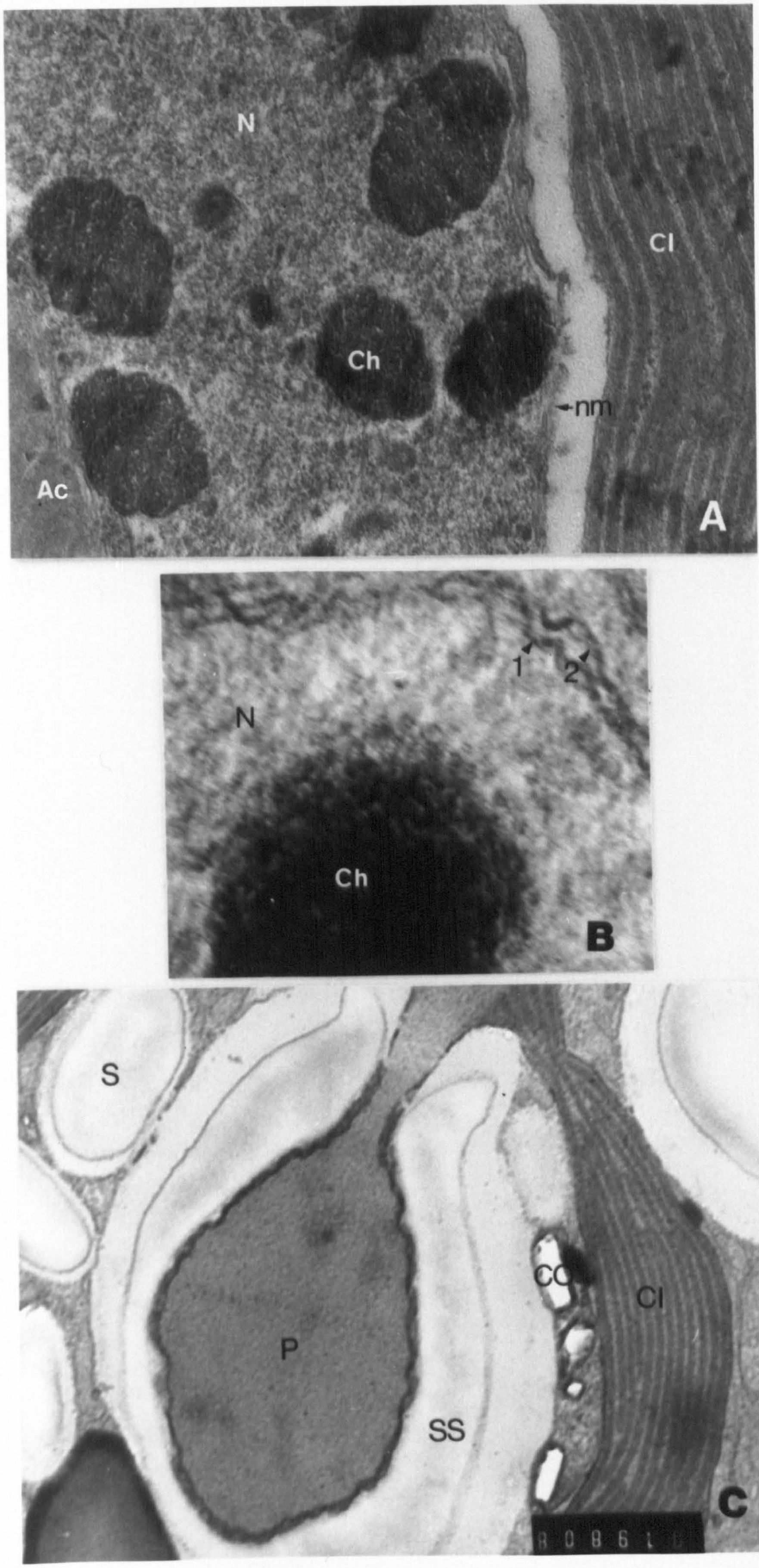
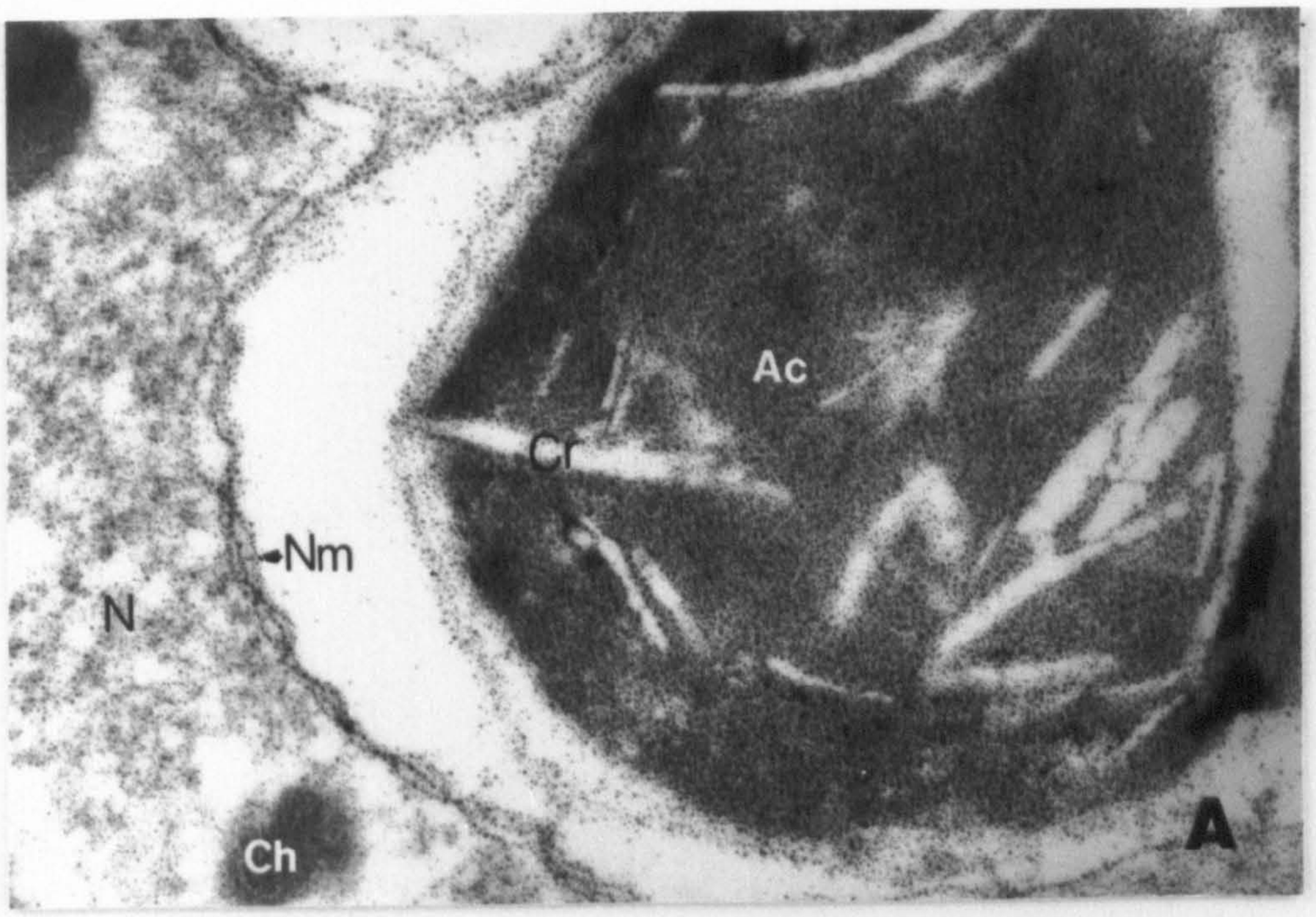

N
Cl
Ch
nm
Ac
A
1
2
N
Ch
B
S
P
SS
CC
Cl
C

Plate 3.6: Transmission electron micrographs showing general features.

(A) The membrane-bound "accumulation body" of a symbiont cell freshly isolated from C. pedunculatus (Lough Hyne) (magnification approx. x64,700).
Ac = "accumulation body"; Cr = crystalline waste; N = nucleus; Nm = nuclear membranes; Ch = chromosome.

(B) Thylakoid and amphiesmal arrangements of a cultured symbiont cell originally isolated from A. viridis (Loch Sween) (magnification approx. x236,250).
pl = plasmalemma; wm = series of wavy membranes; Pe = "pellicle"; pm = peripheral membrane; Cl = chloroplast; arrows 1, 2 and 3 indicate the 3 stacked thylakoid lamellae.

(C) The glycoprotein/mucopolysaccharide 'coat' on the cell surface of a symbiont freshly isolated from A. viridis (Lough Hyne) (magnification approx. x69,400).
(mp = glycoprotein/mucopolysaccharide 'coat'; pe = "pellicle"; Cl = chloroplast.


Ac
Cr
Nm
N
Ch
A


8 2 5
pm
wm
pl
Pe
Cl
3
2
1
B


pe
Cl
mp
C

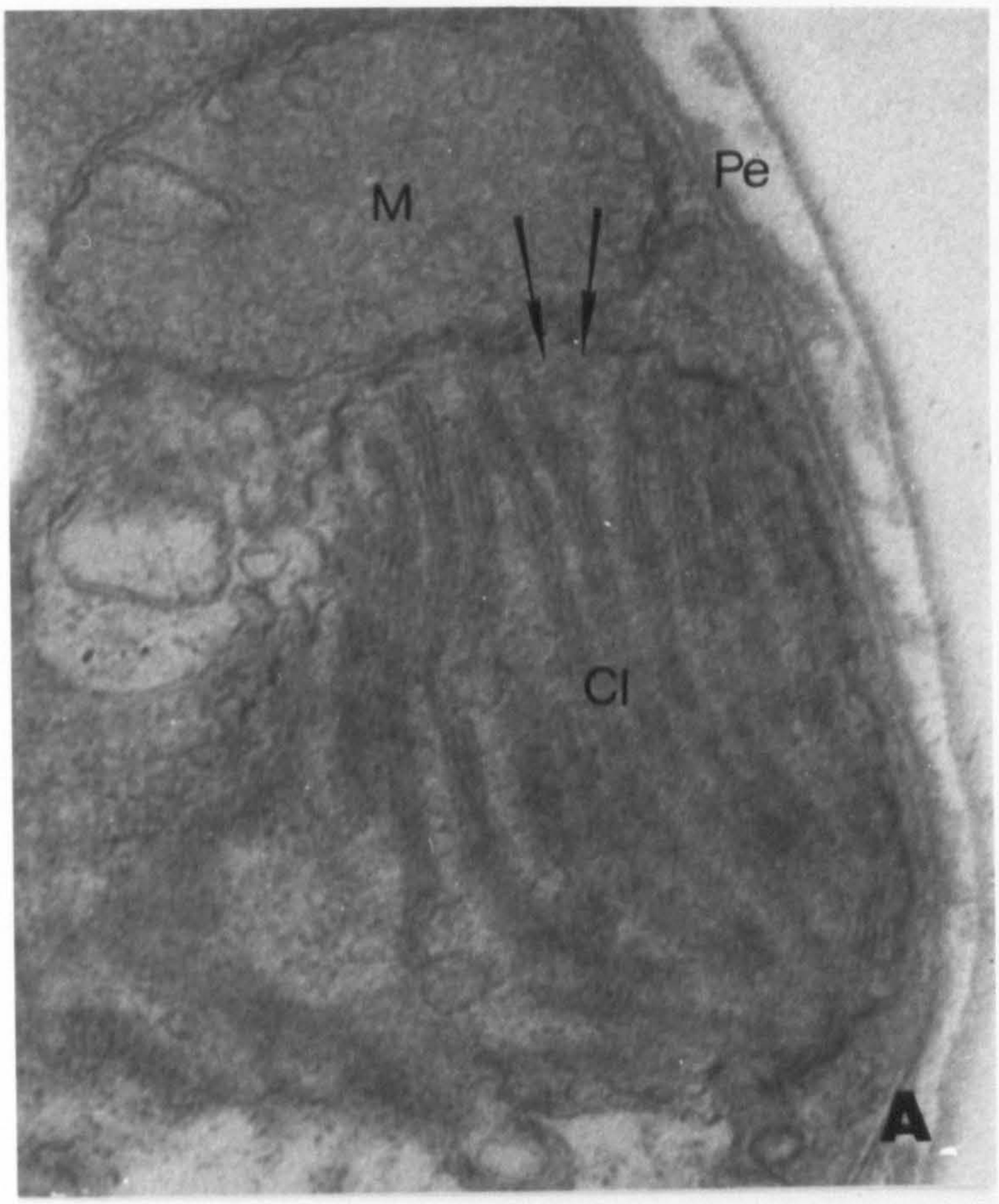
Plate 3.7: Transmission electron micrographs illustrating the presence and absence of peripheral thylakoid lamellae in some 'strains' of Symbiodinium sp..

(A) A chloroplast of a cultured Symbiodinium sp. cell originally isolated from A. pallida showing the absence of peripheral thylakoid lamellae (cf. plate 3.8 (B)) (magnification approx. x94,500).

Cl = chloroplast; M = mitochondrion; Pe = "pellicle"; arrows indicate the absence of peripheral thylakoid lamellae.

(B) A chloroplast of a Symbiodinium sp. cell freshly isolated from A. viridis (Trearddur Bay) showing the presence of peripheral thylakoid lamellae (cf. plate 3.8 (A)) (magnification approx. x94,500).

Cl = chloroplast; Pe = "pellicle"; PT = peripheral thylakoid lamellae.


M
Pe
Cl
A

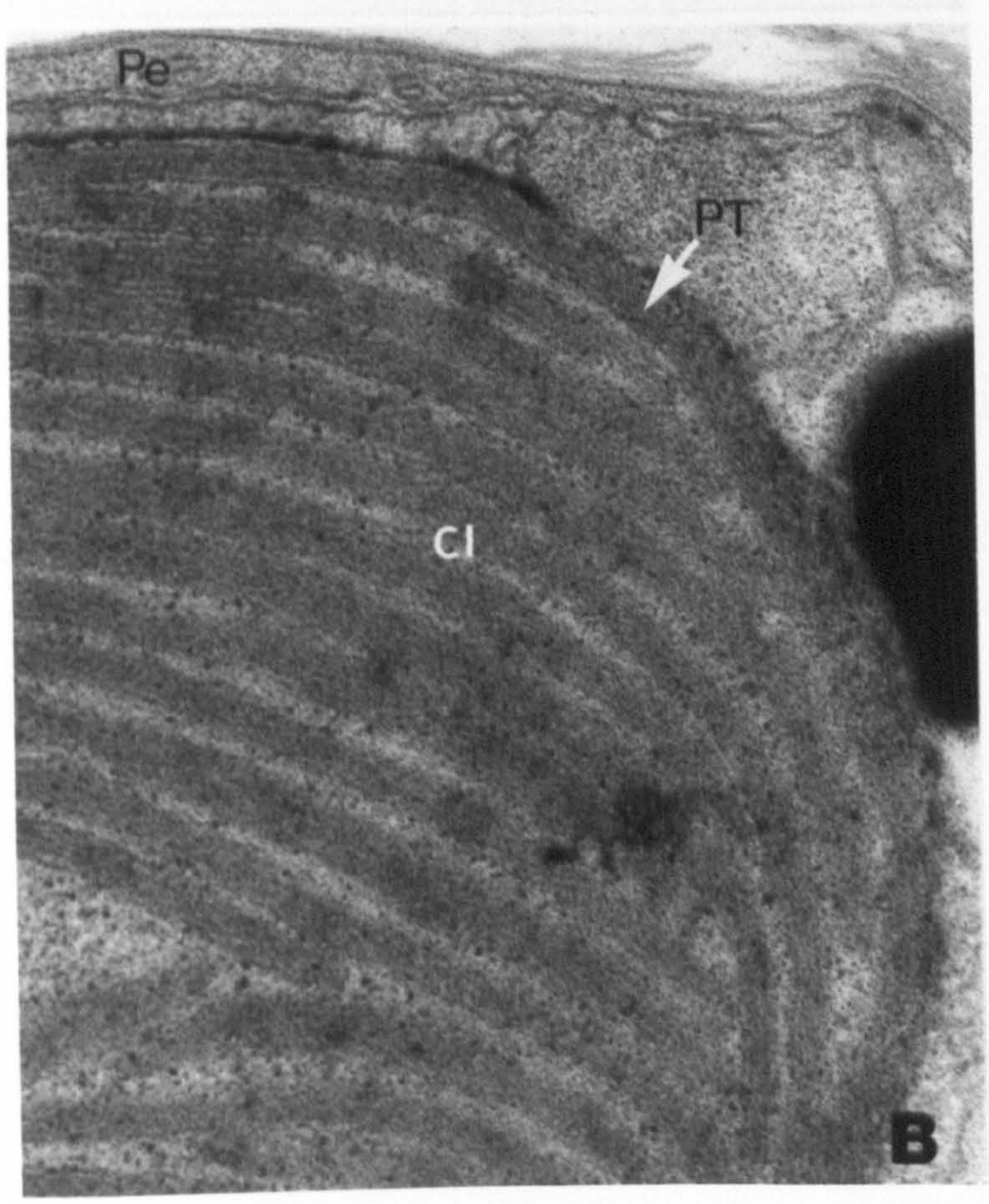

Pe
PT
Cl
B

Plate 3.8: Transmission electron micrographs showing unusual characteristics of symbionts.

(A) A cultured *Symbiodinium* sp. cell originally isolated from *I. sulcatus* showing the unusual thylakoid arrangement (magnification approx. x 54,000).

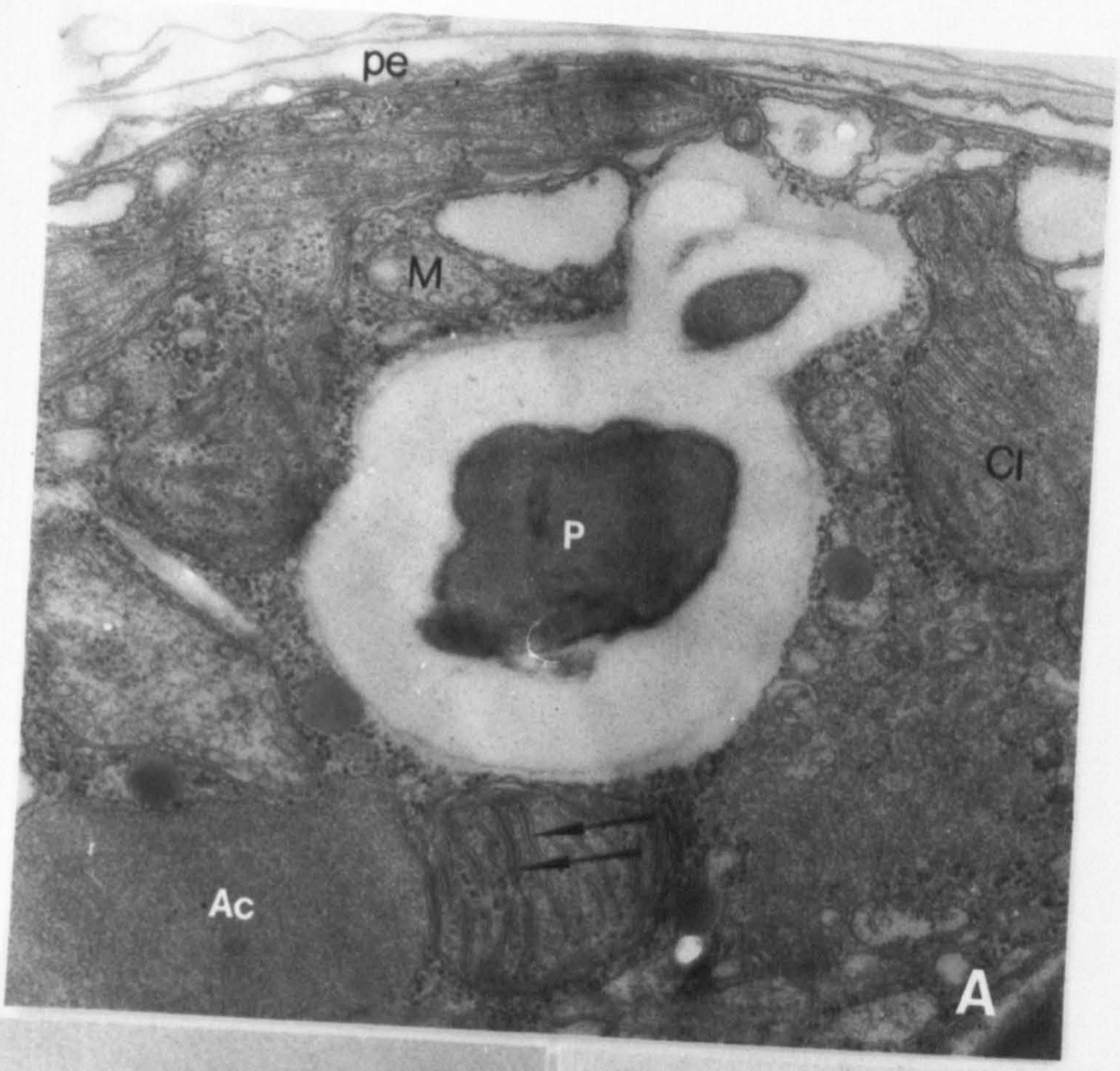
Cl = chloroplast; arrows indicate the thylakoids; P = pyrenoid; pe = "pellicle"; M = mitochondrion; Ac = "accumulation body".

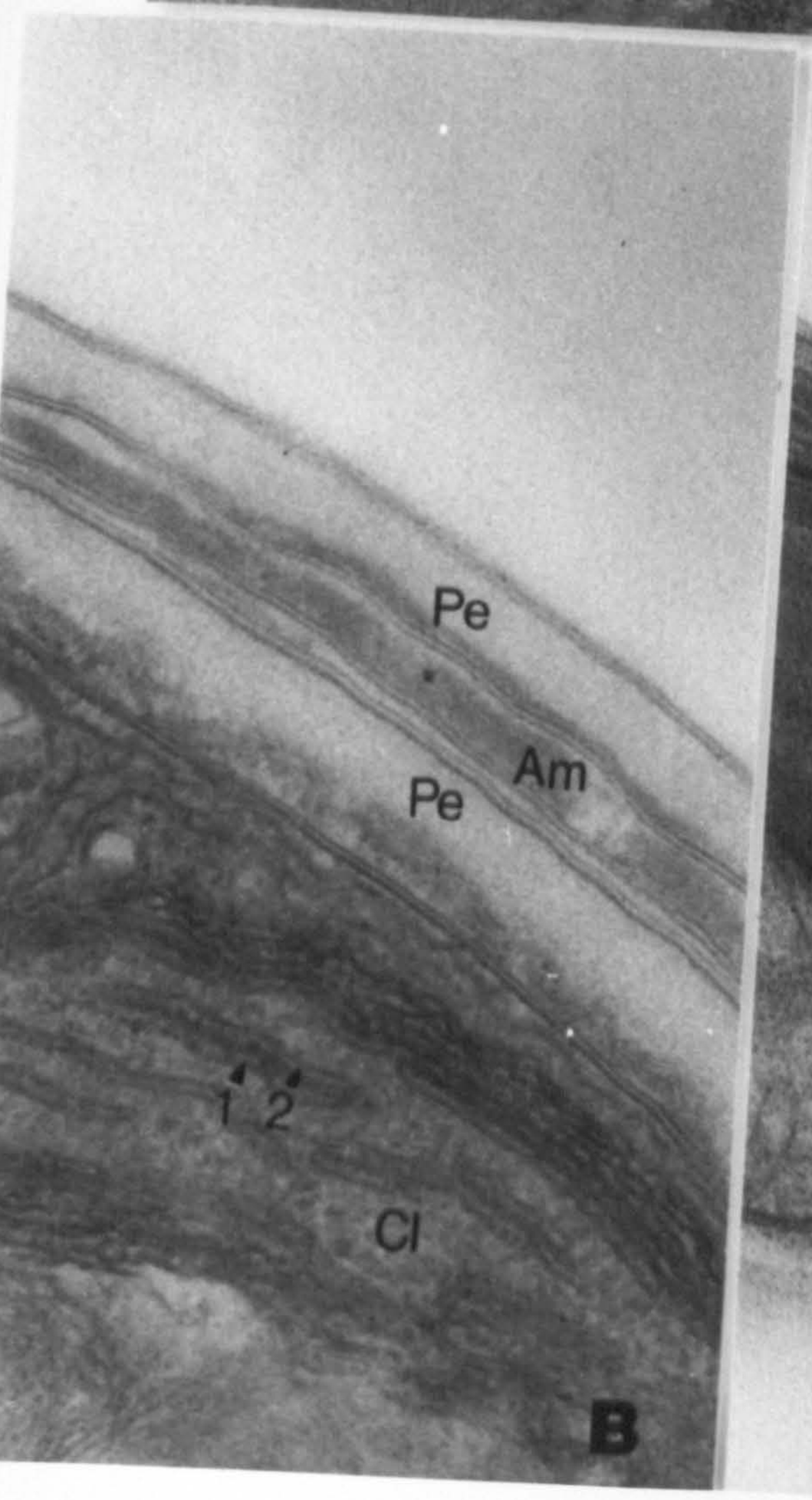
(B) A chloroplast of a cultured *Symbiodinium* sp. cell originally isolated from *I. sulcatus* showing the unusual thylakoid arrangement (magnification approx. x 110,150).

Cl = chloroplast; arrows 1 and 2 indicate the 2 stacked thylakoids; Pe = "pellicle"; Am = amorphous layer. Note that the thylakoids are uniquely (amongst known *Symbiodinium* spp.) stacked in groups of 2. Also note the unusual presence of 2 "pellicles" and an amorphous layer in the amphiesma of the cell. The reasons for this anomalous situation are unknown.

(C) A chloroplast of a symbiont freshly isolated from *A. viridis* (Weymouth Harbour) showing the thylakoid arrangement that predominates in the genus *Symbiodinium* (magnification approx. x94,500).

Cl = chloroplast; arrows 1-3 indicate the 3 stacked thylakoids; pe = "pellicle".


pe
M
P
Cl
Ac
A


Pe
Am
Pe
1 2
Cl
B


pe
1 2 3
Cl
C

Plate 3.9: Transmission electron micrographs showing unusual characteristics of symbionts.

(A) The amphiesma of a symbiont cell freshly isolated from C. pedunculatus (Lough Hyne) showing the absence of a "pellicle" (cf. plate 3.7 (B)) (magnification approx. x126,400).

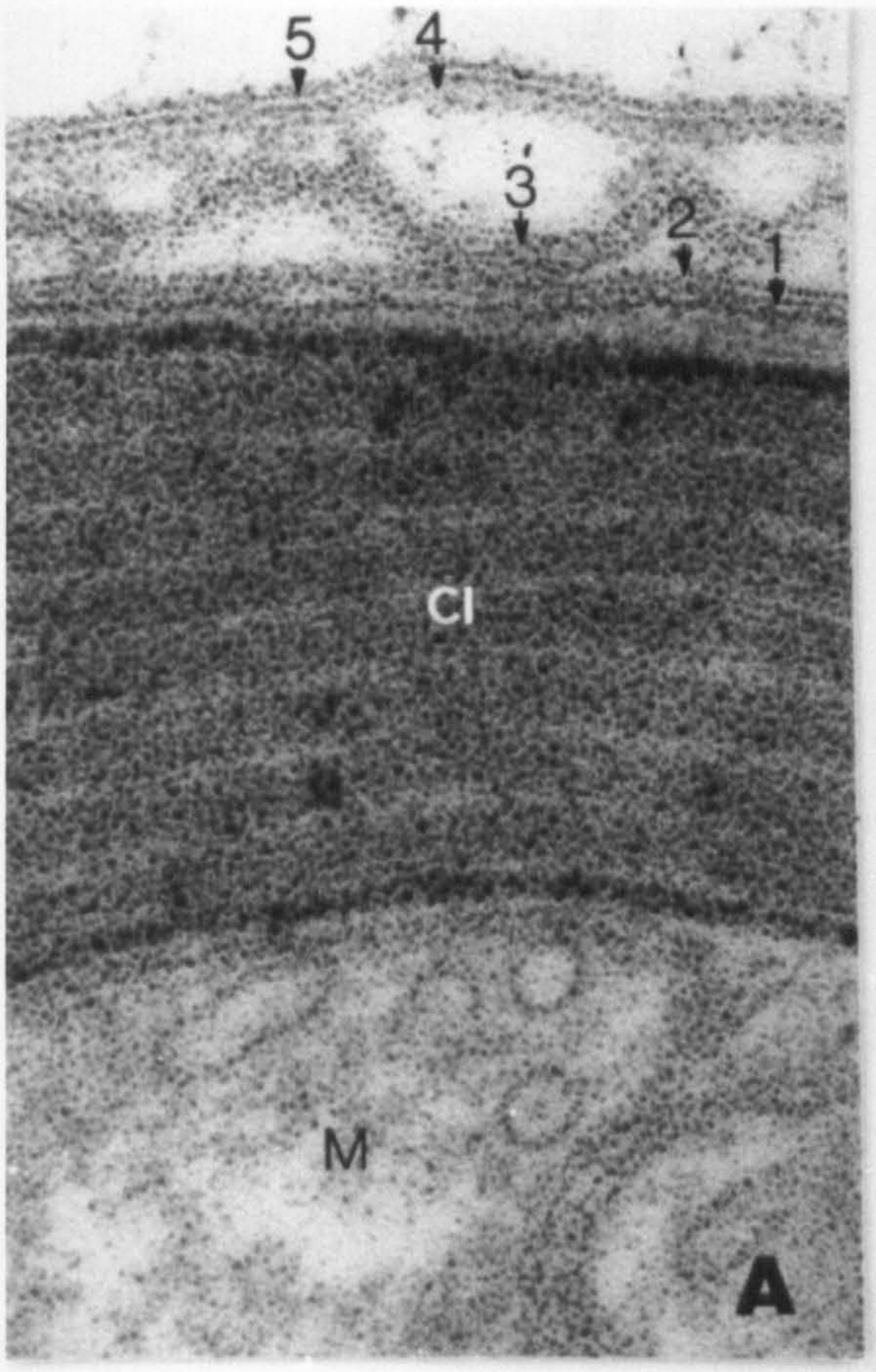
Arrows 1-5 indicate the membranes comprising the amphiesma (arrow 3 refers to an indeterminable number of wavy membranes); Cl = chloroplast; M = mitochondrion.

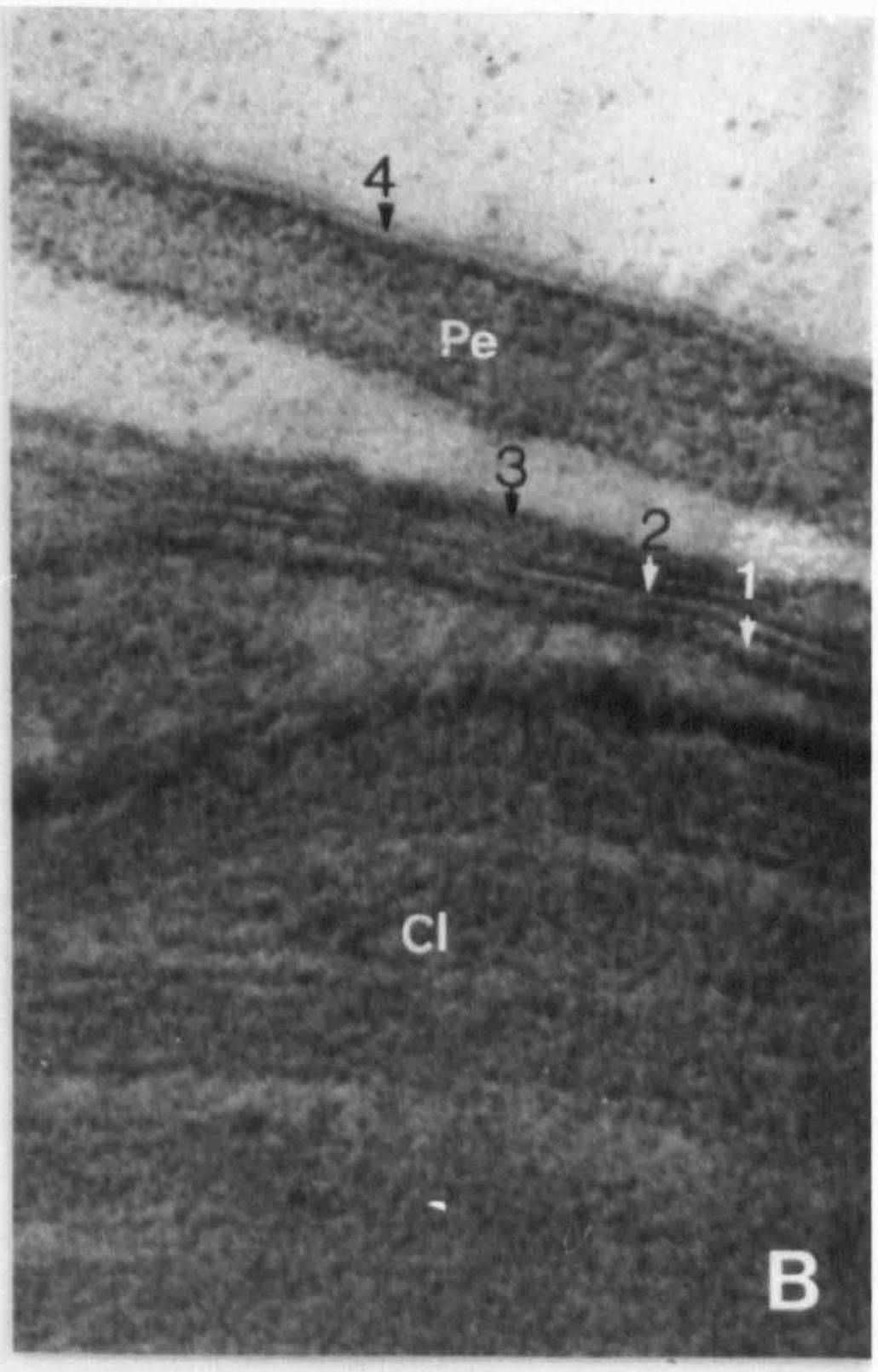
(B) The amphiesma of a cultured symbiont cell originally isolated from C. pedunculatus (Lough Hyne) showing the presence of a "pellicle" (cf. plate 3.7 (A)) (magnification approx. x221,650).

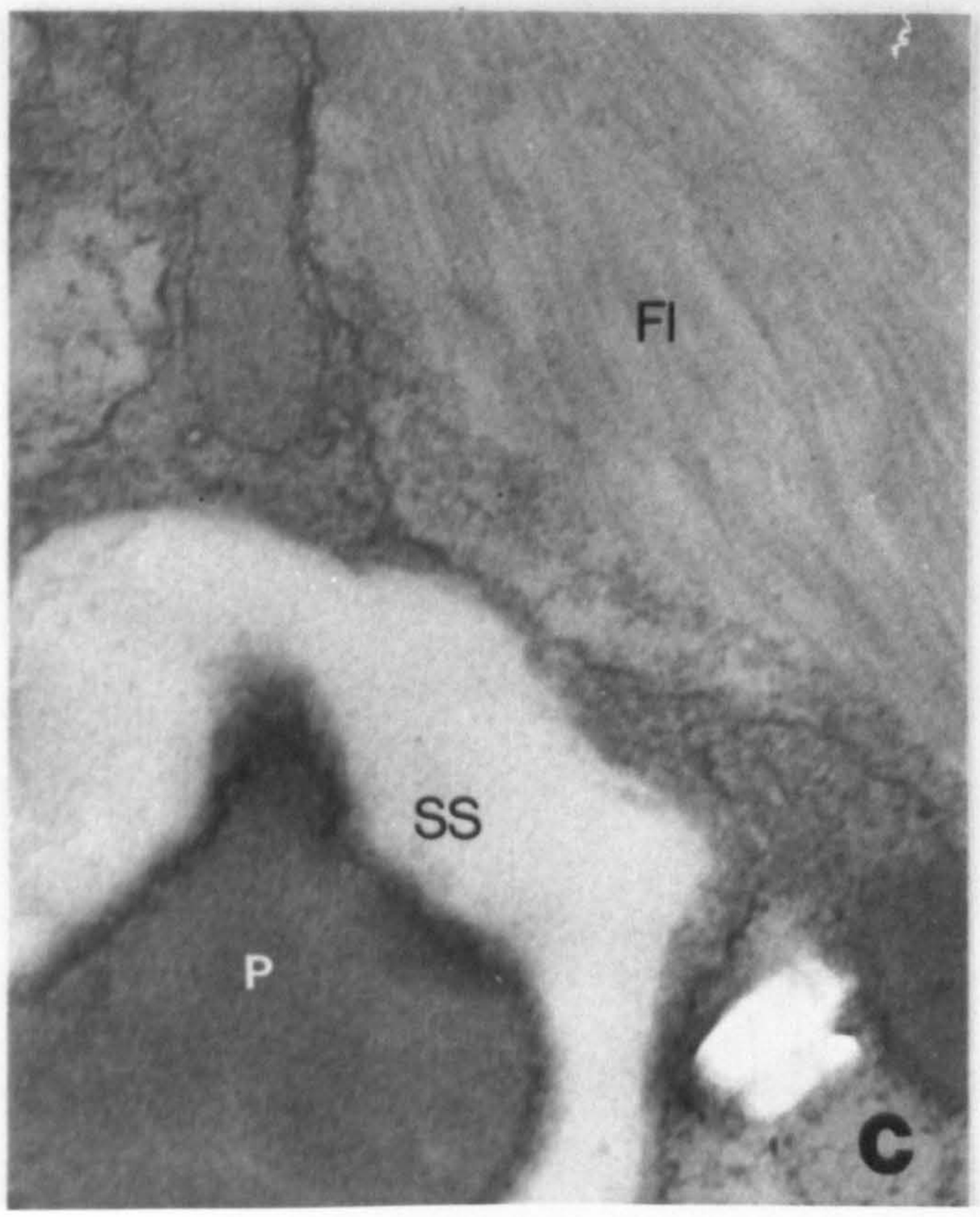
Arrows 1-4 indicate membranes of the amphiesma (arrow 3 refers to an indeterminable number of wavy membranes); Pe = "pellicle"; Cl = chloroplast.

(C) A possible flagellar hair-producing cavity in a cultured symbiont of A. pallida (magnification approx. x94,500).

Fl = flagellar hair-producing cavity; P = stalked pyrenoid; SS = starch sheath of the pyrenoid.


5
4
3
2
1
Cl
M
A


4
Pe
3
2
1
Cl
B


Fl
SS
P
C

3.2.4 Discussion

(a)Location of the symbionts within the animal tissues

The observation that the symbionts are located in the host endoderm is consistent with the view that endosymbionts are invariably localized in the endoderm of sea anemones (Shick 1991). These symbionts are usually concentrated in the tentacles and oral disc, as has been shown in A. pallida and Phyllactis flosculifera (Steele and Goreau, N.I. 1977), and A. elegantissima (Dykens and Shick 1984) where there is little shading by the animal tissue (Shick 1991). The distribution of the symbionts of I. sulcatus throughout the polyp suggests that the column tissues are transparent enough to permit net photosynthesis by the symbionts.

(b)Light microscopical study of symbiont morphology

The structures of the coccoid cells observed under the light microscope do not indicate the presence of different strains or species, however they are consistent with previous light microscopical observations of algae of the genus Symbiodinium. The single stalked pyrenoid surrounded by a starch sheath, the peripheral chloroplast(s), the "accumulation body" and vacuoles have all been been noted in light microscopical investigations of Symbiodinium spp. from Cassiopeia sp. (Freudenthal 1962), A. pallida (Steele 1975) and a further 15 host species (Schoenberg and Trench 1980b). Freudenthal (1962) largely based the taxonomic description of the type species S. microadriaticum upon these observations. Similarly, D.L. Taylor (1974) claimed

that the observation of the single stalked pyrenoid projecting from the inner surface of the chloroplast and encased in a starch sheath, as viewed under light microscopy, was the key taxonomic feature of Gymnodinium (=Symbiodinium) microadriaticum. However, both these taxonomic descriptions stressed the importance of the daily transformation from the coccoid stage to a motile stage when in culture in the recognition of Symbiodinium cells, and this feature is still considered characteristic of the genus Symbiodinium (Trench and Blank 1987).

(c)The presence of motile cells in culture

Motile cells have been reported to appear between 24 hours and one month after the initiation of a culture (Freudenthal 1962; Trench et al. 1981a), and this was confirmed here using A. pallida. The apparent absence of motility in the cell cycle of the cultured symbionts of the British Anthozoa may be due to: (1) Motile cells simply being overlooked, despite rigorous searches; (2) the lack of motile cell production due to some environmental condition during culture; (3) the absence of a motile stage in the life-history of these algae. If the latter is true then these symbionts differ greatly from previously described Symbiodinium strains or species, although the absence of motility probably does not warrant the removal of these algae from the genus Symbiodinium (if all other criteria are met). However, an emendation of the genus description would be required. The reason for the lack of a motile stage of

the British symbionts may be a reflection of the symbiont acquisition method of their host species. A. viridis and A. ballii both possess a 'closed' acquisition system, whereby symbionts are transmitted via the ova (Turner 1988). The situation in C. pedunculatus is less clear because of the brooding nature of host reproduction; oocytes have not been observed, preventing the presence or absence of symbionts to be determined, although symbionts are present in juveniles freshly released from the adult (Turner 1988). Even if symbionts are not present in the ova, it seems unlikely that symbionts would need to be motile to infect juveniles within the coelenteron. Therefore, it is probable that the production of motile cells is not required for the transmission of algal symbionts within these hosts. The method of symbiont acquisition by I. sulcatus is unknown and there are no reports of sexual reproduction in this species. In contrast, Cassiopeia sp., A. pulchella, A. pallida and A. elegantissima, whose symbionts become motile, all possess 'open' symbiont acquisition systems where motile dinoflagellates may infect aposymbiotic larval stages from the ambient environment (Siebert 1974; Trench 1987; Shick 1991). Whilst never observed in the field, infection of aposymbiotic hosts by motile Symbiodinium cells has been demonstrated in the laboratory (Fitt 1984). The possible correlation between symbiont cell cycle and acquisition is deserving of a more extensive investigation.

(d)The amorphous 'matrix' of cultured symbionts

The presence of a sticky material causing cultured Symbiodinium cells to clump was originally described by Freudenthal (1962). The sticky material observed in all the cultures used in this research was suggested to be macromolecules released by the algal cells (Trench, pers. comm.). These exudates have only recently been explored in detail and are believed to contain uronic acids, neutral sugars, sugar amines and all essential amino acids (Markell et al. 1992; Markell and Trench 1993). However, the substantial increase in exudate in degenerating cultures indicated that cell lysis may contribute to this phenomenon. Possible roles for these macromolecules in host-symbiont recognition will be discussed in chapter 4, section 4.4 (c) (p.209).

(e)Dimensions of cultured and freshly isolated symbionts

The use of cell dimensions to characterize living Symbiodinium cells has been widely employed. Single coccoid cells of Symbiodinium have been measured in the range 5.3 - 13 μm (Taylor, D.L. 1969a; Kevin, M.J. et al. 1969; Steele 1975; Loeblich and Sherley 1979; Trench and Blank 1987). However, cell dimensions vary with the stage of the cell cycle (Schoenberg and Trench 1980b). For example, cells of S. pilosum increased from 6 to 12.5 μm between early and late log-phase of culture growth (Domotor and D'Elia 1986). Using the method described in this thesis, Schoenberg and Trench (1980b) standardized the conditions and stage of the

cell cycle at which measurements were taken. These authors concluded that, in the 'strains' they investigated, 2 distinct size classes of Symbiodinium existed. The ranges of these 2 classes were: Group I, 7.48 - 9.34 µm and 6.68 - 8.54 µm in length and width respectively; group II, 10.16 - 11.57 µm and 9.90 - 10.99 µm in length and width respectively. The size classes observed in the present research are similar to the 2 classes observed by Schoenberg and Trench (1980b), with Symbiodinium spp. from A. pallida, Cassiopeia sp. and Z. sociatus being placed in the same groups in both studies. Schoenberg and Trench (1980b) believed the 2 size classes to be indicative of taxonomically different groups of symbionts. However, there was overlap between the size classes into which the British Anthozoa were placed, with symbionts from A. viridis (Lough Hyne) representing the upper limit of class I and the lower limit of class II . Therefore, as was suggested possible by Schoenberg and Trench (1980b), a continuum of cell sizes exists resulting in the inability to make conclusions regarding speciation within the genus Symbiodinium from dimension data alone. It is evident from the 2 size classes that of the British symbionts only those of C. pedunculatus (Lough Hyne) can be placed in size class I with certainty, suggesting that the symbionts of C. pedunculatus may be genetically different to those of A. viridis (Shell Island), A. ballii and I. sulcatus. The fact that the dimensions were not always maintained in culture from when in situ (ie.

symbionts of A. pallida were larger and symbionts of A. ballii and C. pedunculatus were smaller when in culture than when in the host) resulted in 4 size classes. This finding indicated that the host cell environment influences the morphology of the symbionts. This same conclusion was reached by D.L. Taylor (1969a), who observed that the size of non-dividing symbionts of Tridacna sp. increased from 7 µm in situ to 8-12 µm in vitro. Thus, conclusions should not be drawn regarding genetic differences in cell size from symbionts in situ.

The symbionts of geographically isolated populations of A. viridis may also be genetically different. Similarly, mean lengths of Symbiodinium sp. cells from the zoanthid Protopalythoa grandis from Bermudan and Barbadian populations differed by 1.14 µm, although both symbiont populations were assigned to size class II by Schoenberg and Trench (1980b). Domotor and D'Elia (1986) reported variable cell size distributions between the symbionts of different conspecific host populations, although these observations were made on freshly isolated symbionts. The presence of significantly different sized symbionts in different populations of A. viridis is evidence that the same host species may possess genetically different 'strains' of Symbiodinium. Recently, Banaszak et al. (1993) have suggested that individuals of the fire coral, Millepora dichotoma, may also contain one of two distinct taxa of symbiotic dinoflagellates. This suggestion would explain the

different "photoadaptive responses" of co-occurring M. dichotoma colonies reported by Schonwald et al. (1987). In contrast to the findings in the present investigation, D.L. Taylor (1968b) did not note any differences between the symbionts of A. viridis from several locations around Britain, giving a diameter of 12 μm. It is of note that the A. viridis population containing significantly smaller symbionts and the A. viridis population with symbionts nearest to this in size are located in sea lochs, geographically the most isolated sites from which collections were made. It is conceivable that separate strains or species of symbionts may have evolved and persisted in isolation within the boundaries of the lochs.

(f)Scanning electron microscopy (S.E.M.)

Cultured and freshly isolated coccoid symbionts appeared homogenous under the S.E.M.. Micrographs of symbionts of Heteroxenia fuscescens (Benayahu et al. 1989) and A. viridis (Stambler and Dubinsky 1987), and 2 other symbiont 'strains' (Schoenberg and Trench 1980b) also demonstrate this morphological homogeneity. Only S. pilosum has been shown so far to possess a different cell surface in the coccoid stage, with hair-like tufts arising from the cell wall (Schoenberg and Trench 1980b; Trench and Blank 1987).

(g)Transmission electron microscopy (T.E.M.)

The observation of apparently intracellular symbionts in all the host species under investigation here is consistent with the view that the dinoflagellate symbionts of Anthozoa are

TABLE 3.4 CHARACTERISTICS OF THE GENUS SYMBIODINIUM (TRENCH AND BLANK 1987).

GENERAL CHARACTERISTICS OF SYMBIODINIUM
1. Phototrophic marine dinoflagellates found as inter- and intracellular symbionts of invertebrates.
2. Coccoid in the host.
3. Divide only in the coccoid phase to produce 2 daughters *in situ* or 2-4 daughters *in vitro*.
4. Motile cells produced with a characteristic daily rhythm in culture.
MORPHOLOGICAL FEATURES OF COCCOID CELLS
1. A continuous cell wall of varying thickness, which may be readily apparent or reduced depending on the stage of the life history or cell cycle, underlain by a series of membranes.
2. Chloroplast(s), lobulate, one or more in number.
3. Thylakoids arranged in parallel arrays only or in parallel and peripherally, with the thylakoids usually stacked in groups of 3.
4. A single, stalked pyrenoid without invasive chloroplast thylakoids and surrounded by a triple layered chloroplast envelope. One or more stalks are present.
5. One or more mitochondria which are ovoid or reticulate.
6. A "mesokaryotic" nucleus limited by 2 membranes.
7. 1-3 "accumulation" bodies.
MORPHOLOGICAL FEATURES OF MOTILE CELLS
1. Typical gymnodinioid morphology with the hypocone being smaller than or equal in size to the epicone.
2. Characteristic thecal plates in vesicles and with associated microtubules.
3. Longitudinal flagellum and a ribbon-like transverse flagellum.

invariably intracellular (Trench 1987; Shick 1991).

T.E.M. revealed distinct differences between the symbionts of the British Anthozoa and A. pallida. But the cultured and freshly isolated coccoid symbionts of all the host species, with the exception of I. sulcatus, satisfied the ultrastructural characteristics of the most recent emendation of the genus Symbiodinium (Trench and Blank 1987), which are given in table 3.4 (p.104). Trench and Blank (1987) described Symbiodinium cells as "usually" having thylakoids in stacks of 3. The observation of thylakoids mainly in stacks of only 2 in symbionts of I. sulcatus is the first reported deviation in ultrastructure reported for a symbiont which otherwise possesses the ultrastructural features of the genus Symbiodinium. In contrast, LeVay (1989) recorded the thylakoids of the symbionts of I. sulcatus to be in stacks of 3. A similar thylakoid arrangement is shown in electronmicrographs of Scrippsiella chattonii, the dinoflagellate symbiont of Velella velella, although no reference is made to this arrangement (Trench 1981). The variation in thylakoid arrangement was the only aspect of the cell ultrastructure to suggest the presence of more than one genetic entity amongst Symbiodinium sp. from British Anthozoa. But the presence of peripheral thylakoids in the chloroplasts of British Symbiodinium sp. distinguish all these symbionts from those of A. pallida. Peripheral thylakoid lamellae are not uncommon amongst Symbiodinium spp.. Peripheral

thylakoids have been reported in Symbiodinium cells from Tridacna maxima (Schoenberg and Trench 1980b; Trench et al. 1981b), Z. sociatus (Trench and Blank 1987; Blank 1987), A. elegantissima (Blank 1987), Anthopleura xanthogrammica (O'Brien, in Schoenberg and Trench 1980b) and A. viridis (Taylor, D.L. 1968b; Dodge 1973). An absence of peripheral thylakoids has been shown in the symbionts of Cassiopeia sp., M. verrucosa and Ragactis lucida (Trench and Blank 1987).

The number of chloroplasts could not be determined during this study, although Dodge (1973) claimed, without direct evidence, that the symbionts of A. viridis possessed several chloroplasts. However, results of 3 - D cell reconstructions have yet to identify more than 1 chloroplast per cell (Blank 1987; Trench and Blank 1987).

The presence of a single, stalked pyrenoid, together with the chloroplast and thylakoid organisation, was regarded as being essential to the description of Symbiodinium cells by D.L. Taylor (1969a). The stalked pyrenoid was characteristic of Symbiodinium sp. from all the hosts examined in this study. The fact that the chloroplast lamellae did not invade the pyrenoid clearly distinguished these cells from those of the other genus of symbiotic dinoflagellates, Amphidinium (Taylor, D.L. 1974; Trench 1981). Amphidinium spp. normally possess 4 pyrenoids with multiple stalks (Taylor, D.L. 1974), whereas Symbiodinium spp. only have 1 pyrenoid with 1 - 3 stalks on a strain specific basis (Trench and Blank

1987). Only one pyrenoid was ever seen in a single section of any Symbiodinium sp. cell from the British Anthozoa or A. pallida, implying the presence of a single pyrenoid.

The chromosomes were uniquely dinoflagellate in nature, being condensed and showing a series of transverse bands. Differences in chromosome numbers and volumes between symbionts of different host species have been used to support the evidence of seperate species of Symbiodinium. Blank and Trench (1985) demonstrated distinct chromosome numbers, ranging from 26 to 97 +/- 2, and chromosome volumes between Symbiodinium cells isolated from separate host species which remained stable over many years in culture. It was claimed that the differences in chromosome number were not the result of different ploidy levels within the same species due to inconsistencies with the chromosome volume data. But the fact that the chromosome numbers are multiples of one another must lead to some suspicion over these conclusions. Very recently, using both TEM and LM, Udy et al. (1993) showed that chromosome numbers in symbionts of the same host species varied greatly and that the large, condensed regions of DNA normally assumed to be individual chromosomes are infact only sub-units of larger chromosomes. Consequently, these authors concluded that chromosome counts are inappropriate for defining Symbiodinium taxa. However, Udy et al. (1993) did note differences in chromosome form between symbionts of different host species. 3-D reconstructions of the nuclei of the symbionts of the

British Anthozoa could elucidate whether similar differences exist between the chromosomes of these algae.

The presence of assimilatory products stored as crystals and starch was reported briefly by Freudenthal (1962). D.L. Taylor (1968b), using symbionts of A. viridis, produced the first experimental evidence for the exact nature of these entities, identifying the crystals as calcium oxalate. Taylor (1968b) also noted that the large red vacuole enlarged in starved cells and consequently labelled this structure an "accumulation body" rather than an assimilation body. These storage and "accumulation bodies" have been observed in all subsequent ultrastructural accounts of Symbiodinium spp. (eg. Kevin, M.J. et al. 1969; Schoenberg and Trench 1980b; Trench and Blank 1987). The "accumulation body" and calcium oxalate containing vacuoles were observed to become larger in the cultures of all the symbionts under investigation in this research than when in situ. In contrast, the volume and numbers of starch grains present decreased in culture. Identical responses were observed when symbionts of A. viridis were starved in situ by restricting light and heterotrophic nutrition to the host (Taylor, D.L 1969c). Therefore it is likely that the cultured Symbiodinium cells were under a degree of stress when in culture.

No evidence of flagella or flagella basal bodies was observed in any of the symbionts from British hosts, again supporting the view that these symbionts do not possess a

motile stage in their life cycles. Similarly, the possible presence of a flagellar hair-producing cavity in the cultured symbiont of A. pallida is consistent with the motile phase of this alga in culture. Although hair manufacturing cavities have been identified in dinoflagellates (Dodge 1973), there are no records of such a structure being reported before in Symbiodinium cells. Hence this interpretation was made with some reservation.

The observed differences in amphiesmal structure were only obvious in freshly isolated symbionts. This suggests that the lack of a "pellicle" around symbionts from C. pedunculatus and A. pallida may be a result of the host cell environment and/or a more highly integrated symbiosis where boundaries to metabolite movement have been reduced (Smith, D.C. 1974). Therefore, no taxonomic conclusions could be drawn from the absence of a "pellicle" around these symbionts. A future investigation could determine whether the structure of the amphiesma is determined by the host species in which a particular symbiont 'strain' is persisting or whether there is a genetic basis for these observed in situ differences. The presence of a "pellicle" surrounding Symbiodinium cells in situ is uncommon, with the only previously recorded cases being the symbionts of Tridacna spp. (Schoenberg and Trench 1980b) and A. viridis (Taylor, D.L. 1968b). There are no reports of the "pellicle" lacking in culture. The amphiesmal structure, and particularly the number of membranes, was originally

suggested as having potential taxonomic importance (Taylor, D.L. 1968b). But the number of membranes has been shown to vary between symbionts of the same host and in symbionts of different hosts. For example the number of membranes surrounding the symbionts of A. elegantissima vary from a few to many (Trench 1971a; Muscatine et al. 1975). Consequently, little taxonomic significance was attached to the number of amphiesmal membranes observed in the present investigation. The reasons for the elaborate amphiesmal structure of the symbionts in situ remain unknown. Muscatine et al. (1975) claimed that it was unlikely that the membranes offered any resistance to host digestion. But it could be suggested that the series of ruffled membranes provide a greater surface area over which metabolite movement between alga and host (and vice versa) can occur.

The fibrous material observed on the outside of the amphiesma of both cultured and freshly isolated symbionts from all the symbioses under investigation has been the subject of much speculation in recent years. The material has been observed external to the "pellicle" of cultured symbionts of Z. sociatus and C. xamachana (Trench 1987), and in situ in T. maxima (Schoenberg and Trench 1980b). The material has been identified as acid polysaccharide or glycoprotein (Trench et al. 1981a; Markell et al. 1992) and may be involved in host-symbiont recognition. This possible role will be discussed in chapter 4, section 4.4 (a) (pp.203-204).

TABLE 3.5 GROUPS OF SYMBIONTS OF BRITISH ANTHOZOA AND A. PALLIDA BASED UPON MORPHOLOGICAL CRITERIA IN CULTURE.

Group	Host Species	Distinguishing Characteristics
I	A. pallida	Within size class I; Motile phase in culture; No peripheral thylakoid lamellae; Possible flagella-hair producing cavity.
II	A. viridis (Loch Sween and Lough Hyne), C. pedunculatus (Lough Hyne and Netley)	Within size class I; No motile phase in culture; Peripheral thylakoid lamellae.
III	A. viridis (all locations except L. Sween), A. ballii	Within size class II; No motile phase in culture; Peripheral thylakoid lamellae.
IV	I. sulcatus	Within size class II; No motile phase in culture; Peripheral thylakoid lamellae; Thylakoids predominantly in stacks of 2.

The ultrastructural information obtained here confirmed the assignment of the symbionts of the British Anthozoa and A. pallida to the genus Symbiodinium, and suggested that Symbiodinium sp. from I. sulcatus and A. pallida may be distinct genetic entities from the symbionts of the other host species.

(h) General conclusion

The morphological features observed using light and electron microscopy revealed that the symbionts of the British Anthozoa closely resemble those of A. pallida and other previously described tropical and temperate hosts. The symbiotic nature and morphology of these algae clearly indicated that they are all dinoflagellates of the genus Symbiodinium. However, if it is assumed that the differences observed in culture represent genotypic differences (Schoenberg and Trench 1980a), then Symbiodinium cells in different species of British Anthozoa are not genetically homogeneous with regard to morphology. They also demonstrate small morphological differences and different cell cycles from the majority of Symbiodinium spp. described previously. Using the morphological differences observed between cultured Symbiodinium sp. from British Anthozoa and the sub-tropical A. pallida, the symbionts can be assigned to 4 separate groups (table 3.5 (p.111)).

3.2.5 Summary

(i)In situ, freshly isolated and cultured symbionts of A. viridis, A. ballii, C. pedunculatus, I. sulcatus and A. pallida were studied with regard to their positions in the host tissues and morphologies using light microscopy, T.E.M. and S.E.M.. (Cultured symbionts of A. elegantissima, A. pulchella, Z. sociatus and Cassiopeia sp. were also observed under the light microscope).

(ii)The symbionts were restricted to the endoderm in all the hosts investigated.

(iii)All freshly isolated cells were coccoid, whereas cultures of non-British symbionts contained motile and coccoid cells.

(iv)Cell division predominantly resulted in 2 daughter cells, although a triad of cells was observed on one occasion.

(v)The only major structures visible in the coccoid cells under the light microscope were a stalked pyrenoid, a peripheral chloroplast(s), an "accumulation body" and vacuoles.

(vi)Macromolecular exudates, which may be involved in host-symbiont recognition, were observed in cultures of all symbionts.

(vii)The dimensions of the "2-cell stage" in culture revealed 2 size classes with a small degree of overlap. The symbionts of C. pedunculatus and A. viridis (Loch Sween) were in class I, whereas the symbionts of the other British

Anthozoa, including symbionts of A. viridis from other locations, were in class II.

(viii)The cell dimensions were not constant between the in situ condition and in culture, indicating an influence of the host cell environment on symbiont morphology.

(ix)S.E.M. revealed the surface of all coccoid symbionts to be smooth and homogenous.

(x)T.E.M. revealed the intracellular ultrastructure of cultured and freshly isolated symbionts of the British Anthozoa to conform to the type description of the genus Symbiodinium (Trench and Blank 1987).

(xi)The presence of peripheral thylakoid lamellae in the chloroplasts of the British Symbiodinium sp. differentiated them from the symbionts of A. pallida and the majority of previously described Symbiodinium spp..

(xii)The presence of thylakoids in stacks of 2 rather than 3 distinguished Symbiodinium sp. from I. sulcatus from any other descriptions of Symbiodinium spp..

(xiii)In situ, symbionts of C. pedunculatus and A. pallida lacked a "pellicle", in contrast to the symbionts of the other British host species. However, a "pellicle" developed upon culturing. The presence of a "pellicle" in situ is an uncommon feature but of little taxonomic significance.

(xiv)Possible sites of surface recognition were present on both cultured and freshly isolated cells.

(xv)Using all the morphological information available, 4

groups of symbionts were evident of which the symbionts of the British Anthozoa were assigned to 3.

3.3 PIGMENT COMPOSITION OF SYMBIODINIUM SP.

3.3.1 Introduction

The aim of this investigation was to identify the major photosynthetic and accessory pigments of *Symbiodinium* sp. isolated from the British Anthozoa and *A. pallida*.

The major pigments of the dinoflagellates are chlorophylls-*a* and -*c*2, and the characteristic carotenoid, peridinin (Prezelin 1987). However, the use of pigments in the characterization of *Symbiodinium* spp. from different host species has been very limited. D.L. Taylor (1967) described thin-layer chromatography (TLC) plates and produced absorbance spectra for the symbionts of *A. viridis*, concluding that the symbionts unquestionably belonged to the class Dinophyceae. Likewise, the symbionts of clams and corals were identified as dinoflagellates (Jeffrey and Haxo 1968; Jeffrey and Shibata 1969). But the only evidence for 'strain' specific variations in pigmentation was presented by Chang *et al*. (1983): Under identical conditions, *Symbiodinium* cells from 3 different host species exhibited cell volume independent differences with respect to the concentrations of the major pigments and their ratios with one another.

Light quantity and quality must be considered if meaningful comparisons of pigment concentrations and ratios are to be made between symbionts of different host species. Photoadaptation by Symbiodinium spp. may occur by one of 3 known 'strain' specific mechanisms: A change in photosynthetic unit (PSU) size (Falkowski and Dubinsky 1981; Dustan 1982; Chang et al. 1983); a change in PSU number; changes in the activities of CO_2-fixing enzymes or electron transport systems (Chang et al. 1983).

Under natural conditions the dinoflagellate symbionts photoadapt in response to the photic environment of their host, which varies both with depth and microhabitat. Photoadaptation with decreasing light intensity has been shown to result in an obvious increase in the overall chlorophyll-a content of the symbiosis (McCloskey and Muscatine 1984; Berner et al. 1987).

The spectral quality of the light also influences photoadaptation. For example, blue light increased the chlorophyll-a concentration in the symbionts of P. damicornis (Kinzie et al. 1984).

Unfortunately, cultured symbionts were not available in sufficient quantity for use in the work described here, necessitating the use of freshly isolated cells. Consequently, the symbionts were subject to modification as a result of light attenuation by host tissue and self shading. Even so, all the examples of photoadaptation given above were measured over wide ranges of light quantity and

quality. Pigment ratios of marine algae do not vary with spectral quality (Jeffrey and Vesk 1977) and Chang et al. (1983) demonstrated that the absolute and relative quantities of the major photosynthetic and accessory pigments in 3 'strains' of Symbiodinium did not alter with an increase in light intensity from 22 to 57 $\mu E/m^2/s$, although 'strain' dependent changes occurred at higher intensities. Therefore the range of the light quantity and quality to which the in situ symbionts were subjected, as determined by the irradiance source, the light transmission properties of the host tissues and the degree of self shading by the symbionts, was not expected to result in large differences in photoadaptation between the symbionts in different host species.

3.3.2 Materials and methods

(a)Preparation of symbiont pigment extractions

Cultured Amphidinium carterae was obtained (from Dr.I.A.N. Lucas, U.C.N.W.) to act as a standard dinoflagellate. The A. carterae cultures were centrifuged once at x 3000 rpm in a plastic stoppered centrifuge tube and 9 ml 90 % acetone was added to the single algal pellet. Keeping the tube in dim light, the sample was then sonicated in a sonic water bath for 5 minutes to disrupt the cells and placed in a dark box for 18 hours whilst pigment extraction was occurring. No estimate of cell numbers in the pellet was necessary. The resultant pigment extraction was used for analysis of the

absorption spectrum, high-performance (pressure) liquid chromatography (HPLC) and TLC.

Pigment extracts of symbionts from A. viridis (Lough Hyne), A. viridis (Shell Island), C. pedunculatus (Lough Hyne), A. ballii, I. sulcatus and A. pallida were prepared. A. viridis and C. pedunculatus from other locations had not been collected at the time the pigment analysis equipment was available. The tentacles were excised and the symbionts were isolated as described in chapter 2, section 2.3.2 (a) (p.44), except that 0.05 % sodium dodecyl sulphate (SDS) (Sigma Chem. Co.) in FSW was employed. SDS was used in the second wash during all preparations to remove host debris which itself was pigmented. In the preparation of pigment extracts for HPLC the final symbiont pellet was resuspended by vigorous shaking in 3 ml autoclaved FSW. Each 3 ml suspension was then divided up equally amongst 3 separate stoppered centrifuge tubes. The number of cells in each tube was estimated by counting a subsample of the cell suspension on a modified Fuchs Rosenthal type haemocytometer using the x 10 objective of a light microscope. The volume of sample lost to the haemocytometer was accounted for by pipetting the suspension under the coverslip using a 1 ml syringe and recording the volume used. This volume was subtracted from the total 1 ml when the final cell concentration was being calculated to obtain a more accurate estimate of cell number. The cell suspensions were then centrifuged for a final time at x 3000 r.p.m. to ensure that all the remaining

cells were collected. The FSW was decanted off and 90 % acetone added. The volume of 90% acetone added depended on the expected concentration of extracted pigments. Therefore, 9 ml were added to pellets of symbionts from A. viridis and C. pedunculatus, 6 ml were added to pellets from I. sulcatus and A. ballii, and 0.5 ml was added to pellets obtained from A. pallida. Pigments were extracted as described for A. carterae. Symbiodinium sp. samples used for the plotting of absorption spectra were prepared separately to those used for HPLC: To the final pellet obtained from the original washings, 5 ml of 90 % acetone were added and the pigments extracted as described above. No cell counts were required here.

Following the 18 hour extraction period, all the samples were filtered through 0.45 μm Minisart SRP 15 filters (Sartorius GmBH) into 5 ml chromic acid washed glass vials. These vials were surrounded by aluminium foil to prevent light degradation of the pigments. The samples were refrigerated until use.

The subsequent preparation procedures differed depending on whether TLC, HPLC or the plotting of an absorbance spectrum was intended for the particular sample. These procedures are described below.

(b)Plotting of an absorbance spectrum

Maintaining the sample in darkness at all times, the contents of the vial were decanted into a 4 ml HNO_3 acid washed silica cuvette and the absorption spectrum of the

sample plotted following 'blanking' with pure 90 % acetone. The absorbances were determined using a Hewlett-Packard 8452A Diode Array Spectrophotometer. The precise wavelengths at which peaks in the resultant spectrum were observed were determined using the computer attachment of the spectrophotometer. These wavelengths were then compared to known absorption maxima of algal pigments.

(c)HPLC

The method used was an adaptation of that described by Gieskes and Kraay (1983).

Using a 100 µl glass syringe, 100 µl of the pigment extract was injected into a previously set up Varian HPLC system (consisting of a Varian 2020 gradient programmer, a Varian 2050 variable wavelength detector and 2 Varian 2010 HPLC pumps). The syringe was cleaned with acetone before each run. 2 solvents were used: Solvent I, 30 % water in methanol; solvent II, 20 % ethylacetate in methanol. These solvents were pumped through a 30 cm long HPLC column with diameter 3.9 mm in a linear gradient from 25 % of solvent II to 95 % of solvent II over a period of 40 minutes. Detection was performed with the variable wavelength detector set to 440 nm. The HPLC was interfaced with a Hewlett Packard printer on which the chromatogram, including the retention times of the peaks, was recorded. One HPLC run was performed for each sample.

(d)TLC

TLC was performed on the pigments extracted from A. carterae. To obtain as concentrated pigment bands as possible on the TLC plate it was necessary to concentrate the extracted pigments further. The remainder of the pigment extract following HPLC was decanted into a 250 ml round bottomed flask which was wrapped in aluminium foil. The flask was then attached to a Buchi RE111 Rotavapor rotary evaporator and dipped into a Buchi 461 water bath heated to 30 ^{o}C. This temperature was selected to enable the evaporation of the solvent whilst not causing the breakdown of the pigments. The sample was warmed until dry. Following cooling to room temperature, 0.5 ml of 90 % acetone was added to the dried pigment. The concentrated pigment extract was poured into an acid washed 5 ml glass vial wrapped in aluminium foil. Keeping the pigments in dim light, the extract was then pipetted onto a TLC plate (TLC aluminium sheet, silica gel, 20 x 20 cm, layer thickness 0.2 mm). The extract was pipetted using a separate micropipette for each TLC plate. 1 drop of extract was placed alternately on each of 6 spots, which were evenly spaced along a pencil drawn line approximately 2 cm from the bottom of the plate, until all the extract had been used. Transporting the plate in a black plastic photographic bag, the plate was placed in a glass solvent bath. The TLC solvent consisted of 60 hexane : 30 diethyl ether : 20 acetone. This solvent was poured into the bath until approximately level with the bottom of the

plate. The entire solvent bath was covered in black plastic and the TLC plate developed until the solvent was approximately 2 cm from the top of the plate. The final TLC plate was replaced in the black plastic bag.

(e)Identification of pigments on the TLC plate and location of their positions on the HPLC chromatogram.

The major pigment bands of the A. carterae TLC plate were identified. Maintaining the TLC plate in dim light, each distinct band on the plate was scraped off using a spatula and stored frozen in a 5 ml acid washed glass vial surrounded by aluminium foil until required. 5 ml 90 % acetone was then added to each vial, the extract filtered through a 0.45 μm minisart and the peak absorbances of the pigments measured as described for the plotting of the absorption spectra. The pigments were identified from their characteristic peak absorbances and locations and appearances on the TLC plate. Several of the pigments were deemed too dilute to enable their subsequent reanalysis using HPLC, but the more concentrated pigment extracts were injected into the HPLC system and analysed as described in section 3.3.2 (c) (p.120). Therefore, the location of the major pigments on the original chromatogram could be identified. The retention times and corresponding peak positions of these identified pigments were characteristic. Thus, if identical peaks were present on chromatograms of Symbiodinium pigments, the peaks could be identified.

(f)Quantification of the major pigments of Symbiodinium sp..

A crude method was used to compare the concentrations of the 3 major pigments identified. The area of the HPLC peak representing a pigment is proportional to the concentration of the pigment in the extract. Therefore, the areas of the relevant HPLC peaks were calculated by approximating the peaks to triangles and calculating their areas accordingly. The calculated areas were standardized to 6 ml to allow for the different volumes of acetone in which the pigment extractions were performed. The ratios of the pigments to one another were calculated from the standardized areas. But to compare the pigment concentrations between symbionts of different host species it was necessary to also standardize to cell size. To enable this, the average volume of the Symbiodinium cells from each host species was calculated. The volumes were estimated by measuring the diameter of 100 cells, freshly isolated from 5 tentacles or polyps, with the x 25 objective of the light microscope using an ocular micrometer. The average diameter was calculated and converted into the average cell volume, assuming each cell to be a sphere. The average volume was then converted into pg carbon/cell (C) using the following equation, given by Strathmann (1967) and Muscatine et al. (1983):

$$\log C = (\log \text{Volume}) \times 0.866 - 0.46 \quad \text{(Equation 3.1)}$$

Thus, by multiplying the average cell carbon content by the total number of cells in the pellet, the total amount of carbon, in pg/pellet, was calculated. The areas of the HPLC peaks were then standardized to the total amount of symbiont cell carbon contributing to the pigment extract.

The standardized HPLC peak areas were compared statistically between the symbionts of the different host species using a one-way ANOVA and Tukey's test.

3.3.3 Results

(a)Absorption spectra of pigment extracts of Amphidinium and Symbiodinium.

Figure 3.1 (p.129) illustrates the absorption spectrum of *A. carterae*. All the absorption spectra appeared identical and revealed 7 peaks or 'shoulders'. The absorbance peaks and 'shoulders' appeared at approximately 418, 438, 476, 580, 630, 654 and 664 nm. The shape of these absorption spectra was predominantly determined by the presence of chlorophylls-*a* and -*c*. The absorption maxima of chlorophyll-*a* in acetone are at 412, 432 and 660 nm (Prezelin 1987). These values and the relative heights of the peaks approximated to the peaks observed at 418, 438 and 664 nm. Absorption peaks of chlorophyll-*c*2 are at 449, 582 and 629 nm (Prezelin 1987). The peak at 449 nm may have been masked by that of chlorophyll-*a*, however the peaks at 580 and 629 nm were distinctly those of chlorophyll-*c*. The peak absorbances of peridinin are 464 and 474 nm (Deane and

O'Brien 1978). These wavelengths approximated to the 'shoulder' observed at 476 nm. Other pigments were not evident from the absorption spectra.

(b)HPLC

Typical HPLC chromatograms of A. carterae and Symbiodinium sp. from each host species are shown in figures 3.2 (p.129) and 3.3 (p.130) respectively. All the chromatograms showed peaks in identical positions, as marked by the characteristic retention times. 2 major single peaks were observed at approximately 2.4 - 2.5 and 5.4 - 5.8 minutes, and a series of up to 5 large peaks appeared between 22 and 25 minutes. A single sharp peak or shoulder which appeared at approximately 1.9 minutes was also occasionally observed. Other peaks were observed intermittently, but these were small and not investigated further due to them being lost in 'noise' and their retention times not being marked.

(c)Identification of the major pigments and their HPLC chromatogram positions using TLC.

TLC of the pigment extract of A. carterae resulted in the development of 5 distinct bands. From the origin the colours of these bands were 'khaki-green', deep orange, pale yellow, very pale yellow and pale green. The respective identities and absorption maxima of these pigments as derived from analysis using the diode array spectrophotometer were: Chlorophyll-c (444 nm); peridinin (464, 474 nm); diadinoxanthin (410, 430, 456 nm); unidentified xanthophyll (possibly dinoxanthin) (430 nm); chlorophyll-a (412 nm). The

absorption spectra of these pigments are shown in figures 3.4 (p.131). The peaks at 464 and 474 nm represent neoperidinin and peridinin respectively (Deane and O'Brien 1978). The series of peaks attributed to diadinoxanthin indicated contamination of this fraction with other xanthophylls, such as diatoxanthin and pyrrhoxanthin which have been identified in A. carterae (Johansen et al. 1974). However, the colour and position of the pale yellow band corresponded to that described by D.L. Taylor (1967) and Jeffrey (1974) for diadinoxanthin, which is also the second largest component of the carotenoids of A. carterae after peridinin (Johansen et al. 1974).

When the 2 most concentrated orange/yellow pigments extracted from the TLC plate were injected into the HPLC system they produced peaks corresponding to 2 of the large peaks observed previously. The peak occurring at 2.4 - 2.5 minutes represented peridinin and the peak at 5.4 - 5.8 minutes represented diadinoxanthin (figure 3.5 (p.132)). The peaks at 22 - 25 minutes were characteristic of those produced by chlorophyll-a and its derivatives (Gieskes and Kraay 1983), but this was not confirmed using the HPLC because of the diffuse nature of the chlorophyll-a band on the TLC plate. The peak occasionally observed at 1.9 minutes was believed to represent chlorophyll-c as this pigment has a low retention time when these HPLC solvents are used (Gieskes and Kraay 1983).

The identical peaks on the HPLC chromatograms of

Symbiodinium sp. pigment extracts were attributed to the same pigments.

(d)Quantification and comparisons of the concentrations of chlorophyll-a, peridinin and diadinoxanthin.

The average cell diameter, volume and carbon content for Symbiodinium sp. from the different host species, and the carbon contents of the algal pellets are given in table 3.6 (p.133).

The average peak area/pg cell carbon for the pigments of Symbiodinium sp. from the different host species, and their ratios to one another, are given in table 3.7 (p.134).

The results of Tukey's test, comparing the concentrations of chlorophyll-a, peridinin and diadinoxanthin in the symbiont cells of the different host species, are given in appendix 5, tables 1-3 (p.484).

Significant differences between the concentrations of chlorophyll-a in the symbionts of different host species were evident ($F=4.80$, $P=0.020$). More specifically, the concentration of chlorophyll-a in the symbionts of A. ballii and I. sulcatus was significantly greater, being 2.7 and 2.5 times greater respectively, than in the symbionts of A. viridis (Shell Island).

Significant differences were also evident between the concentrations of peridinin in the symbionts of the different host species ($F=4.19$, $P=0.030$). Tukey's test revealed that significant differences existed where the peridinin concentration in the symbionts of A. viridis

(Lough Hyne) was 2.2, 1.8 and 1.4 times the peridinin concentration in the symbionts of A. viridis (Shell Island), A. ballii and C. pedunculatus respectively. Symbionts of I. sulcatus also contained a significantly greater concentration of peridinin than Symbiodinium cells from A. viridis (Shell Is.), with the peridinin being 1.7 times more concentrated.

Finally, the diadinoxanthin concentration was significantly different between the symbiont cells of different host species ($F=8.77$, $P=0.003$). Symbiodinium cells from A. viridis (Lough Hyne) contained 1.8 times the diadinoxanthin concentration of the Symbiodinium cells from A. viridis (Shell Island), a significantly higher concentration.

These differences were reflected in the pigment ratios (table 3.7 (p.134)) where, for example, the ratio of chlorophyll-a : peridinin in symbionts of A. viridis (Shell Island) was 1.9 times that in symbionts of A. viridis (Lough Hyne). In general, the ratios of the pigments in Symbiodinium sp. from the British host species appeared similar. In contrast, although only based on 2 observations, the symbionts of the sub-tropical A. pallida had comparatively low chlorophyll-a concentrations resulting in low chlorophyll-a : carotenoid ratios.

Figure 3.1 Absorbance profile of photosynthetic pigments extracted of A. carterae in 90 % acetone (chl-a = chlorophyll-a, chl-c = chlorophyll-c, p=peridinin, numbers following pigment names are wavelengths (nm) at which peaks occur).

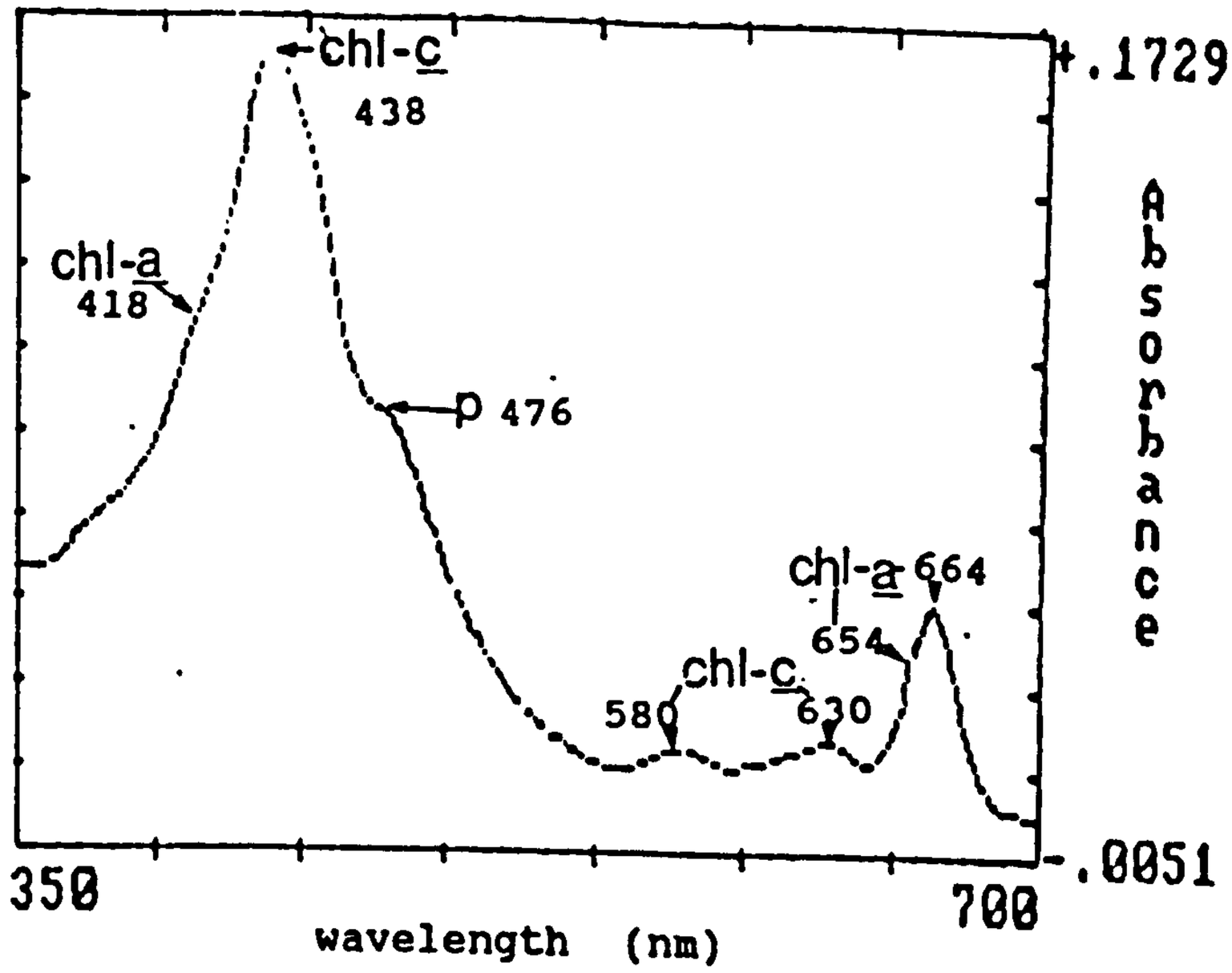


Figure 3.2 HPLC profile (absorbance (Abs) vs. time (T)) of photosynthetic pigments of A. carterae extracted in 90 % acetone (P = peridinin, D = diadinoxanthin, Chl-a = chlorophyll-a complex)

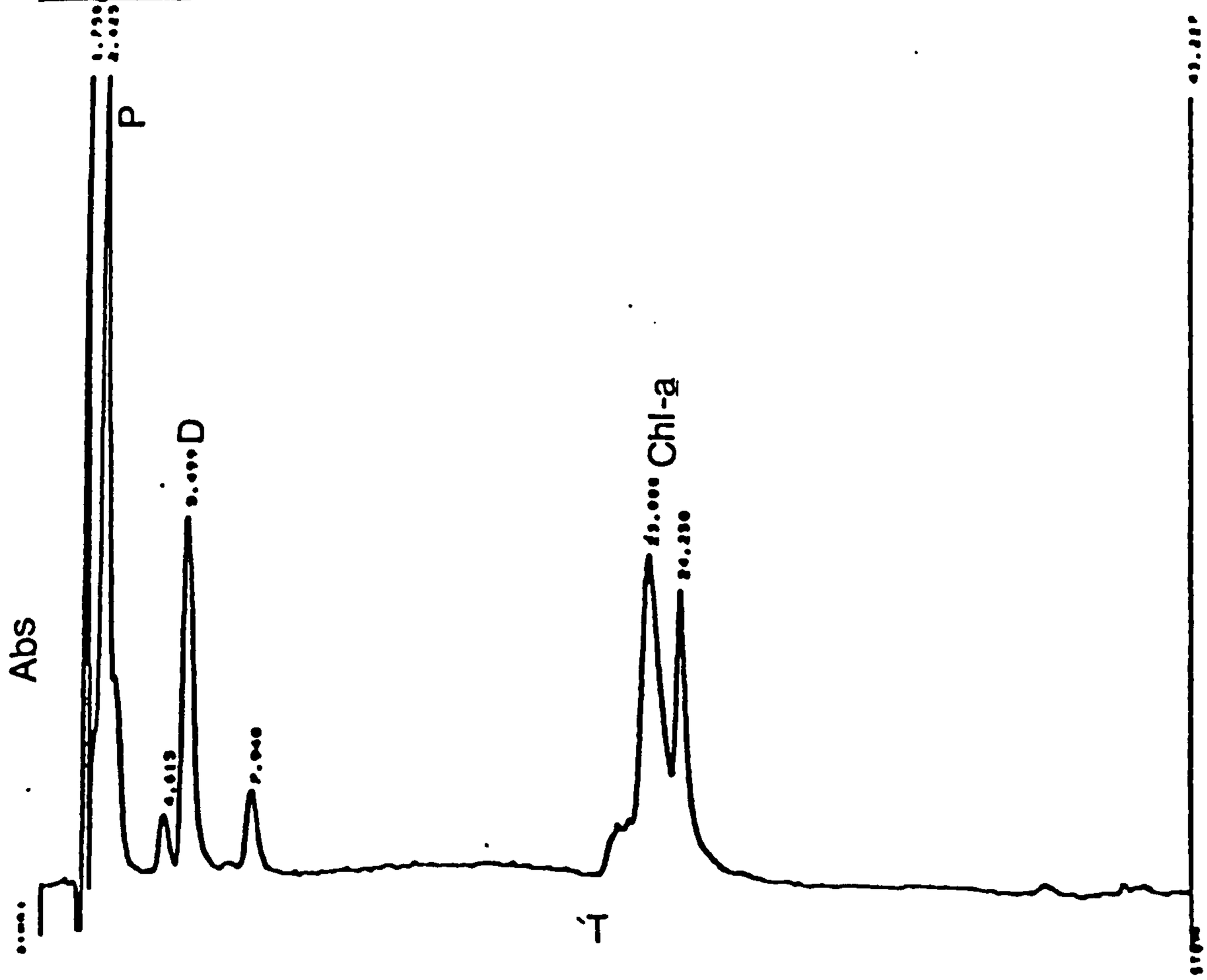


Figure 3.3 HPLC profiles (absorbance (Abs) vs. time (T)) of photosynthetic pigments of symbionts of named host species in 90 % acetone (P = peridinin, D = Diadinoxanthin, Chl-a = chlorophyll-a complex).

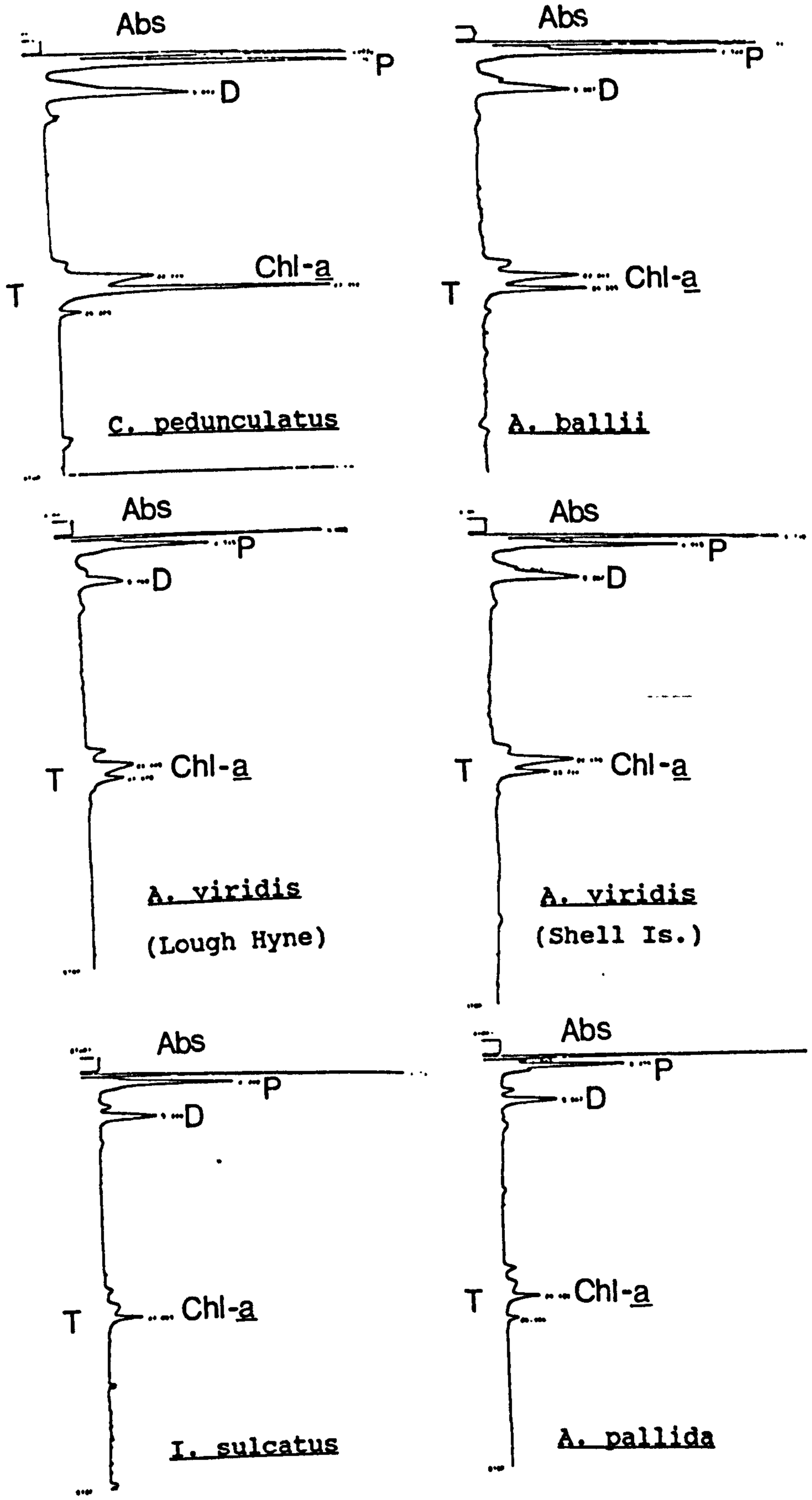


Figure 3.4 Absorbance peaks of named photosynthetic pigments obtained from TLC plates of A. carterae in 90 % acetone.

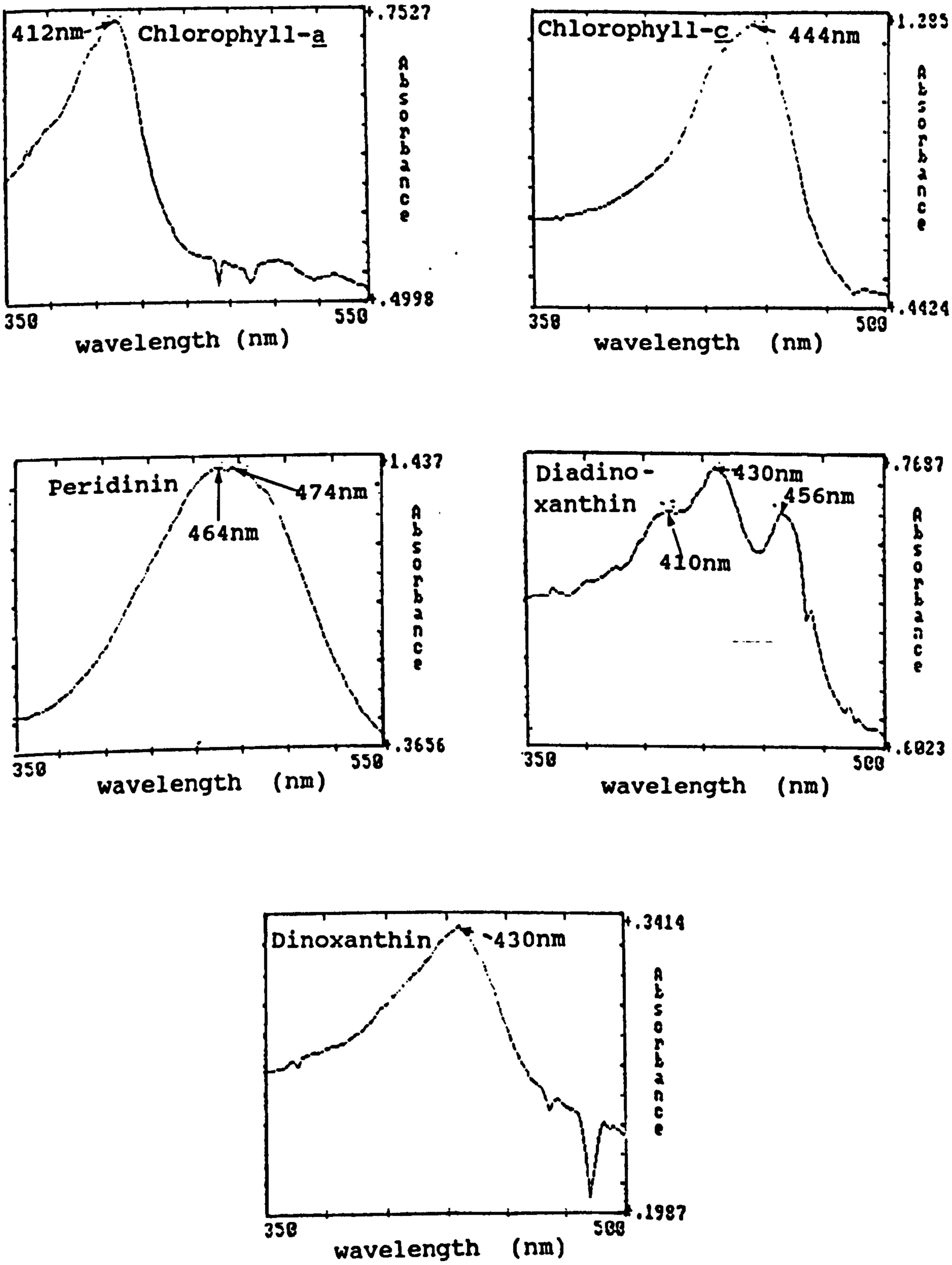


Figure 3.5 HPLC profiles (absorbance (Abs) vs. time (T)) of isolated pigments in 90% acetone.

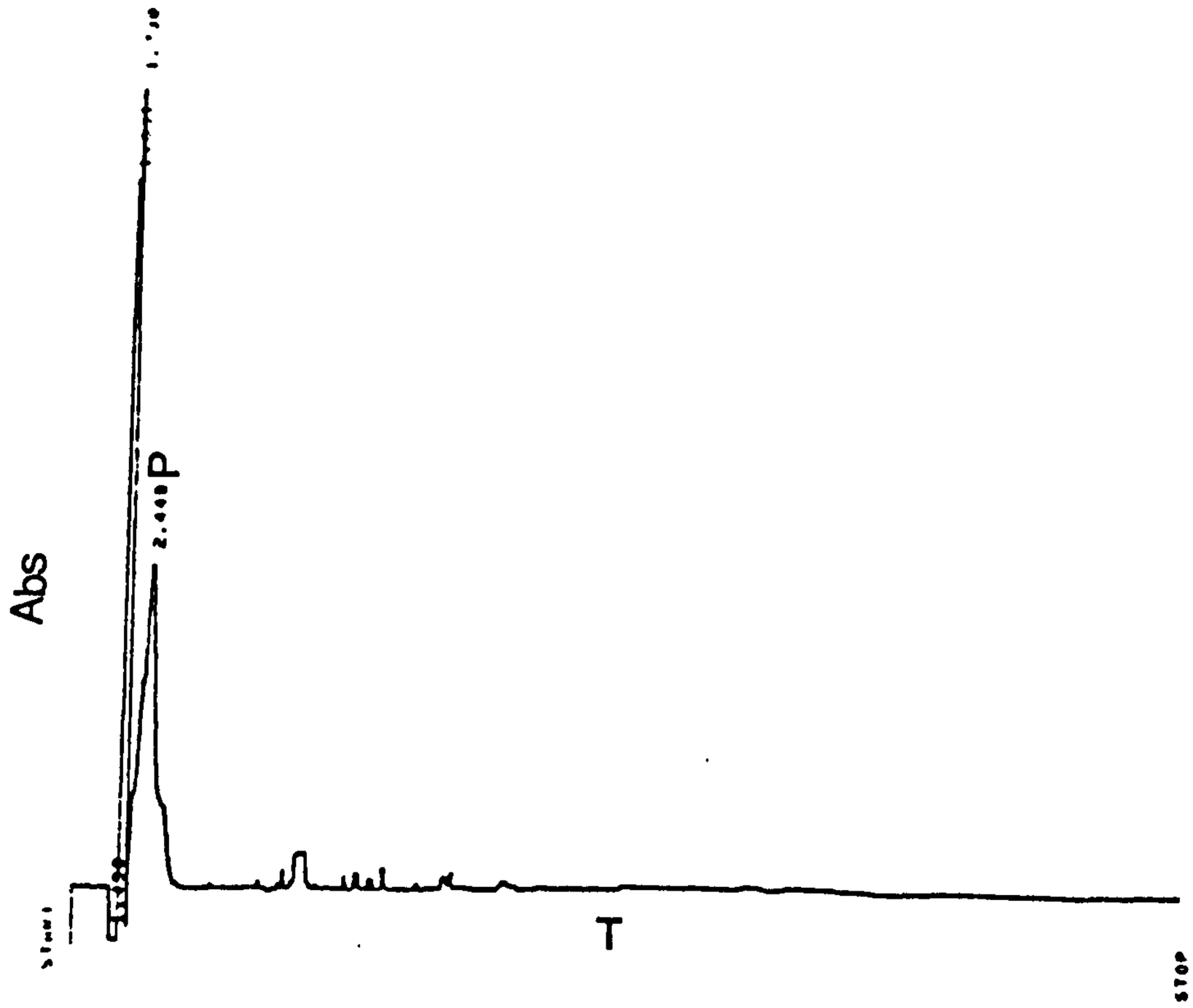


Figure 3.5.1 HPLC profile of peridinin (P).

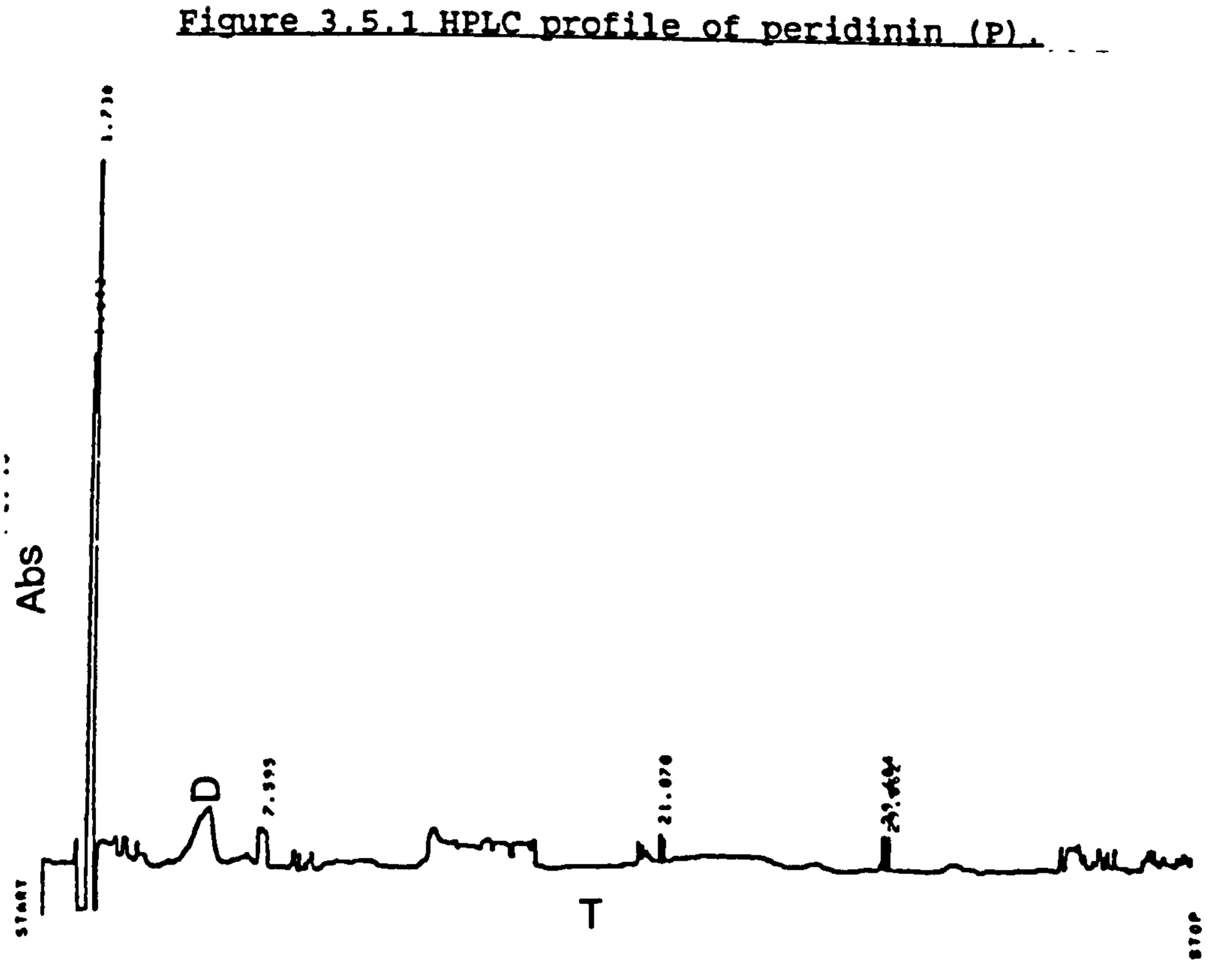


Figure 3.5.2 HPLC profile of diadinoxanthin (D).

TABLE 3.6 CELL DIAMETERS, VOLUMES AND CARBON CONTENTS OF FRESHLY ISOLATED CELLS, AND THE CELL NUMBERS AND CARBON CONTENTS OF THE ALGAL PELLETS USED IN PIGMENT ANALYSES.

Host species	Average cell diameter +/- S.E. (µm) (N=100)	Average cell volume (μm^3) (N=100)	Average cell carbon content (pg) (N=100)	Average number of cells/ pellet (N=3)	Average carbon content of pellet x10⁶ (pg) (N=3)
Cp	8.52 +/- 0.08	323.83	51.75	6095933	3.154
Ab	10.78 +/- 0.11	655.92	95.20	2227533	2.171
AvLH	8.56 +/- 0.10	328.41	52.39	2054067	1.076
AvSI	10.22 +/- 0.11	558.92	83.02	5099600	4.234
Is	11.05 +/- 0.11	706.46	101.70	504867	0.513
Ap	7.80 +/- 0.08	248.47	41.15	538475	0.222

TABLE 3.7 AREAS OF THE HPLC PEAKS STANDARDIZED TO THE CELL CARBON CONTENTS OF THE ALGAL PELLETS.

Host Sp.	Chloro-phyll-a (CHLa) +/- S.E. (mm^2/ pg C) (N=3)	Peridi-nin (P) +/- S.E. (mm^2/ pg C) (N=3)	Diadino-xanthin (D) +/- S.E. (mm^2/ pg C) (N=3)	CHLa:P +/-S.E. (N=3)	CHLa:D +/-S.E. (N=3)	P:D +/- S.E. (N=3)
Cp	1.35 E-6 +/- 2.59 E-7	8.59 E-7 +/- 2.98 E-8	4.44 E-7 +/- 1.43 E-8	1.58 +/- 0.34	3.00 +/- 0.49	1.94 +/- 0.18
Ab	2.23 E-6 +/- 4.27 E-7	7.36 E-7 +/- 1.11 E-7	4.43 E-7 +/- 4.93 E-8	1.50 +/- 0.23	2.48 +/- 0.25	1.65 +/- 0.10
AvLH	1.46 E-6 +/- 1.34 E-7	1.24 E-6 +/- 6.92 E-8	5.77 E-7 +/- 4.84 E-8	1.18 +/- 0.09	2.55 +/- 0.28	2.15 +/- 0.23
AvSI	8.15 E-7 +/- 1.03 E-7	5.53 E-7 +/- 4.18 E-8	3.14 E-7 +/- 4.18 E-8	2.22 +/- 0.86	1.97 +/- 0.50	1.76 +/- 0.06
Is	2.01 E-6 +/- 2.21 E-7	9.33 E-7 +/- 9.44 E-8	5.33 E-7 +/- 2.35 E-8	1.14 +/- 0.36	1.94 +/- 0.25	1.75 +/- 0.21
Ap	2.21 E-6 +/- 4.85 E-7	3.24 E-7 +/- 1.50 E-9	2.03 E-7 +/- 3.0 E-9	0.57 +/- 0.13	0.91 +/- 0.21	1.60 +/- 0.04

** Due to a powercut, one HPLC chromatogram of symbionts of A. pallida was lost. Therefore N=2 for A. pallida.

3.3.4 Discussion

(a)The identity of the pigments.

Comparisons of the absorption spectra and HPLC chromatograms of pigments from the symbionts of the different host species with those from A. carterae revealed clearly that all the symbionts were dinoflagellates.

The 2 most obvious components of the absorption spectra, chlorophylls -a and -c, are characteristic of several of the major marine algal classes. However, the Dinophyceae are the only class to contain just one type of chlorophyll-c, chlorophyll-c2. This feature has been illustrated using Symbiodinium sp. from Tridacna crocea (Jeffrey and Shibata 1969). From this investigation it was not possible to determine which type of chlorophyll-c was present. This is because the absorption maxima of chlorophylls-c1 and -c2 are very close, being 445, 579 and 628 nm for chlorophyll-c1 and 449, 582 and 629 nm for chlorophyll-c2 in ether (Prezelin 1987). If confirmation of the presence of chlorophyll-c2 alone is required, then the chlorophyll-c fraction could be separated using the method of Jeffrey (1972).

The presence of the characteristic dinoflagellate light harvesting carotenoid peridinin was obvious, being approximately two times more concentrated than any of the xanthophylls. Peridinin possesses strong blue-light absorbing characteristics as demonstrated by its absorbance maxima at 464 and 474 nm. Neoperidinin and peridinin have

been shown to be present in Symbiodinium sp. from T. maxima (Deane and O'Brien 1978) and peridinin has also been identified in Symbiodinium spp. from A. viridis (Taylor, D.L. 1967), A. elegantissima (Muscatine 1971), A. pulchella and M. verrucosa (Chang et al. 1983).

The minor xanthophyll fraction of Symbiodinium spp., which may be involved in phototactic and photoprotective responses in and outside the chloroplast, has been less conclusively identified. This fraction warrants more detailed examination, as the minor carotenoids have been suggested as biochemical determinants of taxonomic affinities (Prezelin 1987). D.L. Taylor (1967), using pigments extracted from symbionts of A. viridis, noted two closely associated yellow bands on the TLC plate. The lower of these bands was labelled diadinoxanthin and the upper band was labelled dinoxanthin. Muscatine (1971) recorded 2 similar yellow bands on TLC plates of pigments from the symbionts of A. elegantissima, but did not identify the pigments. Deane and O'Brien (1978) recorded diadinoxanthin in the symbionts of T. maxima but not dinoxanthin and Chang et al. (1983) did not differentiate between any of the xanthophylls.

(b)Evidence for strain or species specific differences in the concentrations of the major pigments .

Despite the small number of replicates used in this pigment analysis, the comparatively low standard errors enable some tentative conclusions to be made concerning the relative concentrations of chlorophyll-a, peridinin and

diadinoxanthin in Symbiodinium sp. from the British Anthozoa.

The large differences observed between chlorophyll-a, peridinin and diadinoxanthin concentrations in Symbiodinium sp. isolated from A. viridis (Shell Island) and the other British host species, and between Symbiodinium sp. from A. viridis (Shell Island) and A. viridis (Lough Hyne), may indicate genetic differences on an inter- and intra-host specific basis. Host specific differences with respect to pigment concentrations have been recorded in cultures of the symbionts of T. maxima, A. pulchella and M. verrucosa (Chang et al. 1983). But despite the considerably higher concentrations of the major carotenoids in the symbionts of A. viridis (Lough Hyne), the ratios of the pigments are consistent with those of the symbionts of the other host species. The observed ratios are all similar to those described by Chang et al. (1983), again demonstrating the similarity between the symbionts of the British Anthozoa and Symbiodinium spp. from tropical hosts.

(c)General conclusion.

D.L. Taylor (1967) placed much emphasis on the similar pigment compositions of the symbionts of several host species when suggesting that Symbiodinium sp. was a worldwide, pandemic species. In spite of this, the use of pigment analysis in the elucidation of the precise taxonomic position of Symbiodinium cells from different host species remains limited beyond assigning the symbionts to the

Dinophyceae. More extensive analysis of the minor carotenoids may reveal differences in the future. However, to remove any doubt over host environment influences upon the pigment composition, cultures providing large quantities of material would be required.

3.3.5 Summary

(i)The major photosynthetic and accessory pigments of freshly isolated symbionts of the British Anthozoa and A. pallida were identified by analyses of their absorption spectra and comparisons with the pigments of a standard dinoflagellate, Amphidinium carterae, using TLC, spectrophotometry and HPLC.

(ii)The pigments identified were chlorophylls-a and -c, peridinin, diadinoxanthin and dinoxanthin, hence confirming these symbionts as dinoflagellates.

(iii)The concentrations of chlorophyll-a, peridinin and diadinoxanthin were compared by standardizing the HPLC peak areas to the carbon content of the symbiont pellet from which the pigments were extracted.

(iv)Significant differences were observed between the pigment concentrations in symbiont cells of different host species. These 1.4 to 2.7 fold differences indicated possible genetic variation on an intra- and inter-host specific basis.

(v)The pigment ratios were similar between symbionts of different temperate host species and to previously

investigated tropical symbionts.

(vi)Because of difficulties in discounting environmental effects on pigment composition *in situ*, the use of freshly isolated material to investigate genetic variation within the genus *Symbiodinium* is limited.

3.4 THE MITOTIC INDEX OF THE SYMBIONTS IN SITU AND IN CULTURE

3.4.1 Introduction

The aim of this work was to measure and compare the mitotic index (MI) of *Symbiodinium* sp. from different host species, both in culture and *in situ*. Therefore any intrinsic differences in symbiont diel division cycles, mitotic indices and hence cell specific growth rates (μ) could be identified, and evidence of host control of symbiont growth could be investigated.

The mitotic index (MI) is defined as the frequency of dividing cells occurring in a population. This has usually been measured by determining the fraction of cells occurring as doublets (eg. Swift *et al*. 1976; Wilkerson *et al*. 1983; Muscatine *et al*. 1984; Stambler and Dubinsky 1987) or the fraction of cells with two nuclei (Weiler and Chisolm 1976; Suharsono and Brown 1992). Originally applied to phytoplankton growth estimates (Swift and Durbin 1972; Lewin and Rao 1975; Weiler and Chisholm 1976; Swift *et al*. 1976),

MI estimates were first made on *in situ* *Symbiodinium* populations by Wilkerson *et al*. (1983), although the technique had been previously applied to the *Hydra*-*Chlorella* symbiosis (McAuley 1981; McAuley 1982). The mathematical relationship between the MI and specific growth rate was revised throughout these studies and equations derived by McDuff and Chisolm (1982) and subsequently used by Wilkerson *et al*. (1983) have now been generally accepted.

In situ MI measurements have proved invaluable in understanding the flux of nutrients and energy through the cnidarian-algal symbioses, where increases in the availability of energy and nutrients are associated with an increase in the MI (Wilkerson *et al*. 1983; Muscatine *et al*. 1989a; Taylor, C.E. *et al*. 1989). This relationship has been implicated in the regulation of the symbiont population by cnidarian hosts (Douglas and Smith, D.C. 1984; Jackson and Yellowlees 1990). Alternative symbiont population regulation mechanisms have also been investigated in the *Hydra*-*Chlorella* symbiosis using comparative measurements of the symbiont MI (McAuley 1982; McAuley 1985).

Despite comparisons of the *in situ* and *in vitro* MI (Wilkerson *et al*. 1983; Muscatine *et al*. 1984; Wilkerson *et al*. 1988), published comparisons between the MI of *Symbiodinium* cells from different host species *in vitro* are uncommon. Even so, different MI were reported for the cultured symbionts of 3 host species by Fitt and Trench (1983), indicating that intrinsic variations in division

behaviour may be present within the genus Symbiodinium. The following describes the measurement of the MI and diel division cycles of freshly isolated and cultured symbionts of the British Anthozoa and A. pallida, and their comparisons.

3.4.2 Materials and methods

(a)Measurement of the mitotic index (MI) using the "two-cell stage"

The MI of freshly isolated symbionts was measured using hosts which had not been fed for 3 days. The following were excised and homogenized: 3 tentacles (each one from a different host individual) of A. viridis (from all 6 locations used in this work) and A. ballii; 3 polyps of I. sulcatus and A. pallida; 3 oral discs of C. pedunculatus (Lough Hyne) and 1 oral disc of C. pedunculatus (Netley). The host animals were all adults with oral discs of the following diameters: A. viridis and A. ballii 3cm, C. pedunculatus 2cm and A. pallida 0.5 cm. The I. sulcatus polyps were approximately 1 - 1.5 mm in height. The use of similarly sized adult hosts negated the possibility of host size (ontogenetic) influences on the MI (Smith, G.J. 1986; Hoegh-Guldberg et al. 1986; Muscatine et al. 1986). The isolation procedure was as described in section 2.3.2 (a) (p.44), except that only one 5 minute centrifugation at x 1200 r.p.m. was performed to remove the bulk of the host material. The symbiont pellet was then resuspended in 3-5 ml

filtered sea water (FSW) depending on the size of pellet obtained. A subsample of the suspension was pipetted onto a Fuchs-Rosenthal type haemocytometer. The percentage of cells in the "2-cell stage" of mitosis was estimated by counting the number of dividing cells in a total count of 500 using the x 10 objective of the light microscope. The MI was measured at 4 hourly intervals over a 24 hour period. The diel division cycle was measured on 2 separate occasions to establish whether the population growth was synchronous or asynchronous. In addition to adult Anthozoa being used in these investigations, freshly settled juveniles of A. ballii (approximately 2 weeks old) were also available. These animals were at a premium, hence just one polyp was squashed under a coverslip on a slide at each time interval. The percentage of dividing cells in a count of 500 cells was then estimated, using one field of view at a time under the x 25 objective. Only one series of measurements was taken using A. ballii juveniles.

Cultured Symbiodinium sp. cells from each host species were harvested, giving 3 ml of each different 'strain' of alga in culture medium (1 ml from each of 3 replicate cultures). Following shaking to separate the cells, the MI was measured using a haemocytometer in an identical manner to that described above. The diel division cycle was again recorded on 2 separate occasions. To ensure that the cultures were of the same age and stage of growth, the first diel division cycle was measured 2 weeks after the addition of fresh

culture medium to 2 month old cultures and the second cycle was measured one day later using the same cultures.

(b)Measurement of the mitotic index (MI) using nuclear division

The feasibility of measuring the MI as suggested by Brown and Zamani (1992) was tested.

The DNA specific Feulgen reaction was employed to visualise the symbiont nuclei. 3 tentacles of A. viridis were excised as described previously. The tentacles were placed in 5 ml glass vials and fixed in 3 : 1 ethanol : glacial acetic acid for 30 minutes. The tentacles were then washed in cold 1N hydrochloric acid (HCL) for 1 minute and subsequently placed in 1N HCL and hydrolysed on a hot plate at 60 ^{o}C for 30 minutes. The HCL was then cooled to 20 ^{o}C for 10 minutes and the tentacles washed in distilled water. The tentacles were immersed in Schiff's reagent for 3 hours and subsequently treated with 3 washes of sulphurous acid (H_2SO_3). The sample was finally washed 3 times in distilled water and mounted in a drop of distilled water on a slide. The stained regions (staining pink /red) were observed under the x 40 and x 100 objectives of the light microscope and the feasibility of measuring the MI from the percentage of symbiont cells possessing 2 nuclei estimated.

(c)Calculation of the cell specific growth rate and doubling time of the symbiont population.

The specific growth rate and doubling time of the symbiont populations were estimated using the equations of McDuff and

Chisholm (1982) and Wilkerson et al. (1983).

The specific growth rate (μ), a measure of the amount of carbon used daily to synthesize new algae (Muscatine et al. 1983), was calculated using the following equation:

$$\mu = 1 / t_d \ln (1 + f_i) \quad \text{(equation 3.2)}$$

where t_d = the duration of cytokinesis and f_i = the average daily MI in an asynchronously dividing population of algae. The value of f_i was taken from the pooled average of the 2 replicate series. Unfortunately, t_d has never been measured directly for any Symbiodinium spp. in situ, but t_d has been estimated from phased populations of Symbiodinium sp. as being approximately 11 hours (Wilkerson et al. 1983) and subsequently applied to asynchronously dividing populations (Wilkerson et al. 1983; Steen and Muscatine 1984; Hoegh-Guldberg et al. 1986). Estimates of μ will only be significantly affected if t_d is less than 5 or 6 hours (Muscatine 1990). A value of t_d of 7 hours was used in the case of cultured Symbiodinium sp. (Wilkerson et al. 1983).

The doubling time (Dt) was subsequently estimated using the equation:

$$Dt = \ln 2 / \mu \quad \text{(equation 3.3)}$$

(d)Statistical comparisons of the mitotic index (MI) of the cultured and in situ symbiont populations of different host species and conspecific hosts from separate geographical locations.

The mitotic indices measured over the diel division cycle were found to be normally distributed. Thus a Student's t-test was performed to compare the 2 replicates of each diel division cycle measured. If the 2 series were not significantly different they were pooled for further statistical analysis. Inter-host specific variation in the MI was then investigated using the Kruskal - Wallis test followed by multiple comparisons. Comparisons of the MI of the symbionts of A. viridis from different locations were made using a one-way ANOVA. The mitotic indices of Symbiodinium sp. from C. pedunculatus from Lough Hyne and Netley were compared using the Student's t-test, as were the mitotic indices of the in situ symbionts and the cultured symbionts of the same host species.

3.4.3 Results

(a)MI measured using the "two-cell stage"

The diel division cycles of the cultured and in situ symbiont populations are shown in graphs 3.3-3.14 (pp.148-153). From these graphs it can be clearly seen that all the symbiont populations exhibited asynchronous division patterns. The 2 replicate series of in situ and cultured diel division cycles of the symbionts of each host species

were not significantly different (P>0.05) (appendix 6, table 1 (p.486)). Therefore, the 2 series were pooled. The average MI over the 24 hour diel division cycle, the specific growth rate (μ) and the doubling time (Dt) of the population for the cultured and *in situ* symbionts of all the host species from each location are given in table 3.8 (p.154). Comparisons between the MI *in situ* and *in vitro* of symbionts from the same host species and location (appendix 6, table 2 (p.487)) demonstrated that the MI in culture was significantly greater than the MI in the host in all cases (P<0.0001). Significant differences were evident between the cultured symbionts of different host species (H=36.21, P<0.0001). Subsequent multiple comparisons of the MI of the cultured symbionts of different host species revealed no significant differences between the symbionts of any of the temperate Anthozoa (P>0.05), however the MI of the temperate symbionts *in vitro* were all significantly higher than the MI of *Symbiodinium* sp. from *A. pallida* (appendix 6, table 3 (p.487)). Significant differences were also evident between the MI of *in situ* symbionts of different host species (H=34.0, P<0.0001). *In situ*, the symbionts of *C. pedunculatus* (Lough Hyne) possessed a higher MI than the symbionts of *A. viridis* (Shell Island), *I. sulcatus* and *A. pallida*, and the MI of the symbiont populations of *A. ballii* and *A. viridis* were significantly higher than the MI of the symbionts of *A. pallida* (appendix 6, table 4 (p.488)). The MI of the symbionts of *C. pedunculatus* (Lough Hyne) and *C.*

pedunculatus (Netley) were not significantly different in situ (T = 0.54, P = 0.59), but the MI of Symbiodinium sp. from C. pedunculatus (Lough Hyne) was higher than the MI of symbionts of C. pedunculatus (Netley) in culture (T=2.94, P=0.009). There were no significant differences between the MI of the symbionts of A. viridis from any of the different locations either in situ (F=1.65, P=0.156) or in culture (H=11.72, P=0.020) (although significance was indicated by the Kruskal-Wallis statistic, multiple comparisons revealed no significant differences (appendix 6, table 5 (p.488)).

The diel division cycle of the symbionts of juvenile A. ballii was also asynchronous (graph 3.14 (p.153)), however the average MI over the 24 hour period was 10.49 %. This value was 7.3 times greater than the MI of the symbionts in the adult A. ballii. The specific growth rate = 0.22/day and the doubling time = 3.18 days for the symbiont population of the A. ballii juveniles.

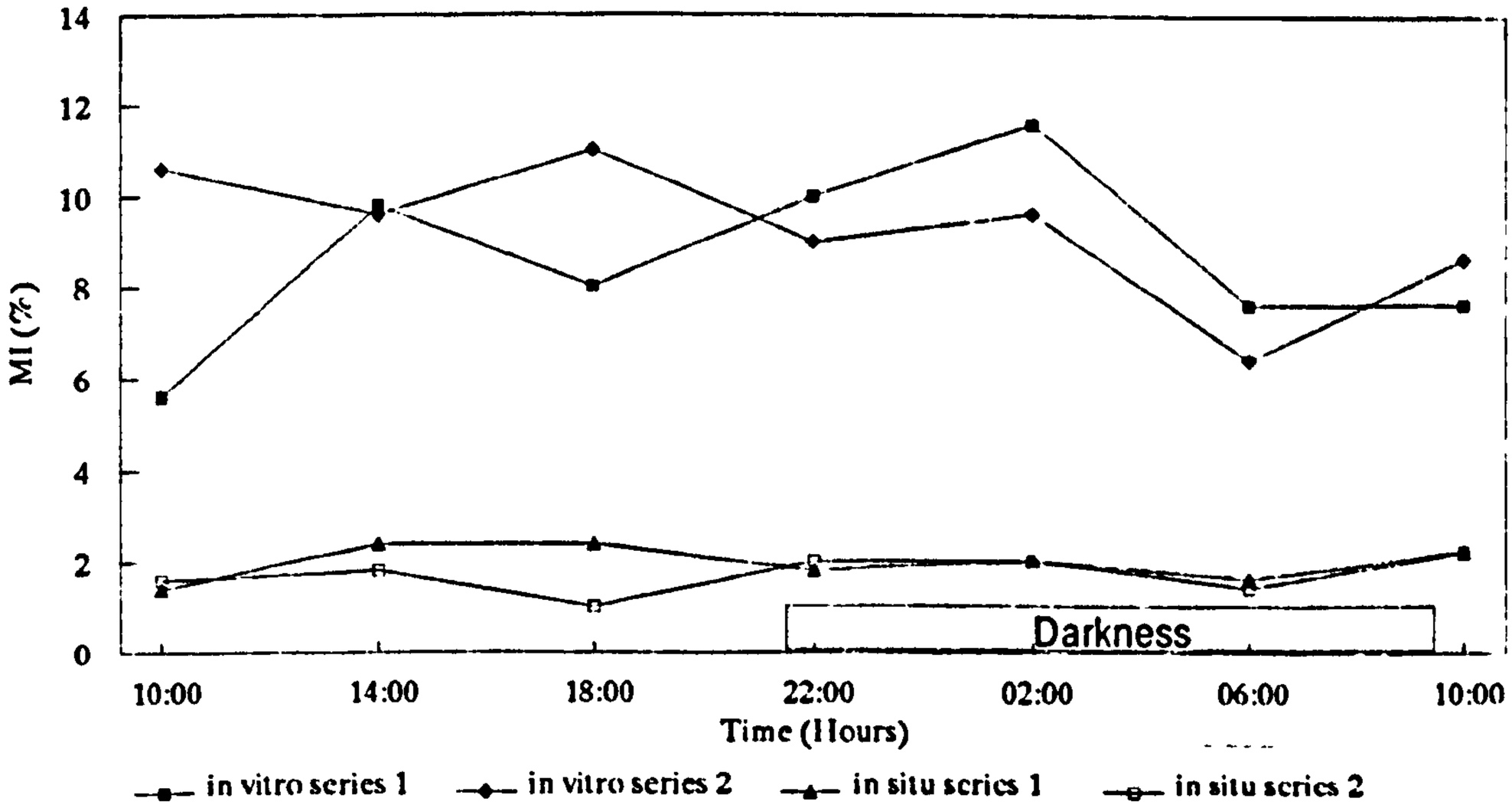
(b)The mitotic index (MI) measured using nuclear staining

As a result of the Feulgen staining technique, the symbiont nuclei appeared pink. But the nucleus was often masked partly or completely by other cell inclusions making it impossible to determine whether the nucleus was dividing. The presence of host cell nuclei taking up stain in freshly isolated samples may also have led to confusion with symbiont nuclei. This technique was therefore discontinued.

Graphs 3.3–3.14: Diel Division cycles of Symbionts (MI=% cells dividing in a count of 500 cells)(N=1).

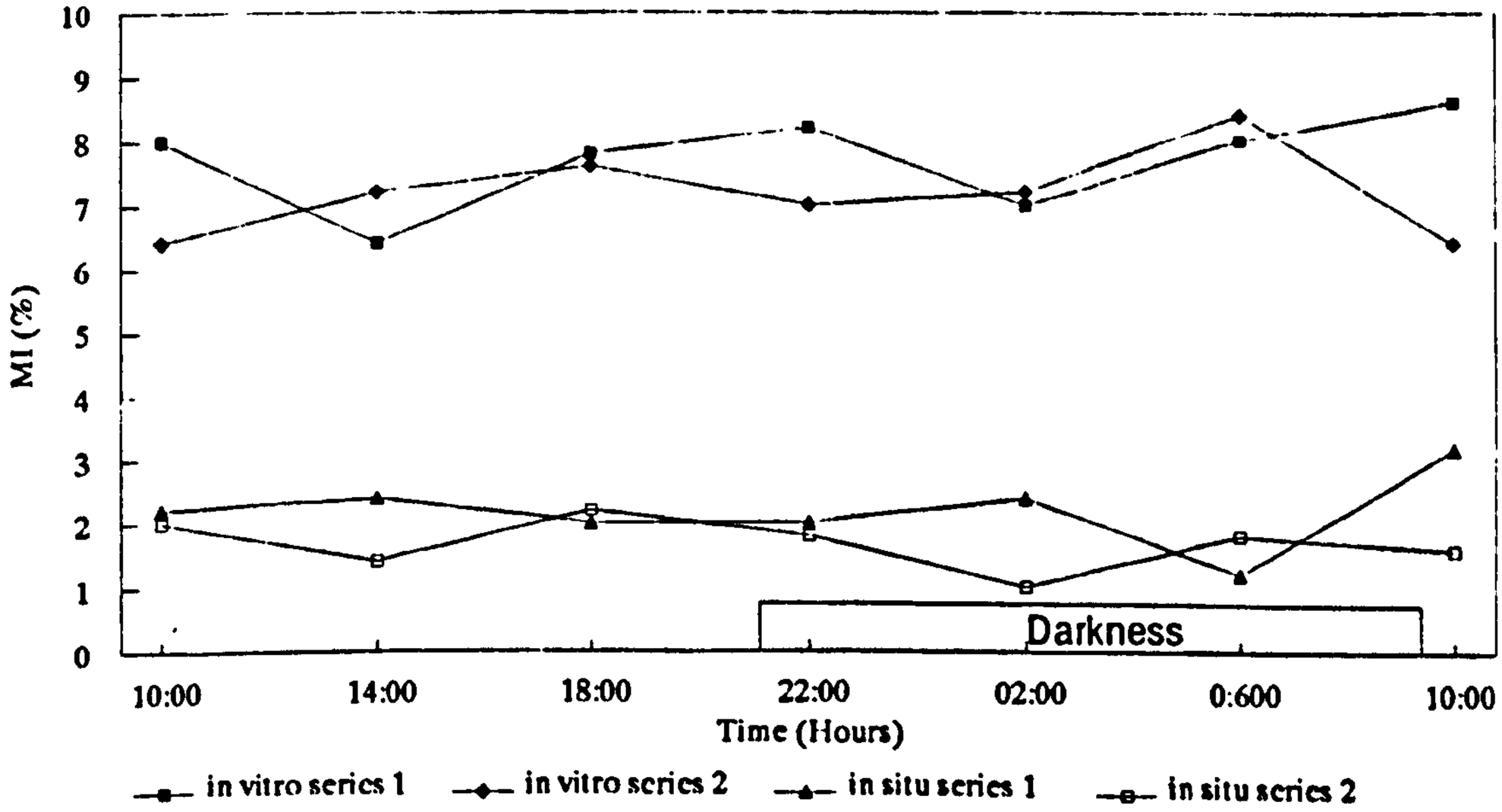
Graph 3.3

Host Species: C. pedunculatus (Lough Hyne)



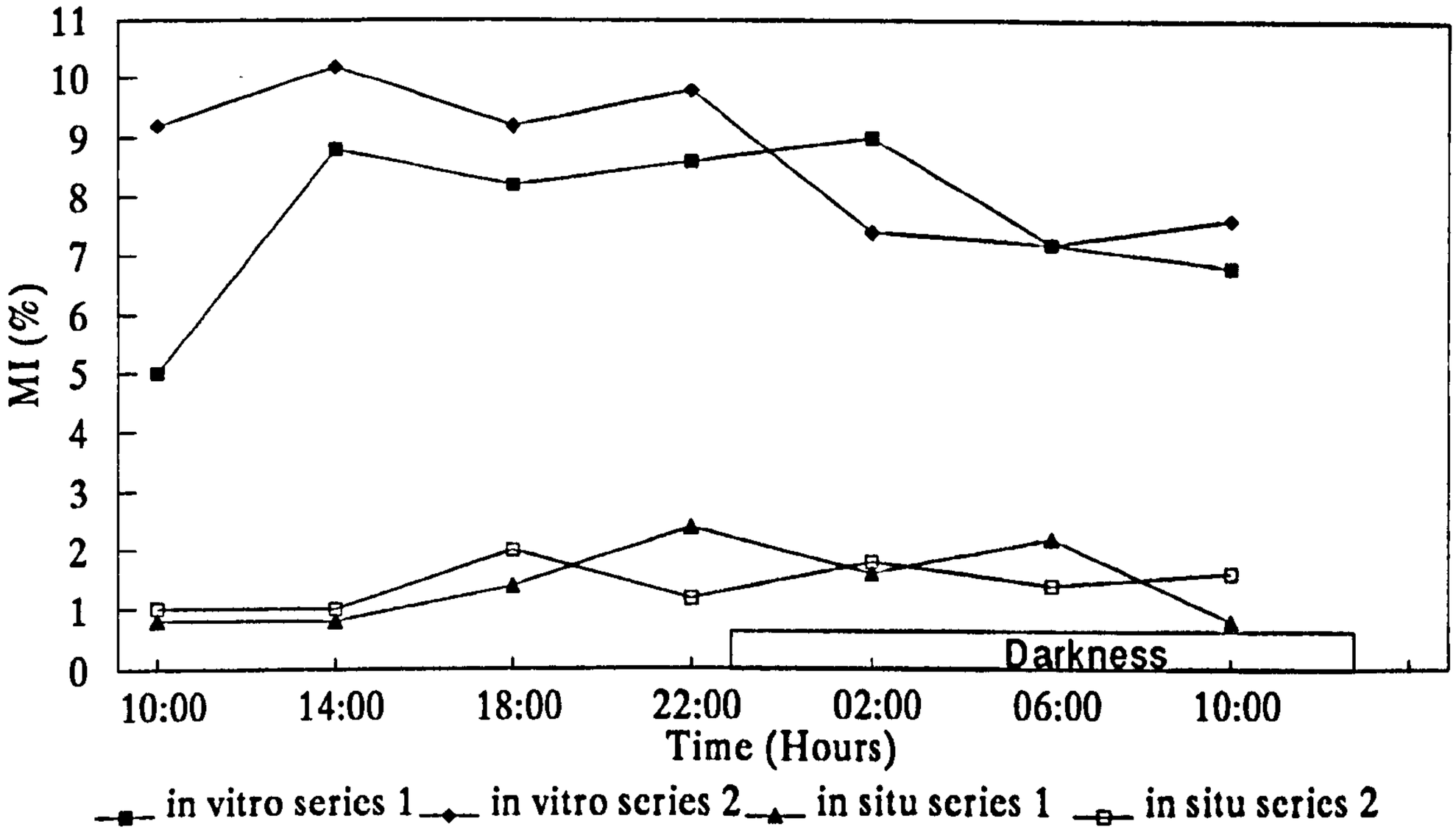
Graph 3.4

Host Species: C. pedunculatus (Netley)



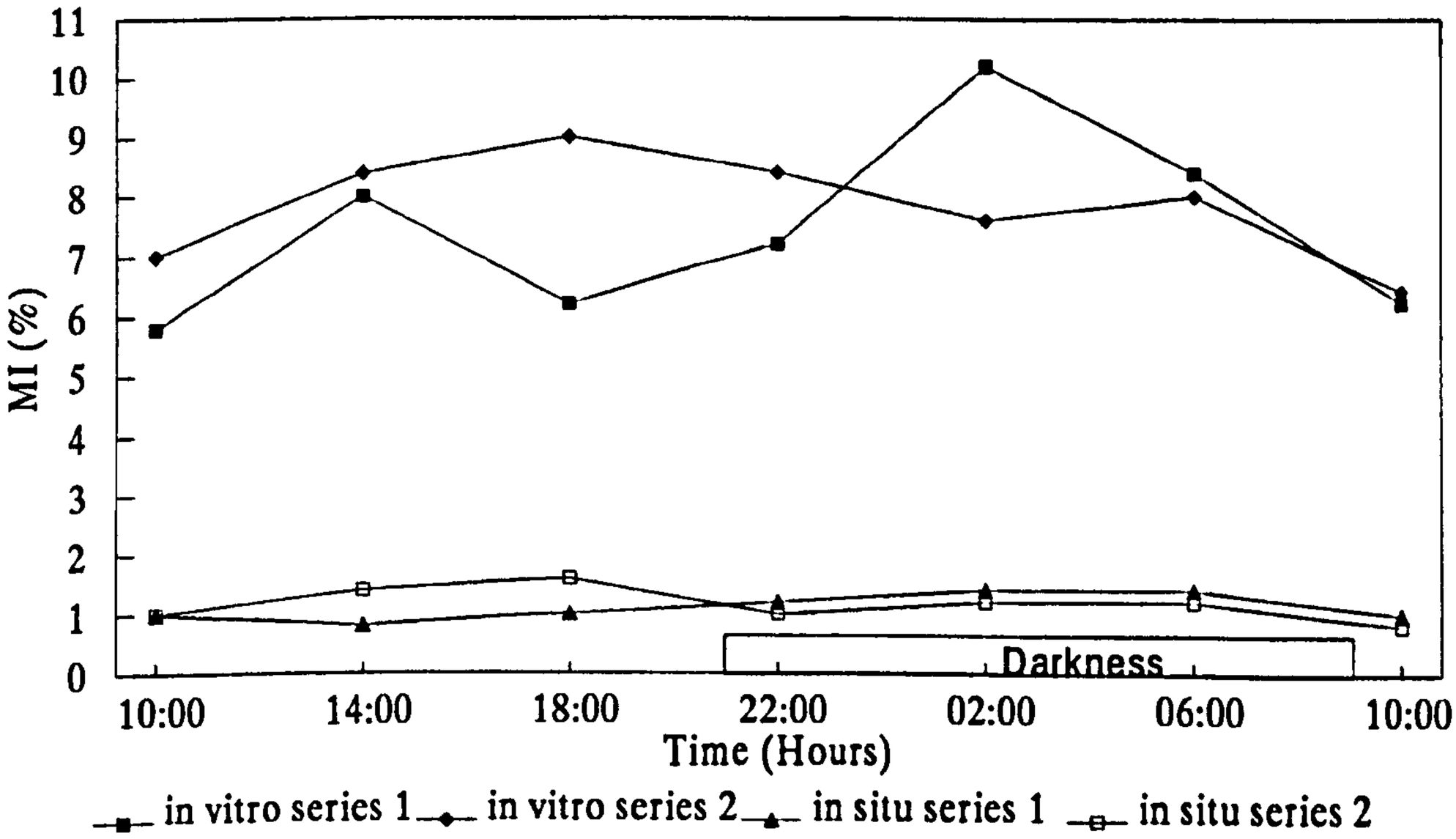
Graph 3.5

Host Species: A. ballii

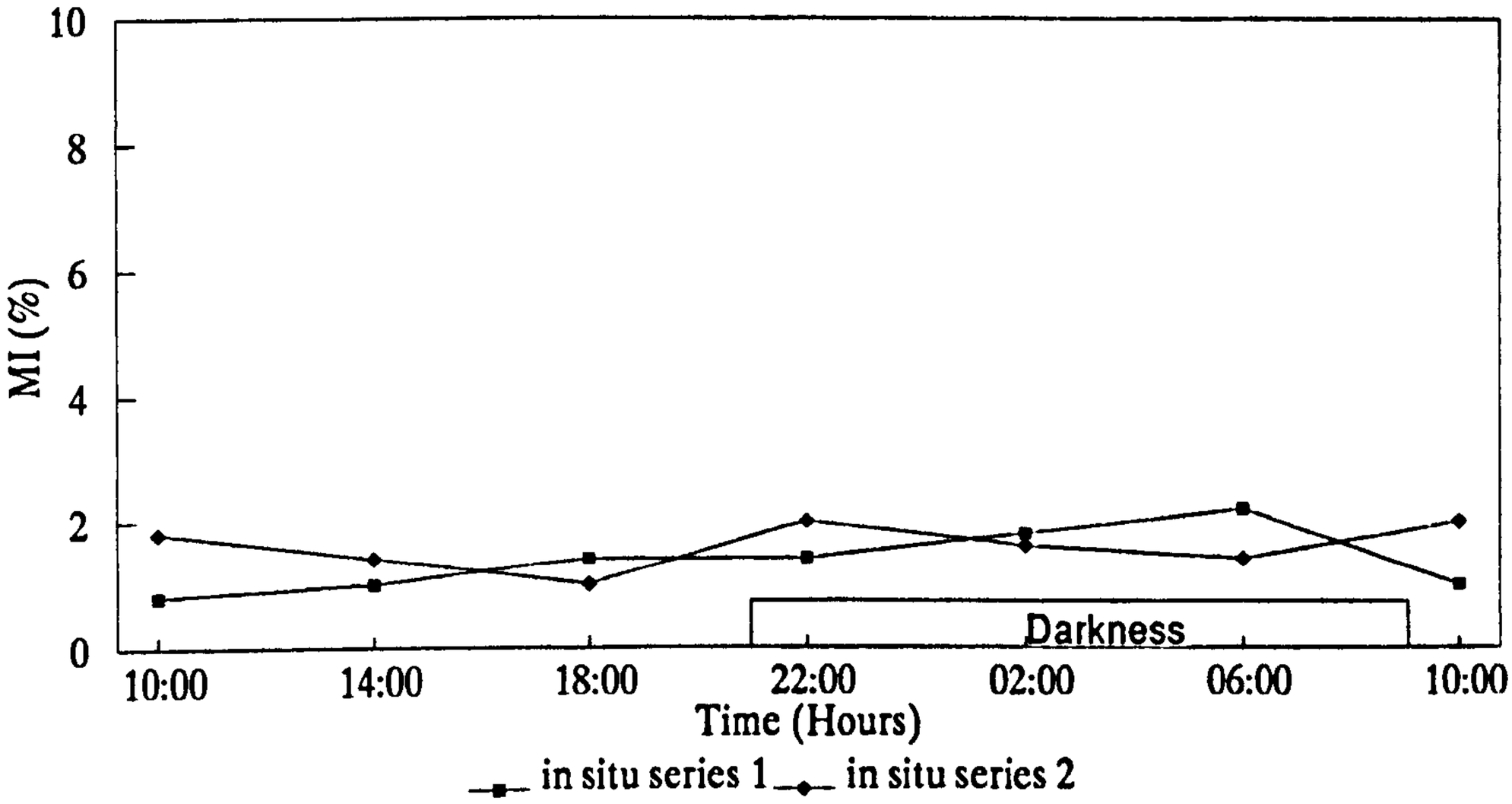


Graph 3.6

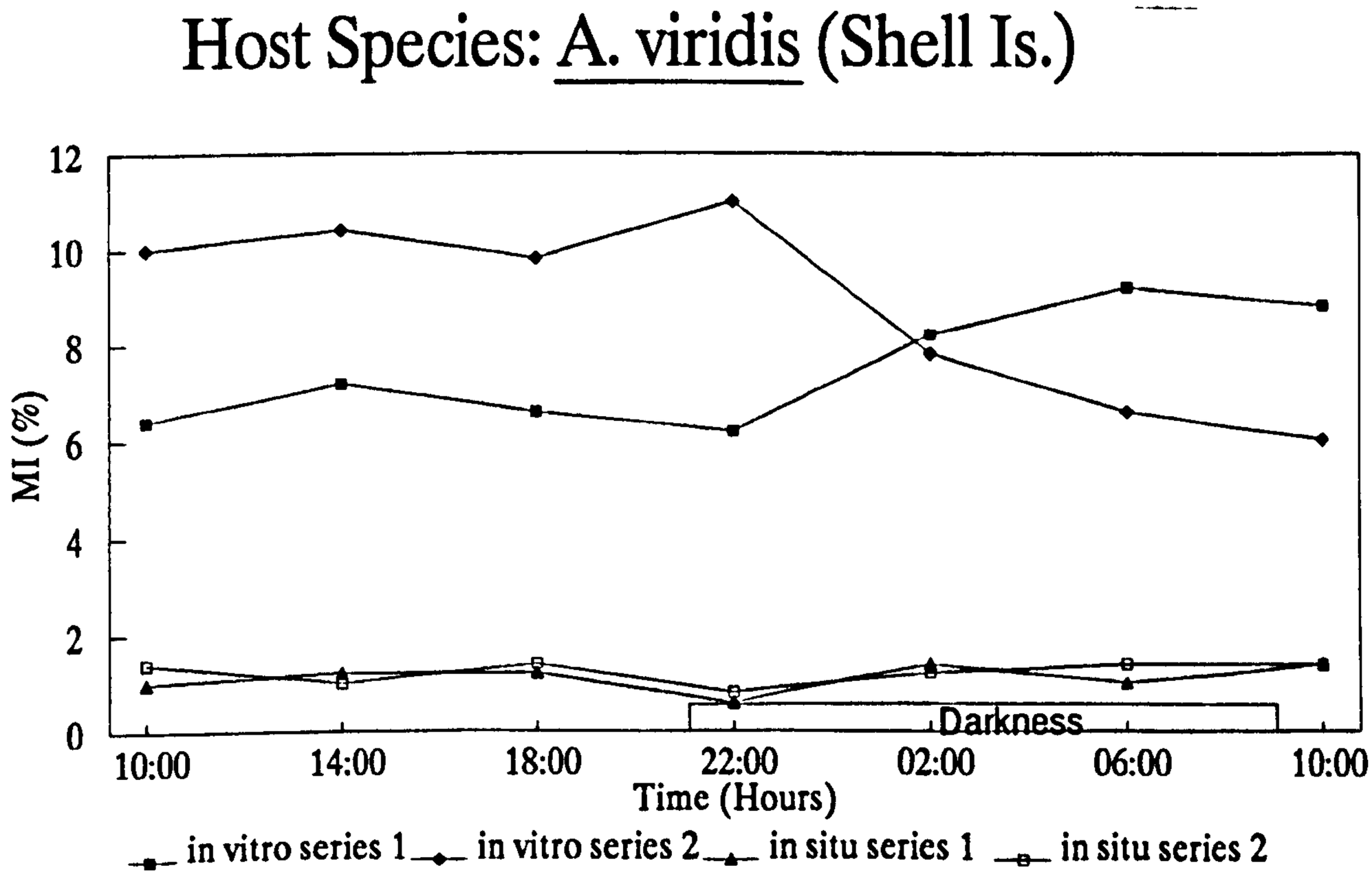
Host Species: I. sulcatus



Graph 3.7

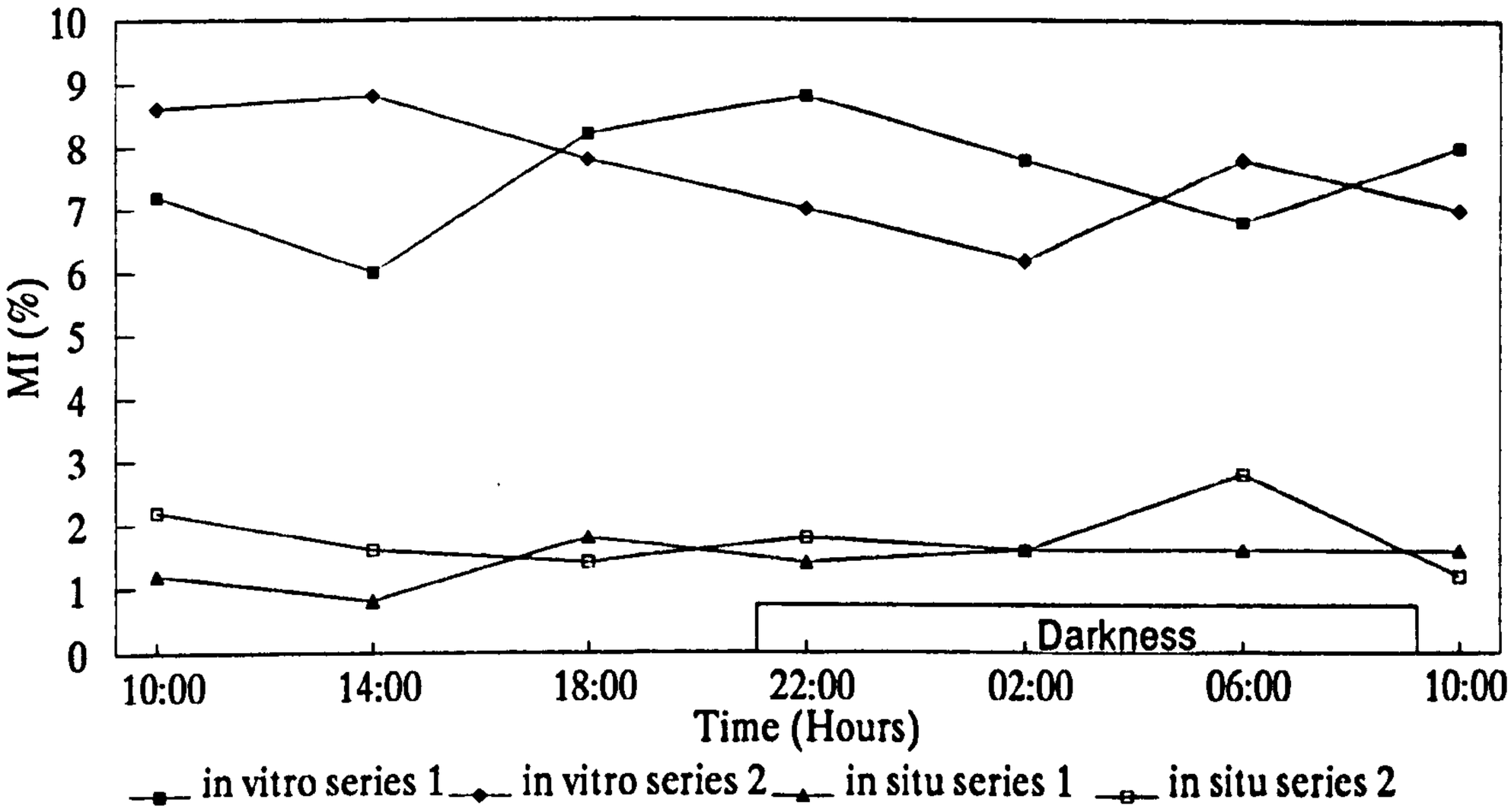


Graph 3.8



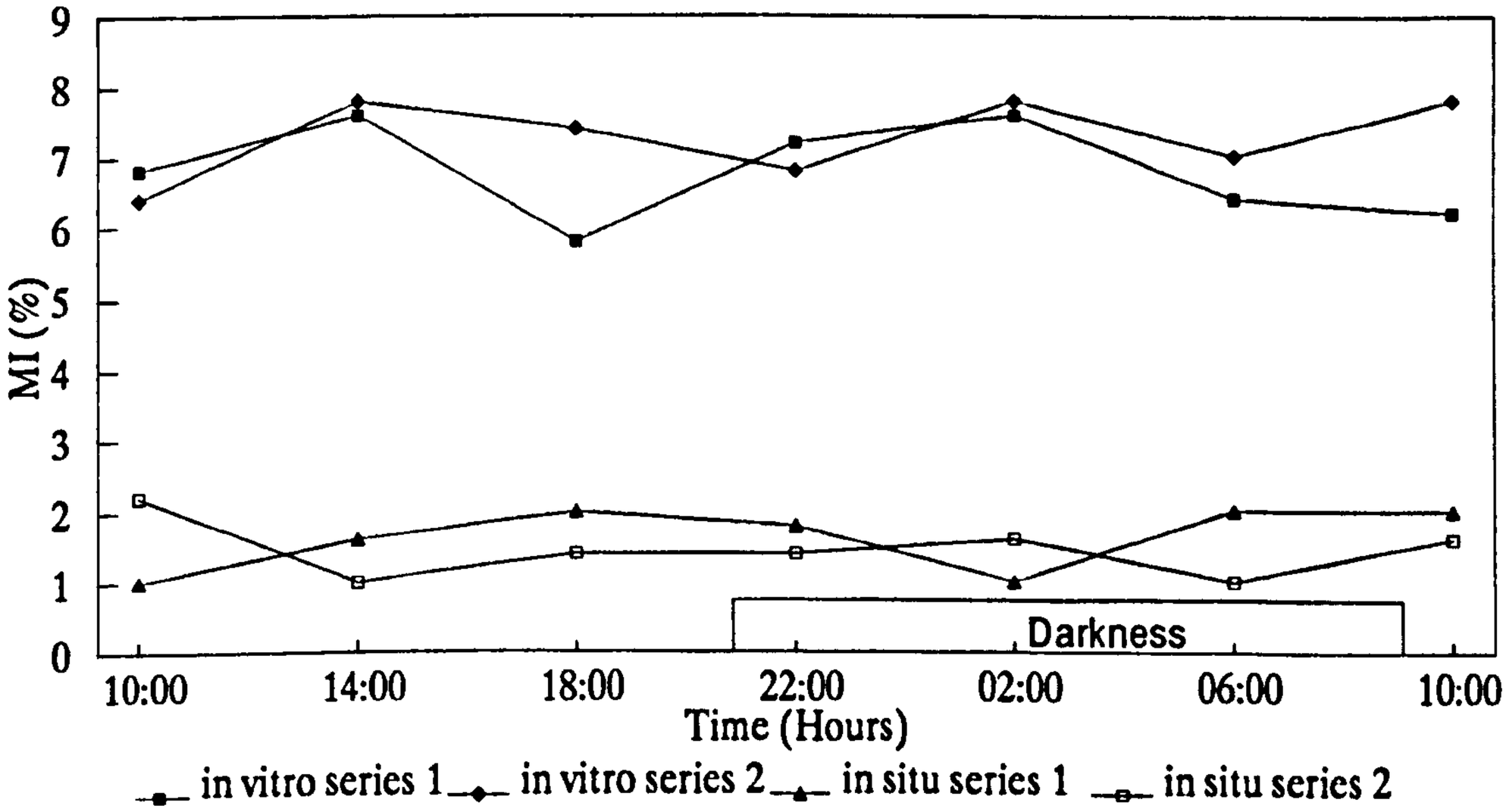
Graph 3.9

Host Species: A. viridis (Bembridge)

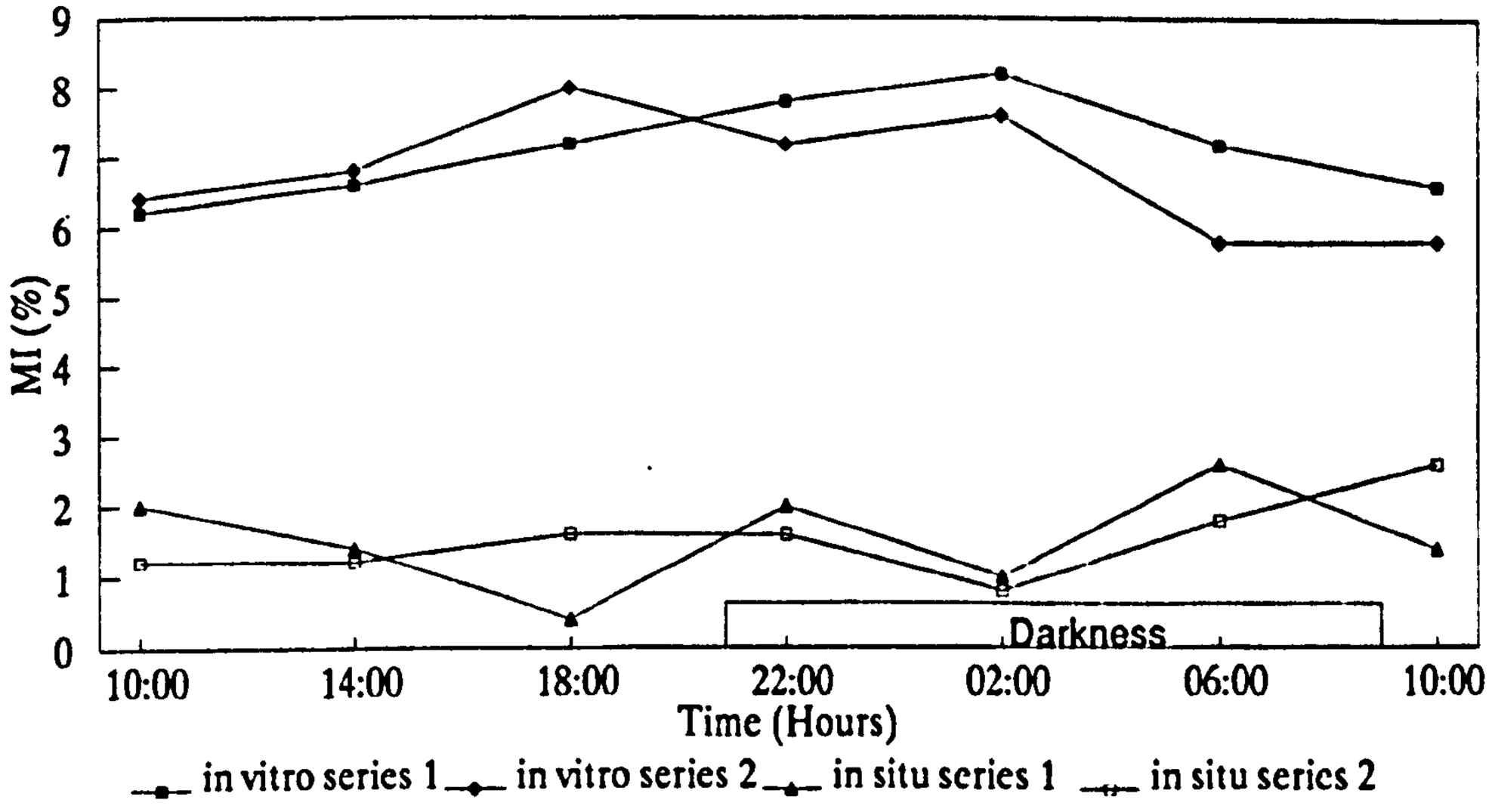


Graph 3.10

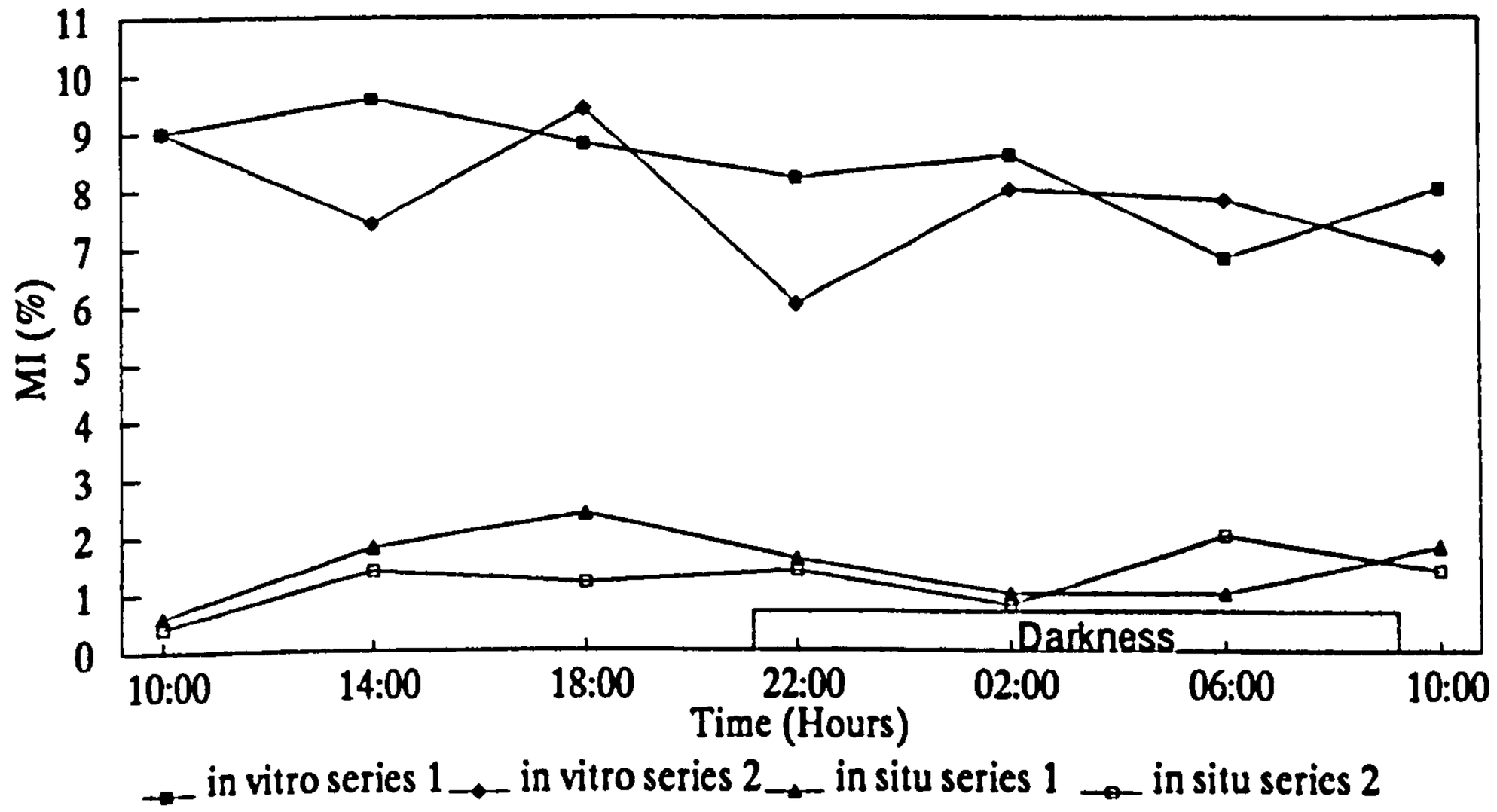
Host Species: A. viridis (L. Sween)



Graph 3.11

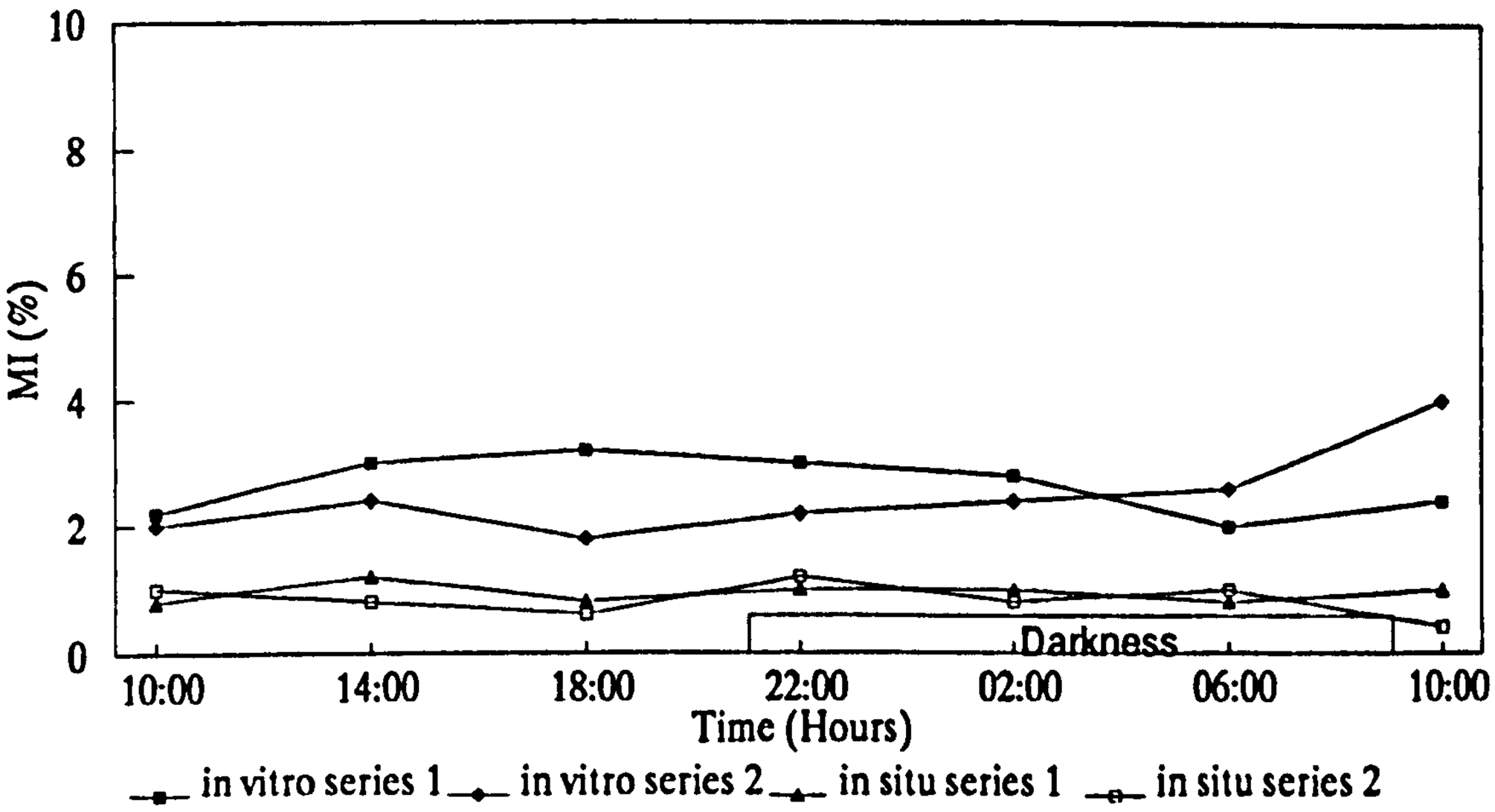


Graph 3.12



Graph 3.13

Host Species: A. pallida



Graph 3.14

Host Species: A. ballii (juvenile)

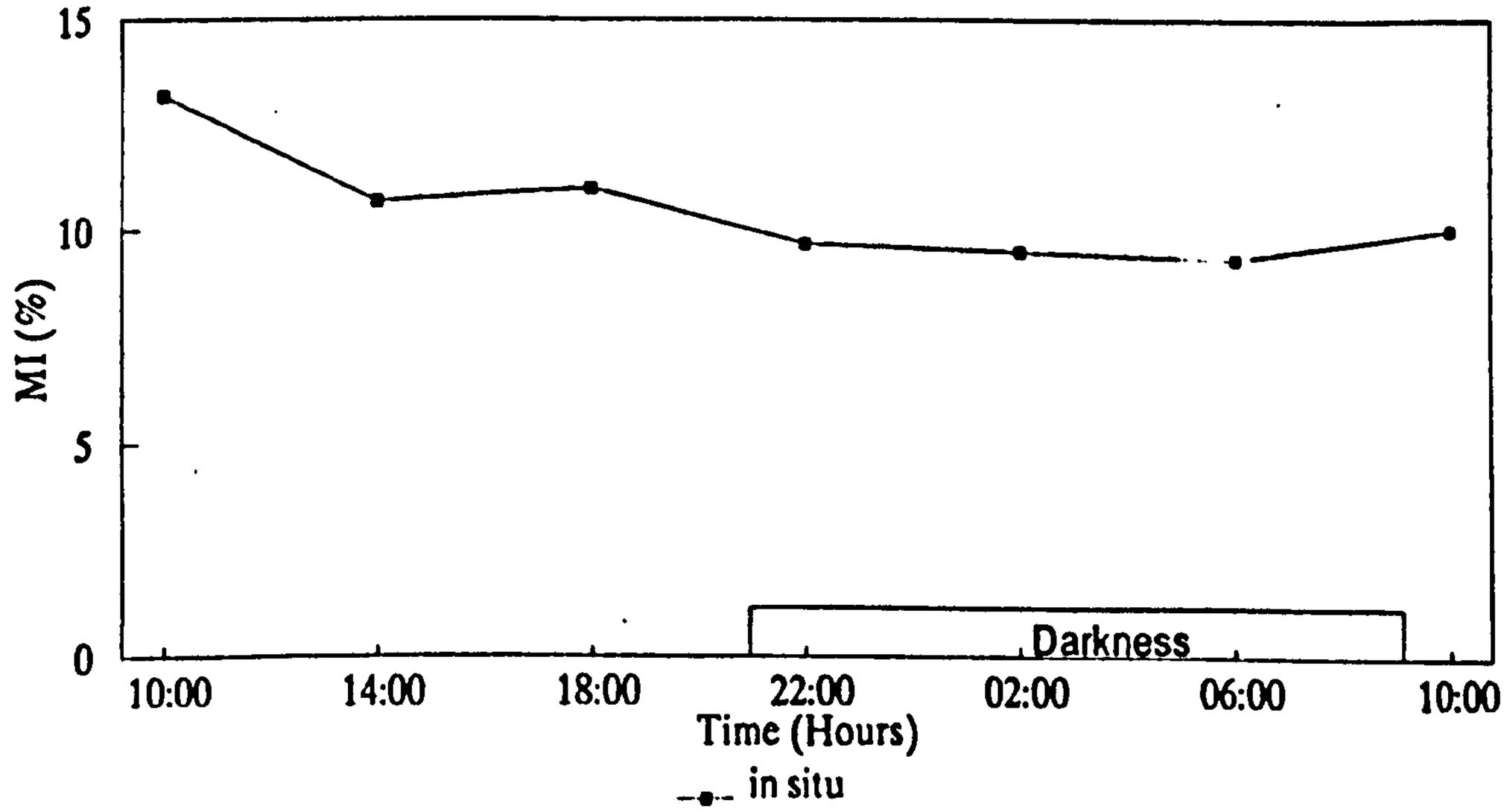


TABLE 3.8 AVERAGE MITOTIC INDICES (MI), SPECIFIC GROWTH RATES (μ) AND DOUBLING TIMES (Dt) OF THE IN SITU AND CULTURED SYMBIONT POPULATIONS

HOST SP.	Average MI *in situ* +/- S.E. (%) (N=14)	Average specific growth rate (μ) *in situ* (day^{-1})	Average doubling time (Dt) *in situ* (days)	Average MI *in vitro* +/- S.E. (%) (N=14)	Average specific growth rate (μ) *in vitro* (day^{-1})	Average doubling time (Dt) *in vitro* (days)
Cp	1.84 +/- 0.11	0.040	17.3	8.93 +/- 0.46	0.293	2.37
CpNt	1.94 +/- 0.15	0.042	16.5	7.44 +/- 0.20	0.246	2.82
Ab	1.43 +/- 0.14	0.031	22.4	8.16 +/- 0.37	0.269	2.58
Is	1.14 +/- 0.06	0.025	27.7	7.63 +/- 0.33	0.252	2.75
Ap	0.89 +/- 0.06	0.019	36.5	2.57 +/- 0.16	0.087	7.97
AvLH	1.49 +/- 0.12	0.032	21.7	-	-	-
AvSI	1.17 +/- 0.07	0.025	27.7	8.16 +/- 0.46	0.269	2.58
AvBm	1.61 +/- 0.13	0.035	19.8	7.57 +/- 0.24	0.250	2.77
AvLS	1.54 +/- 0.11	0.033	21.0	7.04 +/- 0.18	0.233	2.97
AvTB	1.54 +/- 0.17	0.033	21.0	6.96 +/- 0.21	0.230	3.01
AvWH	1.34 +/- 0.15	0.029	23.9	8.10 +/- 0.28	0.267	2.60

3.4.4 Discussion

(a)The phasing of the diel division cycle.

Asynchronisation of diel division cycles is common amongst endosymbiotic dinoflagellates previously investigated. In situ, Symbiodinium spp. populations of the anthozoans S. pistillata (Wilkerson et al. 1983; Muscatine et al. 1989a), A. pulchella, A. elegantissima (Wilkerson et al. 1983) and Z. sociatus (Steen and Muscatine 1984), and the nudibranch Pteraeolidia ianthina (Hoegh-Guldberg et al. 1986) all exhibit asynchronous division patterns. Zamani and Brown (1992) also measured an asynchronous division cycle for the symbionts of A. viridis, however the MI was only measured over a period of 12 hours and so conclusions regarding the diel division pattern of A. viridis cannot be drawn from their data. The asynchronous division patterns of the symbionts of these hosts, and the hosts investigated in this work, are probably related to the relatively constant nutrient concentrations in shallow coastal waters (cf. oceanic waters) (Wilkerson et al. 1983).

Phased division cycles are common amongst phytoplanktonic species (Swift and Durbin 1972; Lewin and Rao 1975; Weiler and Chisholm 1976), but have been reported infrequently amongst endosymbiotic populations. In the field, phased division of symbionts occurs in the jellyfish Mastigias sp. when the host periodically swims into the nutrient rich chemocline, resulting in a peak MI of 11% (Muscatine and Marian 1982; Wilkerson et al. 1983). But evidence of phasing

in the symbiont populations of sessile hosts is less conclusive: Steen and Muscatine (1984) reported "slight" phasing in the division of the symbionts of the zoanthid Palythoa variabilis and Stambler and Dubinsky (1987) concluded that the diel division cycle of the symbionts of A. viridis was phased with a peak of 3 % at midnight. These measurements were made under laboratory conditions and cannot therefore be accounted for by changes in ambient nutrient levels. However, neither of these assumptions of phasing is conclusive, as they are based on just one series of measurements. Results from this work show that diel division cycles which appear phased on one occasion may peak at different times the following day. This research conclusively demonstrated that, like the symbionts of most other Anthozoa, the diel division cycle of the symbionts of A. viridis in situ and under constant environmental conditions is asynchronous.

The asynchronous division patterns of the symbionts of the host species under investigation here were also maintained in culture, when the symbionts were no longer under the possible influence of host control. Hence, it is concluded that the intrinsic pattern of cell division in the symbiont populations of the British Anthozoa and A. pallida is asynchronous.

(b)The mitotic index (MI) in temperate and tropical waters.

Although the MI of symbionts of the coral S. pistillata has occasionally been measured as >3 % (Patton and Burris 1983;

Muscatine et al.. 1989a) (cf. Wilkerson et al. 1983; Muscatine et al. 1984), symbiont division rates in temperate waters have usually been found to be higher than in the tropics. The MI of in situ symbionts of A. pulchella from tropical waters, which are characteristically low in dissolved inorganic nutrients, was reported to be 0.76 %. In contrast, the MI of Symbiodinium sp. from A. elegantissima, from temperate waters with a relatively high dissolved inorganic nutrient concentration, was 4 % (Wilkerson et al. 1983). Symbiodinium sp. freshly isolated from A. viridis from the Mediterranean coast of Israel also possessed a relatively higher MI, which ranged from 0.8 % to 3 % (Stambler and Dubinsky 1987). These authors, using the relevant equation for a phased division cycle, estimated the division time (t_d) for symbionts of A. viridis as 1.5 hours and the doubling time (Dt) as 23.45 days. This value of Dt was similar to the doubling time of 19.8-27.7 days calculated here for the in situ symbionts of A. viridis. In situ MI values of >1 % were measured for the symbionts of all the host species of British Anthozoa in this work. This was in contrast to the average in situ MI of 0.89 % of Symbiodinium sp. in A. pallida. Thus these observations were consistent with those of previous investigations.

The higher MI noted here in some of the temperate symbioses compared to the MI of the symbionts of A. pallida was in spite of all the Anthozoa being maintained in sea water from the same origin and consequently of the same dissolved

inorganic nutrient concentration. In light of this, it is possible that either the host species differentially regulate the growth rates of their symbionts or that the symbionts of different host species possess different intrinsic division rates.

(c)The mitotic index (MI) in culture and evidence for intrinsic differences between the symbionts of different host species.

Whether the significantly lower MI of the symbionts of A. pallida in culture represents an intrinsic difference or a 'strain' specific lack of a vitamin or nutrient is unclear. Due to these same possibilities, it is also difficult to draw conclusions with regard to the the similar MI values of the cultured symbionts of the British Anthozoa. But differences between growth rates of Symbiodinium 'strains' in culture have been concluded as representing intrinsic differences in other studies (Fitt and Trench 1983a).

Previously published specific growth rates (μ) of cultured Symbiodinium spp. have ranged from 0.29-0.39/day (Fitt and Trench 1983a; Domotor 1982, in Muscatine et al. 1984). Therefore the specific growth rates of the cultured British Symbiodinium sp., which ranged from 0.23-0.29/day, approached the lower end of this range.

(d)Possible mechanisms by which symbiont growth is suppressed in situ.

Three mechanisms of symbiont growth control by the host have been proposed to explain the suppression and control of

symbiont growth rate *in situ*: (1)Host determined changes in the percentage of fixed carbon directed into symbiont growth; (2)host limiting of the dissolved inorganic nutrient supply to the symbionts; (3)host chemical inhibition of symbiont mitotic division.

The fixed carbon translocation control hypothesis is based upon the assumption that of the total net carbon added daily to the symbiont population, a fraction is directed into algal growth and the remainder is translocated to the host (Muscatine *et al*. 1983; Muscatine *et al*. 1984). In the *Hydra* - *Chlorella* symbiosis the ability of the host to control algal growth has been linked to the algal capacity for maltose release at pH 4-5, with the host inducing symbiont division by a temporary alkalinization of the perialgal vacuole (Douglas and Smith, D.C. 1984). But, in contrast to the likely situation in anthozoans (Miller and Yellowlees 1989), the perialgal vacuole in *Hydra* sp. has recently been demonstrated not to be acidic, thereby disputing the hypothesis of Douglas and D.C. Smith (1984) (Rands *et al*. 1992a). Experimentally demonstrated links between *Symbiodinium* cell division rates and translocation are limited. Investigating ontogenetic influences on symbiosis carbon flux using the sea anemone *Aulactinia stelloides*, G.J. Smith (1986) revealed that the symbionts divided more rapidly and released less photosynthate in juvenile hosts than in adults.

The supply of dissolved inorganic nutrients to the symbionts

may be limited by the host. Recent investigations have suggested that Symbiodinium spp. cells are phosphorus limited in situ. The relatively high phosphorus-related enzyme activities of freshly isolated Symbiodinium cells and the lower phophorus concentration of the perialgal vacuole than the host cell cytoplasm in corals imply that the host may restrict phosphorus uptake by the symbionts at the perialgal vacuole (Jackson et al. 1989; Jackson and Yellowlees 1990).

Evidence for a host produced mitosis inhibitor does not exist for Symbiodinium - invertebrate symbioses, although a host prey derived division factor has been implicated in the control of Chlorella sp. division by hydra (McAuley 1985).

As is evident from the above examples, little is known of the symbiont growth control mechanism(s) in anthozoans. All the above hypotheses suggest that symbiont growth is under the direct control of the host. But control of division rate by the symbionts themselves cannot be discounted. The higher MI of the symbionts in juvenile A. ballii, where the symbiont population was still establishing itself, than is seen in the adults may reflect density determined self-regulation of division rate by the symbiont population itself. But it is possible that any host inhibition of symbiont divison is relaxed whilst the symbiont population is being established. Higher symbiont division rates in juvenile hosts than in adults have been reported in the jellyfish Mastigias sp. (Muscatine et al. 1986) and the

anemone A. stelloides (Smith, G.J. 1986).

(e)The accuracy of determining the mitotic index (MI) by the 'two-cell stage method'.

The accuracy of estimating the MI from freshly isolated cells using the percentage of cells appearing as doublets, has recently been questioned. The percentage of cells undergoing mitosis was shown to increase when the symbionts of A. viridis were expelled into the coelenteron (Suharsono and Brown 1992). But under non-stressful conditions the numbers of symbionts released by the host are small (Hoegh-Guldberg et al. 1987). As a result, any changes in MI due to symbiont release were negligible in this work. Brown and Zamani (1992) compared the MI of symbionts of A. viridis when measured using the percentage of symbiont cells with 2 mitotic figures (ie. those in karyokinesis) and the percentage of cells appearing as doublets, concluding that the doublet method underestimates the MI by 3 - 5 fold. However, attempts to utilize the recommended method of Brown and Zamani (1992) found the method to be unreliable.

(f)General conclusion.

It is evident that the symbiont populations of the British Anthozoa divide asynchronously and at a low rate, with an in situ MI of >1 % typical of temperate Symbiodinium cells. In culture, the division patterns and rates of Symbiodinium sp. from the different temperate host species showed no differences indicative of the presence of 'strains' of Symbiodinium.

3.4.5 Summary

(i)The mitotic indices (MI) of cultured and *in situ* symbionts of *A. viridis* (from 6 different geographical locations), *A. ballii*, *C. pedunculatus* (Lough Hyne and Netley), *I. sulcatus* and *A. pallida* were measured by estimating the percentage of doublet cells in a sample of 500 at 4 hourly intervals over 24 hours.

(ii)All the symbiont populations divided asynchronously, reflecting the constant concentration of dissolved inorganic nutrients in their environment.

(iii)The MI of symbionts from all the host species were significantly higher in culture than *in situ*. The mechanism of symbiont growth regulation is unknown.

(iv)The MI of the temperate symbionts *in situ* was higher than that of the subtropical symbionts of *A. pallida*, agreeing with previously published MI of temperate and tropical *Symbiodinium* spp. populations.

(v)The average MI of the cultured symbionts of the temperate host species were not significantly different and therefore did not indicate the presence of different strains of *Symbiodinium*.

(vi)The average *in vitro* MI of the symbionts of *A. pallida* was significantly lower than the MI of the temperate symbionts, indicating that *Symbiodinium* sp. from *A. pallida* is different from the symbionts of the British Anthozoa.

(vii)Significant differences were observed between the MI of the symbionts of different temperate host species *in situ*,

suggesting differential degrees of growth limitation.

(viii)Measuring the MI by estimating the percentage of cells undergoing karyokinesis using the Feulgen staining technique was unreliable.

3.5 GENERAL DISCUSSION

It is clear from the data presented that, whilst the symbionts of the British Anthozoa belong to the genus *Symbiodinium*, they may not be genetically identical on an inter- or intra-host specific basis. This conclusion is predominantly based upon morphological criteria in culture. All the British *Symbiodinium* sp. may also be genetically distinct from all other reported anthozoan symbionts because of the possible absence of a motile stage in their life history. The symbionts of the British Anthozoa were all clearly distinct from the symbionts of *A. pallida*. The symbionts of this host species have recently been named as the species *S. bermudense* (Trench unpubl., in Banaszak *et al*. 1993). But no 'type description' of this species has yet been published and so symbionts of *A. pallida* will be continued to be referred to as *Symbiodinium* sp. in this thesis.

Whilst the differences in morphology indicate the presence of different strains of *Symbiodinium* amongst the British Anthozoa, more detailed information at the genetic level is required to clarify the situation. 3-D reconstruction of

symbiont cells, as described by Blank and Trench (1985) and Blank (1987), would enable accurate counts of chromosomes, pyrenoid stalks and chloroplasts, and estimations of the volumes of cellular inclusions. Detailed ultrastructural information obtained from 3-D reconstructions is now an integral part of Symbiodinium classification (Trench and Blank 1987). Schoenberg and Trench (1980a) used starch gel electrophoresis to investigate the isoenzyme patterns of Symbiodinium cells from seventeen host species, identifying 12 symbiont strains. However, these authors focussed on just four isoenzymes and only hesitantly related the electrophoretic properties of the enzymes to genetic relatedness. The most useful method of determining the genetic relationship between algae of the genus Symbiodinium proposed to date is the characterization of small ribosomal subunit RNA (ssRNA) genes. These genes contain sufficient evolutionary information for the measurement of close and distant phylogenetic relationships (Medlin et al. 1988). SsRNA gene sequencing has recently resulted in the naming of a new diatom species previously thought to be a strain of Skeletonema costatum (Medlin et al. 1991) and has successfully been applied to the genus Symbiodinium (Rowan 1991; Rowan and Powers 1991a; Rowan and Powers 1991b). Rowan (1991) and Rowan and Powers (1991a; 1991b) revealed that Symbiodinium cells isolated from 16 cnidarian host species yielded six distinct ssRNA genes. Different individuals of seven species of Caribbean coral were found to contain

genetically different algae. Individuals of the same host species contained identical symbionts, except for 2 species of coral. These contained 2 symbiont ssRNA genes, suggesting the presence of 2 types of algae or a polymorphism within one alga. Certain hosts (eg. Cassiopeia frondosa and A. pulchella) belonging to different taxa were symbiotic with genetically indistinguishable symbionts. Some of the genetic differences between these 'strains' were thought to be large enough to indicate the presence of different species of Symbiodinium. But, as acknowledged by Rowan and Powers (1991b), ssRNA evolves slowly and therefore two symbionts which possess identical ssRNA gene sequences may not in fact be the same. Thus sequencing of ssRNA genes is only useful for identifying genetic differences. For example, S. pilosum and S. microadriaticum possess distinctly different morphologies and yet are indistinguishable with regards to their ssRNA sequences. The application of 3-D ultrastructural studies and ssRNA gene sequencing to the symbionts of the British Anthozoa could prove to be a major step in the elucidation of their genetic relationships with one another and with tropical Symbiodinium spp..

That the symbionts of the British Anthozoa apparently differ on an intra- and inter-host specific basis raises the question of to what degree 'strains' of algae can establish a symbiosis with a host species which is different to the one with which they are associated in the field. This question is addressed in the following chapter.

CHAPTER 4

THE UPTAKE AND PERSISTENCE OF 'HOMOLOGOUS' AND 'HETEROLOGOUS' SYMBIONTS

4.1 Introduction

The specific objectives of this investigation were:

(a) To acquire aposymbiotic Anthozoa.

(b) To investigate whether symbionts released by symbiotic Anthozoa may infect other individual hosts in the field.

(c) To establish if the anemone C. pedunculatus exhibits a specificity for Symbiodinium isolated from other species of British Anthozoa and the sub-tropical A. pallida.

(d) To investigate the uptake of 'homologous' and 'heterologous' symbionts by aposymbiotic C. pedunculatus, with the aim of establishing whether symbiosis specificity is in part determined by surface recognition.

(e) To investigate the persistence of 'homologous' and 'heterologous' symbionts in reinfected aposymbiotic C. pedunculatus, with the aim of establishing if symbiosis specificity is partly determined by intracellular events.

(f) To investigate whether symbiont proliferation in the host was related to the mitotic index (MI).

(g) To investigate the possibilities of labelling symbionts of different origins for 'multiple reinfections' of a host.

(h) To reinfect C. pedunculatus for future carbon budget investigations.

The terms 'homologous' and 'heterologous', which will be

used throughout this thesis, refer to the host and symbionts originally being involved in the same (ie. the same host species from the same location) or different associations respectively.

In tropical waters, the common occurrence of temporarily aposymbiotic anthozoan hosts as a result of "bleaching" (Goreau, T.F. 1964; Jokiel and Coles 1977; Brown and Howard 1985; Gates 1990; Gates et al. 1992) and an 'open' symbiont acquisition system (Kinzie 1974; Trench 1987; Benayahu et al. 1989; Shick 1991) provides opportunity for infection by 'heterologous' symbionts and the establishment of novel symbioses. In contrast, aposymbiotic hosts are very rare in temperate waters; the zoanthid Zoanthus alderi, currently known from just one colony, may in fact be aposymbiotic I. sulcatus (Manuel 1981), and no published records of naturally occurring aposymbiotic individuals of the other species of British Anthozoa exist. Under optimum conditions, anthozoans are believed to 'farm' their symbionts by exocytosis, thereby maintaining a constant symbiont density (Taylor, D.L. 1969c; Steele 1977). Released Symbiodinium cells may then infect a host by either becoming motile, swimming towards their potential host in response to chemical stimuli (possibly ammonia) (Kinzie 1974; Fitt and Trench 1983b; Fitt 1984), or being ingested by an "intermediate host" which is in turn ingested by the anthozoan (Fitt 1984). Infection of hosts by Symbiodinium cells may also occur as a result of photosynthetically

TABLE 4.1 APOSYMBIOTIC HOST SPECIES WHICH HAVE BEEN USED TO INVESTIGATE THE UPTAKE AND PERSISTENCE OF 'HOMOLOGOUS' AND 'HETEROLOGOUS' SYMBIONTS.

Host species	Authors
Cnidaria: Anthopleura elegantissima; Pseudopterogorgia bipinnata; Aiptasia pulchella; Hydra sp.; Aiptasia pallida; Cassiopeia xamachana.	Trench (1971a); Kinzie (1974); Kinzie and Chee (1979); Jolley and Smith,D.C. (1980); Schoenberg and Trench (1980c); Trench (1981); Trench et al. (1981a); Colley and Trench (1983); Fitt and Trench (1983b and c).
Mollusca: Tridacna squamosa	Fitt and Trench (1981)
Platyhelminthes: Convoluta roscoffensis; Amphiscolops langerhansi; Amphiscolops sp.; Haplodiscus sp..	Provasoli et al. (1968); Taylor, D.L. (1971); Trench and Winsor (1987).
Protozoa: Trichosphaerium AM-I-7	Rogerson et al. (1989)

active symbionts being passed in the faecal material of predators of symbiotic hosts (Muller-Parker 1984). Therefore, possibilities arise whereby British symbiotic anthozoans could be infected by symbionts from a different host species. The significant differences in size of the symbionts of different host species and the consistently different ultrastructure of symbionts from I. sulcatus suggest that if 'heterologous' symbionts are ingested they do not persist when in competition with 'homologous' symbionts, indicating specificity (as defined in chapter 1, section 1.2.4 (p.11)) between host and symbiont.

Several species of aposymbiotic host have been used previously to investigate the specificity of amphidinioid and gymnodinioid dinoflagellate-invertebrate symbioses (table 4.1 (p.168)). All these investigations revealed differences between the uptake and persistence of closely related symbionts when inoculated into the same host species, indicating specificity. The mechanism through which cnidarian - algal symbiosis specificity is mediated has been reviewed by Trench (1988). Research investigating the recognition mechanism has concentrated on the possibilities of cell surface recognition (Pool 1975, in Muscatine et al. 1975; Jolley and Smith, D.C. 1980; Trench et al. 1981a; Markell et al. 1992) and recognition based upon intracellular factors (McAuley and Smith, D.C. 1982; Colley and Trench 1983; Fitt and Trench 1983c; Colley and Trench 1985; Markell and Trench 1993). However, it has been

proposed that symbiosis specificity is the result of a series of mechanisms of recognition (Schoenberg and Trench 1980c; Smith, D.C. 1980; Trench 1981). D.C. Smith (1980) suggested seven stages in the colonisation of hosts by symbionts at which selection of symbionts leading to specificity could occur: Transmission; entry; countering host defences; positioning; providing net advantage to the host; surviving in the host nutrient environment; regulation of symbiont population by the host. This research therefore investigated the specificity of aposymbiotic C. pedunculatus with the symbionts of other species of British Anthozoa and A. pallida. It was aimed to determine whether the symbionts exhibited different patterns of uptake and persistence, and whether any observed specificity was determined by surface recognition or intracellular interactions.

4.2 Materials and Methods

(a)Obtaining aposymbiotic Anthozoa

Because aposymbiotic individuals cannot be collected from the field, attempts were made to obtain aposymbiotic C. pedunculatus (Lough Hyne), A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Island), I. sulcatus and A. pallida in the laboratory. To initiate the release of symbionts by temperate hosts, animals were shocked, as suggested by Buchsbaum Pearse (1974) for the temperate A. elegantissima. 10 temperate symbiotic anthozoans, which had been starved for 3 days, were placed in a sea water filled pyrex beaker

positioned in a water bath at 30, 31 and 32 ^{o}C. The sea water was aerated vigorously at all times and a lid placed on the beaker to prevent evaporation. The animals were maintained at the elevated temperature for 48 hours, changing the water after 24 hours. Following this they were returned to 21 ^{o}C in a 'blacked-out' aquarium containing millipore filtered sea water. 20 A. pallida were cold shocked at 4 ^{o}C for 4 hours as suggested for tropical A. pulchella by Muscatine et al. (1991). Otherwise A. pallida were treated as above. All the Anthozoa were fed once every 4 weeks, thereby reducing the heterotrophic input which may be used to maintain the symbiont population in the dark (Steen 1986; D'Elia and Cook 1988; Cook et al. 1988). This method was attempted as heat stress greatly increases the rate of symbiont egestion compared to dark treatment alone (Buchsbaum Pearse 1974). The return of heat stressed Anthozoa to their original ambient temperature is essential for the release of symbionts from the host, permitting ciliary and muscular action to propel symbiont pellets from the pharynx to the external medium (Gates et al. 1992).

Approximately 250 dark treated C. pedunculatus (Lough Hyne) were available at the beginning of this investigation. These anemones had been placed as unshocked juveniles in a dark box three years prior to use. Fresh sea water flowed through the dark box continually at the ambient temperature. Water was not permitted to flow from tanks containing symbiotic anthozoans into the dark box. Following the

experimental use of all these aposymbiotic individuals, approximately 120 juvenile C. pedunculatus were collected from Lough Hyne during August 1992. Freshly collected adult C. pedunculatus, which brood larger numbers of juveniles than laboratory maintained animals (Manuel 1981), were gently squeezed to release the juveniles. These newly released juveniles were placed immediately into a dark box containing sea water, thereby inhibiting light to the already small population of symbionts (Turner 1988). The juveniles were fed every 2 months with Artemia, in addition to food they obtained from the running sea water.

(b)Checks of aposymbiosis prior to experimental use

It was essential to establish the degree of aposymbiocity of the Anthozoa before experimental use. A subsample of two tentacles from each of 10 individuals of dark treated A. viridis, A. ballii and adult C. pedunculatus, and 5 whole polyps of juvenile C. pedunculatus (dark treated I. sulcatus and A. pallida were not available) were squashed under a coverslip on a slide. These were viewed using the x 25 objective of the light microscope. Both normal and epifluorescence illumination were used. Final checks of aposymbiocity before experimental use were made by placing all the anemones to be reinfected, plus 5 additional juvenile C. pedunculatus for subsequent microscopical examination, in 100 ml transparent polystyrene containers filled with filtered sea water (FSW) and maintained at 80 $\mu E/m^2/s$ on a 12 hour light : dark cycle and 21 ^{o}C. These

Anthozoa were fed with Artemia once weekly and the water changed every 2 days. After 2 weeks, the colour of the polyp was noted for any indication of 'browning' due to the presence of Symbiodinium cells, and tentacles or polyps (as appropriate) were squashed and observed as above. Any individual which appeared to continue to possess symbionts was replaced in the 'blacked out' aquaria.

(c)Cross infection between aposymbiotic and symbiotic C. pedunculatus

Five C. pedunculatus, which had been dark treated for 3 years, were placed in each of two 200 ml plastic bowls containing FSW. Another five symbiotic juvenile C. pedunculatus were placed in one of the bowls. The animals were illuminated from above by a warm white fluorescent light at 80 $\mu E/m^2/s$ on a 12 hour light : dark cycle and maintained at 21 ^oC for 2 months. The water was changed every 2 days. After the 2 month period, the previously dark treated polyps (distinct due to their still paler pigmentation) from both the experimental and control treatments were permitted to settle in petri dishes half filled with FSW. The anemones were relaxed by adding an equal amount of 7.5 % $MgCl_2.6H_2O$ in FSW to the sea water in the petri dish. The anthozoans were relaxed for approximately 1 hour, after which they were insensitive to a seeker. The polyps were then squashed and observed using the x 25 objective of the light microscope under normal and epifluorescent conditions to establish whether a symbiont

population had been established.

(d)Use of host tissue maceration to investigate the phagocytosis of reinfected symbionts

Because of the inability to observe whether inoculated symbionts have been phagocytosed by the host or are only in the coelenteron, a method of dissociating the endodermal cells was sought. Firstly, the method of David (1973) for the maceration of hydra cells was employed. Maceration fluid composed of glycerin : glacial acetic acid : distilled water (1:1:13) was made up. A juvenile symbiotic C. pedunculatus was placed in 0.5 ml of macerating fluid in a test tube for approximately 1 hour. Following gentle shaking, the animal cells dissociated after this period. A drop of the cell solution was then observed under the light microscope with the x 40 objective and the symbionts and associated endodermal cells observed. Secondly, a method to stain the animal nucleus of the endodermal cell around the symbiont was investigated. The method, as described by Gates and Muscatine (1992), involved the maceration of the animal in 0.5 ml 0.05 % trypsin (Sigma Chemical Co.) in calcium-free sea water for 1 hour, with periodic shaking. The resultant cell suspension was then pipetted onto a slide and a coverslip overlaid. Following this, the sample was stained for 30 minutes with 0.04 ml of DNA specific fluorochrome Hoechst 33258 (bisBenzimide) solution (5 mg/ml distilled water) in 9.96 ml 0.1 M sodium phosphate buffer with 3 % sodium chloride and 0.004 % calcium chloride. The sample was

observed under epifluorescence at 450-490 nm, which was expected to result in the stained nuclei fluorescing blue.

(e)Estimate of the symbiont uptake saturation density by aposymbiotic C. pedunculatus

Aposymbiotic 'juvenile' C. pedunculatus (Lough Hyne) were used as the experimental hosts for the continuation of the reinfection work because of the inability to obtain aposymbiotic hosts of other species (chapter 4, section 4.3 (a) (p.183-184)) and the large available numbers of juvenile C. pedunculatus. Due to host size influencing the numbers of symbionts phagocytosed and persisting (Trench et al. 1981a), it was ensured that all aposymbiotic C. pedunculatus used experimentally were of similar size. The parameter of oral disc diameter was used as an indication of size as suggested by Trench et al. (1981a) and Colley and Trench (1983). Oral disc area was linearly related to animal biomass, with, for example, a two-fold increase in disc area being accompanied by a two-fold increase in polyp protein content (appendix 7 (pp.489-494)). All aposymbiotic animals used had oral disc diameters of approximately 2-3 mm.

The saturating density and volume of reinfecting symbionts was established using 'homologous' symbionts. The anthozoans used here were from the completely aposymbiotic 3 year dark treated population. 5 aposymbiotic C. pedunculatus were settled in each of 5, 100 ml transparent polystyrene containers containing 90 ml FSW for 2 days at 21 ^{o}C. The anemones were illuminated at 80 $\mu E/m^2/s$ on a 12 hour light :

dark cycle. Symbiodinium sp. was freshly isolated from symbiotic C. pedunculatus by homogenizing excised oral discs in 10 ml FSW in a glass tissue grinder and centrifuging for 10 minutes at x 1200 r.p.m.. The algal pellet was then resuspended in 5 ml FSW and centrifuged for 5 minutes at x 1200 r.p.m.. The resultant pellet was adjusted to give a final volume of 1 ml wet packed cells in the graduated centrifuge tube. Using a Fuch's Rosenthal haemocytometer, the cell density of this pellet was estimated to be 10^8 cells/ml. The wet packed cells were then diluted 10, 100 and 1000 fold with FSW to give a final volume of 1 ml. Therefore the approximate final densities of these suspensions were 10^7, 10^6 and 10^5 cells/ml. Using a 1 ml hypodermic syringe, 0.1 ml of a different density algal suspension was then pipetted directly onto the oral discs of the aposymbiotic C. pedunculatus in each of the 4 experimental containers, until all 1 ml had been used. The animals in the fifth container were the control. Once the animals had expanded again, 0.1 ml of a heavy Artemia suspension was pipetted onto each oral disc (including the control animals) to induce phagocytosis of the symbionts; symbiont rejection by the host was observed when reinfection occurred without Artemia (pers. obs.). Whilst the anemone's tentacles were withdrawn, the sea water in the containers was replaced with fresh FSW, thereby removing Artemia and algae that had not been caught by the polyps. The inoculated C. pedunculatus were then left for 4 hours (Colley and Trench 1983). Following this period,

the water in the containers was decanted to remove any egested material, and the containers were half filled with fresh FSW. Once the animals had fully expanded, 7.5 % $MgCl_2$ in FSW was slowly added to the containers until they were full. The animals were relaxed in this solution for 1 hour. Each animal was then placed on a slide and its oral disc diameter (not including the tentacles) was measured using an ocular micrometer on the x 10 objective of the light microscope. Since neither of the endodermal cell maceration techniques were satifactory (chapter 4, section 4.3 (c) (pp.185-186)), the polyps were then dissected by cutting longitudinally and washing out any remaining algal cells or Artemia in the coelenteron with a seeker and pipette. Finally the polyps were homogenized in 1 ml FSW and the algal number estimated using the Fuch's Rosenthal haemocytometer. The density of symbionts (symbionts/mm oral $disc^2$) was compared using the Kruskal-Wallis test and multiple comparisons to locate the inoculation density at which uptake is saturated. These statistics, and all others discussed in this chapter were performed at the 5 % level.

(f)Uptake and short term persistence of 'homologous' and 'heterologous' symbionts by aposymbiotic C. pedunculatus: An investigation of host-symbiont surface recognition

The aposymbiotic C. pedunculatus used here had only been dark treated for 5 months. Consequently, occasional residual symbionts were observed in squashes. Because of the short term nature of the measurements and the high density of

symbionts being inoculated into the host animal, the residual symbionts were not expected to influence the results.

5 aposymbiotic C. pedunculatus were settled in each of 21, 100 ml polystyrene containers under the same conditions as used previously. As a consequence of the findings of the saturation density investigation, 0.2 ml wet packed cells were isolated from C. pedunculatus (Lough Hyne), A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Island), I. sulcatus and A. pallida using the homogenization and centrifugation procedure described in the preceding section. To account for any symbiont differences within hosts of the same species, 5 tentacles from 10 individuals of A. viridis and A. ballii, 3 complete oral discs of C. pedunculatus, 30 polyps of I. sulcatus and 10 complete A. pallida were collected for symbiont isolation. The resultant pellets were resuspended in 1 ml FSW to give a final concentration of 2 x 10^7 cells/ml. This concentration was twice the approximate saturation density when reinfected with Symbiodinium cells isolated from C. pedunculatus. Thus, the different volumes of the symbiont cells isolated from the different host species, and consequently the different numbers of Symbiodinium cells that were present in the algal pellets, were accounted for. The aposymbiotic C. pedunculatus were reinfected as described in the previous section, resulting in 3 containers each containing 5 aposymbiotic Anthozoa reinfected with symbionts from one of the 6 original host

species. The animals in the final 3 containers were fed Artemia but were not reinfected and therefore acted as controls. In addition to the FSW changes immediately after feeding with Artemia and after 4 hours, the water was changed after 2 days. There was no possibility of water movement and hence cross infection between host animals between different containers. 5 replicates of each reinfection and 5 control animals were sampled after 4 hours, 2 days and 4 days, and the symbiont densities (cells/mm oral $disc^2$) estimated as in the investigation of the uptake saturation density. In order to compare the biomass of cells phagocytosed and persisting, the number of cells was multiplied by the average cell carbon content determined in chapter 3 (table 3.6 (p.133)) to give pg cell carbon/mm oral $disc^2$. Significant tests (one-way ANOVA followed by Tukey's test or the Kruskal-Wallis test followed by multiple comparisons where appropriate) were performed between the densities and biomasses of symbionts from different host species present in the reinfected Anthozoa at each time interval.

(g)Long term persistence of 'homologous' and 'heterologous' symbionts by aposymbiotic C. pedunculatus

Aposymbiotic C. pedunculatus used for long term persistence work had been dark treated for 3 years and therefore contained no symbionts. Aposymbiotic animals were prepared and reinfected exactly as described for the uptake and short term persistence investigation, with the exception that 4

containers, each with 5 replicate animals, were required for each of the 6 different reinfections and the control. The water was changed every 2 days and the animals were fed weekly with Artemia. The symbiont densities and biomasses were estimated and analysed as described before (except that it was not necessary to wash out the coelenteron) at 2, 4, 6 and 8 weeks.

(h)Reinfection of aposymbiotic C. pedunculatus for carbon budget work

Animals used here had been dark treated for 3 years and were completely aposymbiotic. C. pedunculatus were reinfected as before, with 17 individuals being reinfected with symbionts from each host species and 5 control animals remaining uninfected. The reinfected animals were maintained under the same environmental conditions and feeding regime as described for the long term reinfected C. pedunculatus. After 9 months, 5 replicate animals were used for radioisotopic work, 5 for respirometry and 7 for measurements of the symbiont mitotic index (MI). The MI was measured by counting the number of dividing symbionts in a total of 500 cells, in 1 animal every 4 hours over a 24 hour period. The average cell carbon content of the 'heterologous' and 'homologous' symbionts 9 months after reinfection was evaluated simultaneously with the MI measurements: The average diameter of 100 cells from a total of 5 replicates of each reinfection was measured, and equation 3.1 applied (chapter 3, section 3.3.2 (f)

(p.123)). The symbiont density (symbionts/mm oral disc^2) and symbiont biomass (pgC/mm oral disc^2) in the animals involved in the respirometry work were subsequently determined. The 'homologous' symbiont density and MI after 9 months were compared to the symbiont density and MI in similarly sized (2-3 mm), permanently symbiotic C. pedunculatus. The symbiont density and MI in these animals were estimated using the same sample sizes and methodologies as applied to the reinfected C. pedunculatus. The differences between the densities, biomasses and MI of 'homologous' and 'heterologous' symbionts in C. pedunculatus were compared using one-way ANOVA and Scheffe's test.

(i)Measurement of MI in 'homologous' and 'heterologous' reinfections after three weeks

As a result of the similar MI of the 'homologous' and 'heterologous' symbiont populations after 9 months (chapter 4, section 4.3 (g) (pp.198-199)) and their differing rates of symbiosis establishment (chapter 4, section 4.3 (e)(f) (pp.187-198), the MI of a 'homologous' and a 'heterologous' symbiont population were measured 3 weeks after the reinfection of aposymbiotic C. pedunculatus (3 year dark treated). The 'heterologous' symbionts were isolated from A. viridis (Shell Island) due to the symbionts from this host being the slowest to establish a stable symbiosis with C. pedunculatus (cf. the rapid establishment of the symbiosis with 'homologous' symbionts). It was therefore possible to investigate whether the comparatively

slow establishment of Symbiodinium sp. from A. viridis (Shell Is.) was, in part, because of a low MI and whether the MI changed over time in the host cell environment. 7 aposymbiotic C. pedunculatus were reinfected with each symbiont 'strain' and maintained for 3 weeks as described previously. Following this period, the MI was measured using 1 animal every 4 hours as described earlier. The average MI was compared with the MI of the same 'strain' in the reinfected C. pedunculatus after the 9 month period, and between these 2 'strains' after the 3 week period using Student's t-test.

(j)Investigation of methods of vital labelling of symbionts for 'multiple reinfections'

A vital labelling technique to observe competition between symbionts isolated from different host species when reinfected into the same host animal was investigated.

The cellulose stain 'Cellufluor' (Calcofluor White M2R) (Polysciences, Inc.), which has been used previously to distinguish between infected symbionts and the original population (McAuley and Smith, D.C. 1982), was used to label C. pedunculatus symbionts. An oral disc of a symbiotic C. pedunculatus was excised, homogenized and centrifuged once at x 1200 r.p.m. for 10 minutes. The resultant pellet was then resuspended in 10 µg/ml 'Cellufluor' in distilled water for 1 minute, maintaining the sample in the dark, and then centrifuged again at x 3000 r.p.m. for 5 minutes. The algal pellet was resuspended in FSW and a subsample observed with

a Leitz Orthoplan light microscope under epifluorescence at 345-400 nm. The stained cell walls were expected to fluoresce blue.

A second fluorescent stain, 'Fluorescein' (sodium salt) (Sigma Chemical Co.) was also used to stain isolated symbionts. The symbiont isolation and staining procedure was as above, using 10 µg/ml 'Fluorescein' in distilled water. The stained cells were again observed under epifluorescence at 400 nm and were expected to fluoresce white.

4.3 Results

(a) Acquisition of aposymbiotic Anthozoa

The most successfully achieved aposymbiotic species of Anthozoa was C. pedunculatus (plate 4.1 A (p.184)). C. pedunculatus proved to be an ideal experimental animal because of the large numbers of juveniles that could be obtained and the small symbiont population of each juvenile. Heat stressing juveniles frequently resulted in death of the animals. But, placing the juveniles in the dark rendered C. pedunculatus almost aposymbiotic after 5 months and completely aposymbiotic after 2 years.

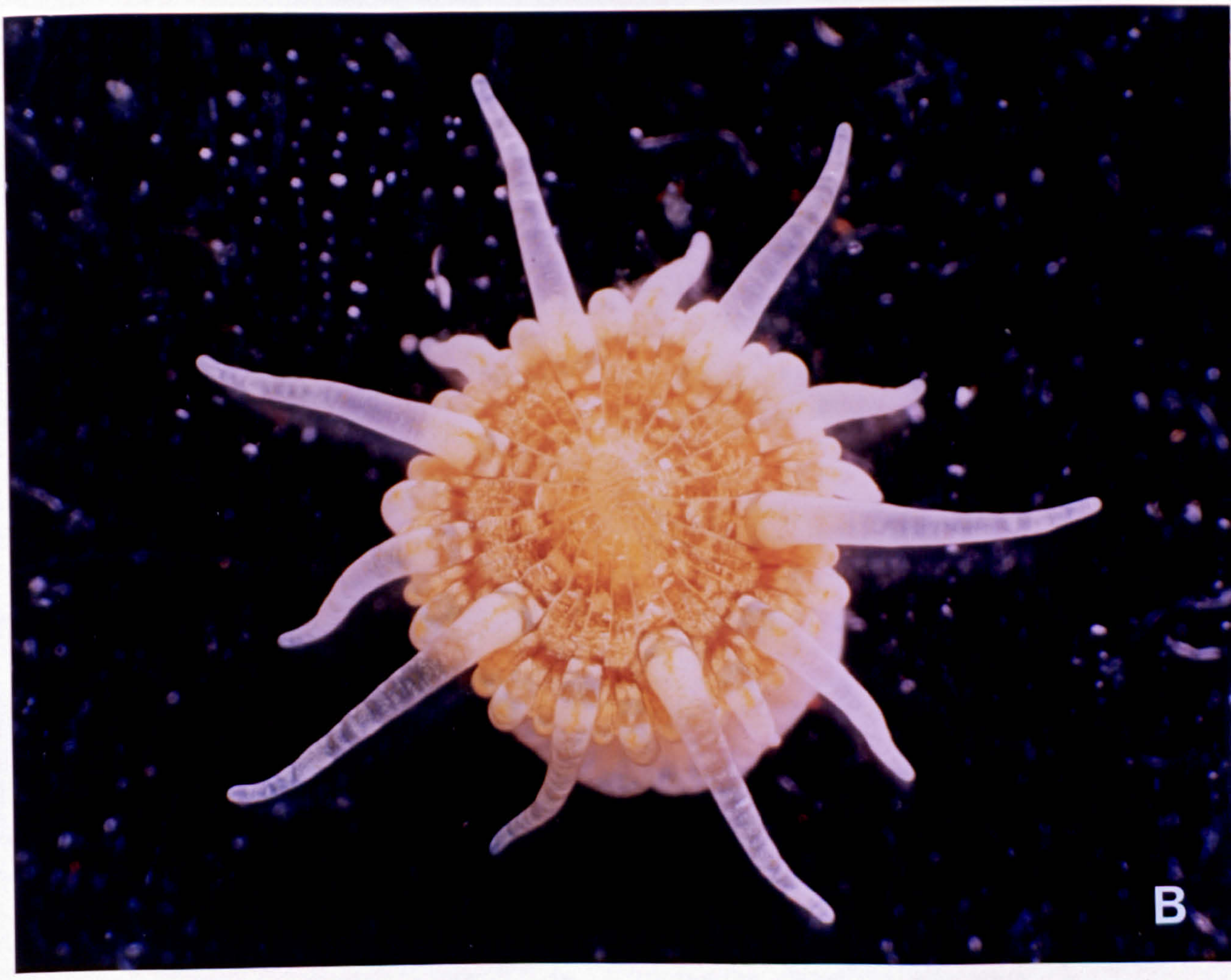
Frequent death of all host species heat shocked above 30 ^{o}C occurred. Animals shocked at 30 ^{o}C or A. pallida shocked at 4 ^{o}C survived and were observed to release clumps of symbionts (identified under the light microscope) wrapped in mucus during the temperature treatment. Within several months of being placed in the dark most A. viridis and all

Plate 4.1: Aposymbiotic and Reinfected C. pedunculatus.

(A) Aposymbiotic C. pedunculatus (after 3 years in the dark) (scale bar = 1.0 mm).

(B) Formerly aposymbiotic C. pedunculatus 9 months after reinfection with symbionts isolated from A. viridis (Shell Island). Note brown colouration (cf. plate 4.1 (A)). (scale bar = 1.0 mm).


A


B

I. sulcatus and A. pallida died, although loss of colouration due to expulsion of symbionts was evident. A. ballii survived in most cases and approached aposymbiocity after 8 months. When placed in the light however, all dark treated A. ballii reestablished large symbiont populations within 2 weeks. The dark treated A. ballii and surviving A. viridis are still being maintained in the laboratory for future investigation.

(b)Cross infection between symbiotic and aposymbiotic C. pedunculatus

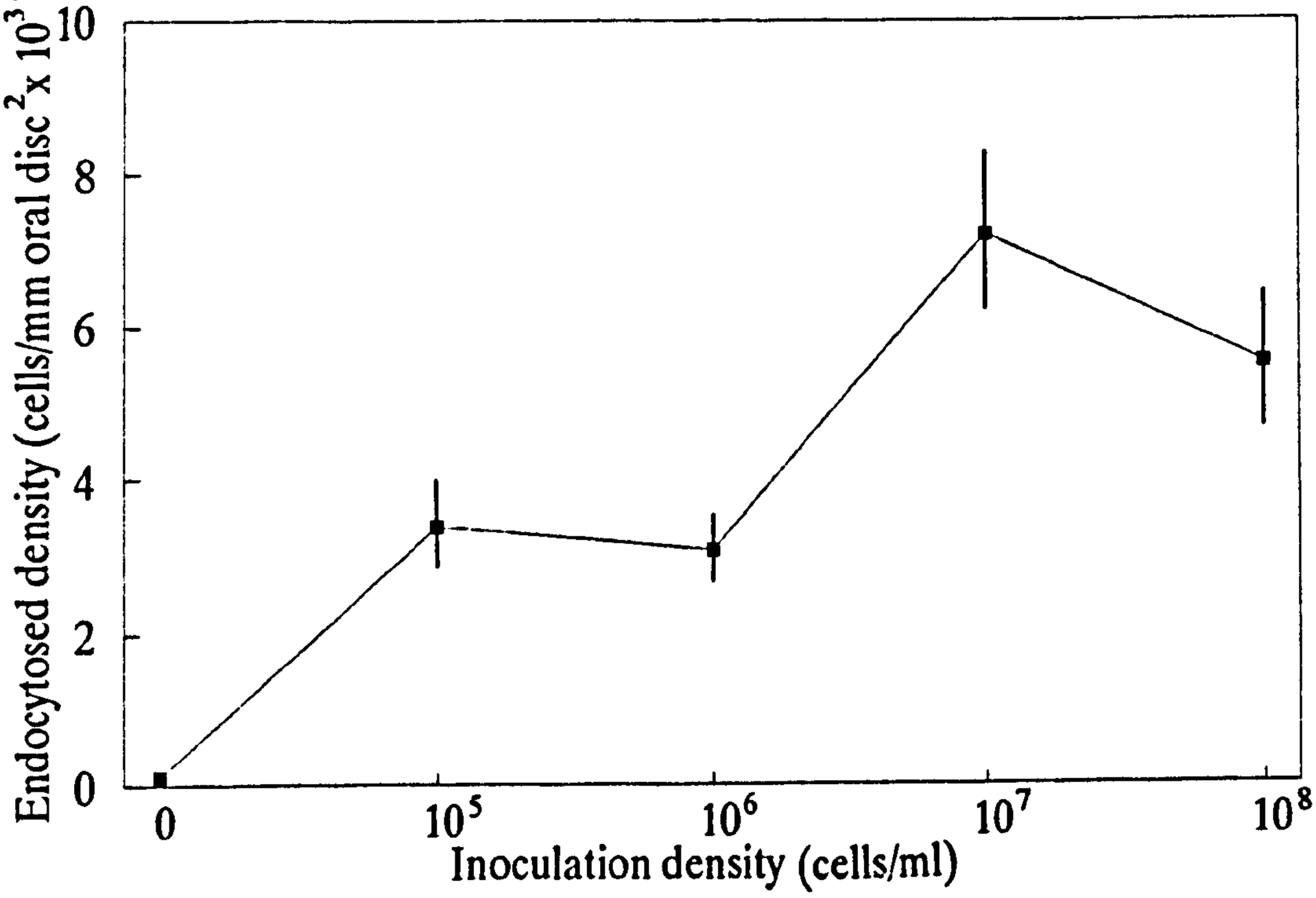
All previously aposymbiotic C. pedunculatus in the vicinity of symbiotic individuals were brown in colour after 2 months. When squashed and viewed under the light microscope all these previously aposymbiotic Anthozoa clearly possessed a dense population of symbionts within their endoderms. In contrast, the control aposymbiotic anemones still appeared completely white and no symbionts were evident under the light microscope.

(c)Use of host tissue maceration to establish phagocytosis of symbionts

Simple host maceration using the method of David (1973) enabled the clear observation of component cells. But it appeared that there was only 1 symbiont cell per endodermal cell, with the symbiont cell being very tightly bound by the endodermal cell. Thus, in most cases the presence or absence of the endodermal cell enclosing a symbiont was difficult to establish. The nuclear staining technique of Gates et al.

Graph 4.1: The saturation of Symbiodinium cell uptake by aposymbiotic C. pedunculatus

(+/- S.E.) (N=5).



(1992) was used in an attempt to elucidate this matter by identifying the animal nucleus in close proximity to the symbiont cell. Endodermal cell nuclei fluorescing blue were clearly visible on several occasions. Because the visibility of the animal cell nucleus depended on its position relative to the symbiont cell, it was decided that the method could not be applied to quantitative measurements of phagocytosis.

(d)Estimation of the saturation density of symbiont uptake

The number of symbionts phagocytosed by anemones exposed to different inoculation densities were significantly different ($H=9.08$, $P=0.029$), although multiple comparisons did not reveal specific significant differences (appendix 8, table 1 (p.496)). It was evident that symbiont uptake when inoculated with 10^5 and 10^6 Symbiodinium cells/ml was approaching being significantly lower than uptake when inoculated with 10^7 cells/ml. Therefore, uptake saturation appeared to occur when between 10^6 and 10^7 'homologous' symbionts/ml were available. The different densities of symbionts present in the anemones after 4 hours of maintenance in different density algal suspensions are shown in graph 4.1 (p.186).

(e)Uptake and short term persistence of 'homologous' and 'heterologous' symbionts

The densities of symbionts present in the reinfected C. pedunculatus following initial uptake and after 2 and 4 days, and in the control animals over the same period, are given in appendix 8, table 2 (p.496). These values are

illustrated in graphs 4.2-4.8 (p.190).

The densities of the different strains of Symbiodinium (cells/mm oral disc^2) were significantly different after 4 hours ($F=3.72$, $P=0.012$) and 2 days ($H=15.46$, $P=0.009$) but were not significantly different after 4 days ($F=2.17$, $P=0.092$). Results of the more detailed statistical comparisons between the cell densities of the different 'strains' of symbionts at each time interval are shown in appendix 8, tables 3 and 4 (pp.497-498). From these tables it is evident that, with the exception of symbionts originally isolated from A. ballii which were endocytosed to a lesser extent than symbionts from I. sulcatus, similar numbers of all the other Symbiodinium 'strains' were phagocytosed by similarly sized animals.

However, after 2 days the densities of symbionts from A. viridis (Shell Is.) and I. sulcatus were significantly lower than the density of 'homologous' symbionts, and the densities of symbionts from A. ballii and A. viridis (Lough Hyne) were approaching being significantly lower than the 'homologous' symbionts.

Despite the densities of the 'heterologous' and 'homologous' symbiont populations not being significantly different after 4 days, graphs 4.2-4.8 (p.190) indicate that the 'homologous' symbionts were still persisting at a higher density.

The uptake and persistence over 4 days of 'homologous' and 'heterologous' symbionts, expressed in terms of symbiont

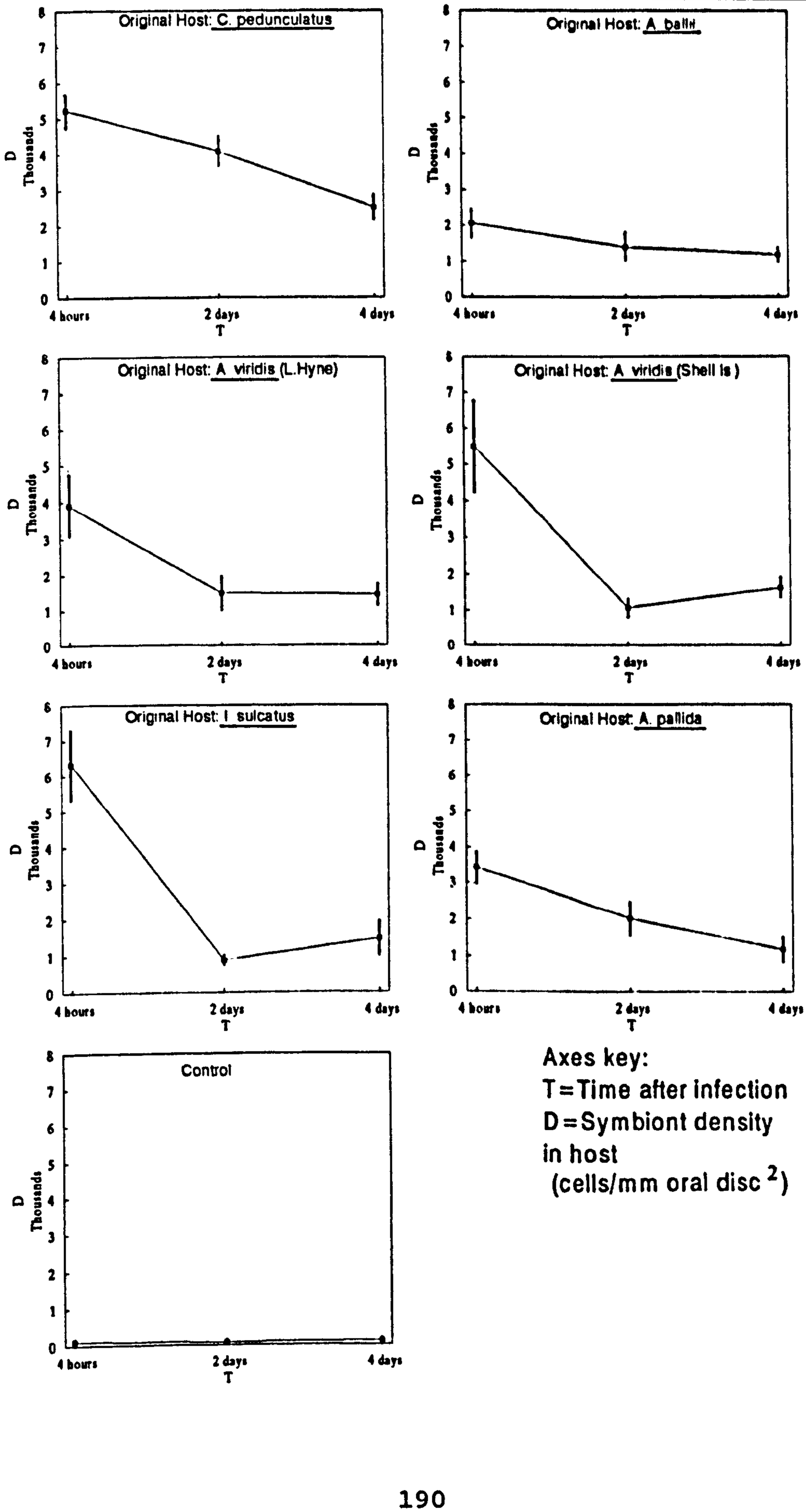
biomass, is given in appendix 8, table 5 (p.498). These values are illustrated in graphs 4.9-4.15 (p.191).

When the uptake of 'homologous' and 'heterologous' symbionts was compared with respect to biomass (pg cell C/mm oral disc^2), uptake of Symbiodinium cells from different origins was again significantly different (H=15.14, P=0.01). The biomass of endocytosed symbionts originally from I. sulcatus was significantly greater than the biomass of endocytosed symbionts originally isolated from A. pallida (appendix 8, table 6 (p.499)).

Significant differences between the biomasses of the 'homologous' and 'heterologous' symbiont populations were present after 2 days (H=13.72, P=0.018), with the biomass of persisting 'homologous' symbionts being significantly greater than the biomass of persisting symbionts originally isolated from A. viridis (Lough Hyne). The biomass of persisting 'homologous' symbionts was also approaching being significantly greater than the biomass of persisting symbionts originally isolated from A. pallida and A. viridis (Shell Is.) (appendix 8, table 7 (p.499)).

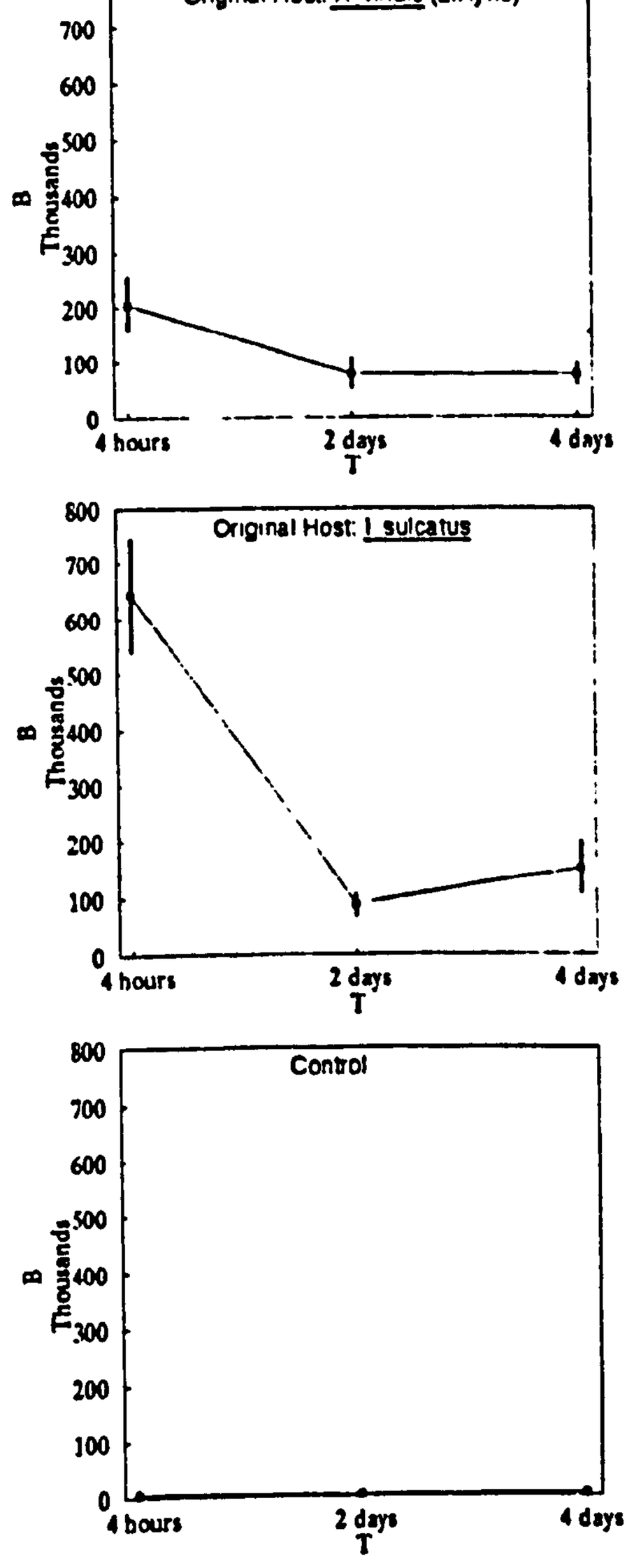
4 days after reinfection the biomass of all 'homologous' and 'heterologous' symbionts present was not significantly different (F=1.85, P=0.145).

Graphs 4.2 – 4.8: Densities of 'homologous' and 'heterologous' symbionts over 4 days following endocytosis by aposymbiotic C. pedunculatus. (N=5) (+/– S.E.).



Axes key:
T = Time after infection
D = Symbiont density in host (cells/mm oral disc ^{2})

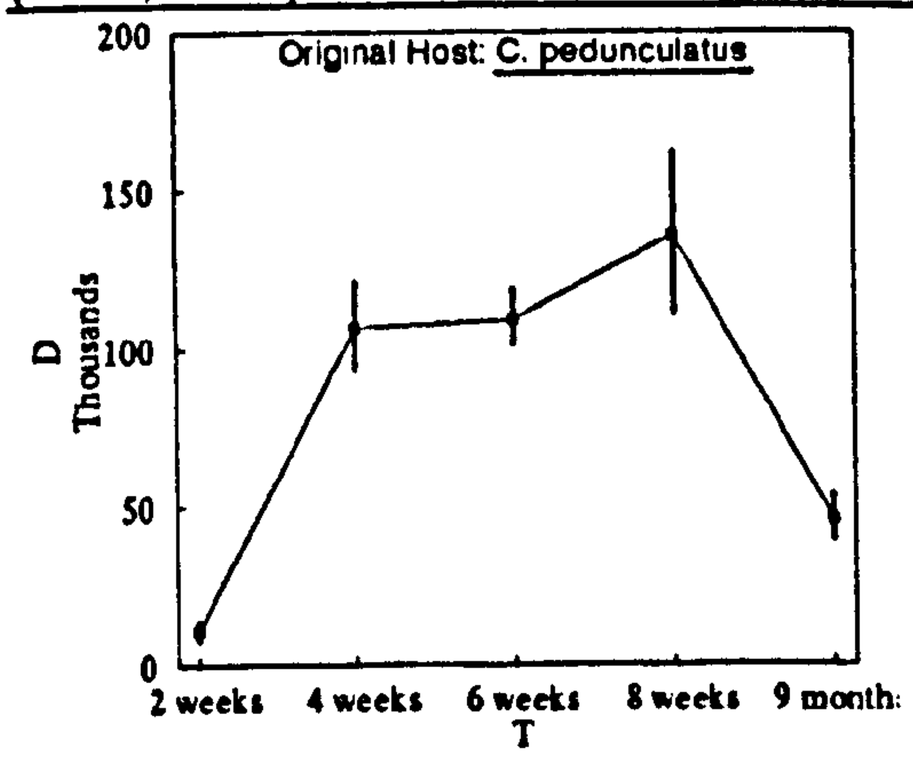
Graphs 4.9 – 4.15: Biomass of 'homologous' and 'heterologous' symbionts over 4 days following endocytosis by aposymbiotic C. pedunculatus. (N=5) (+/- S.E.).

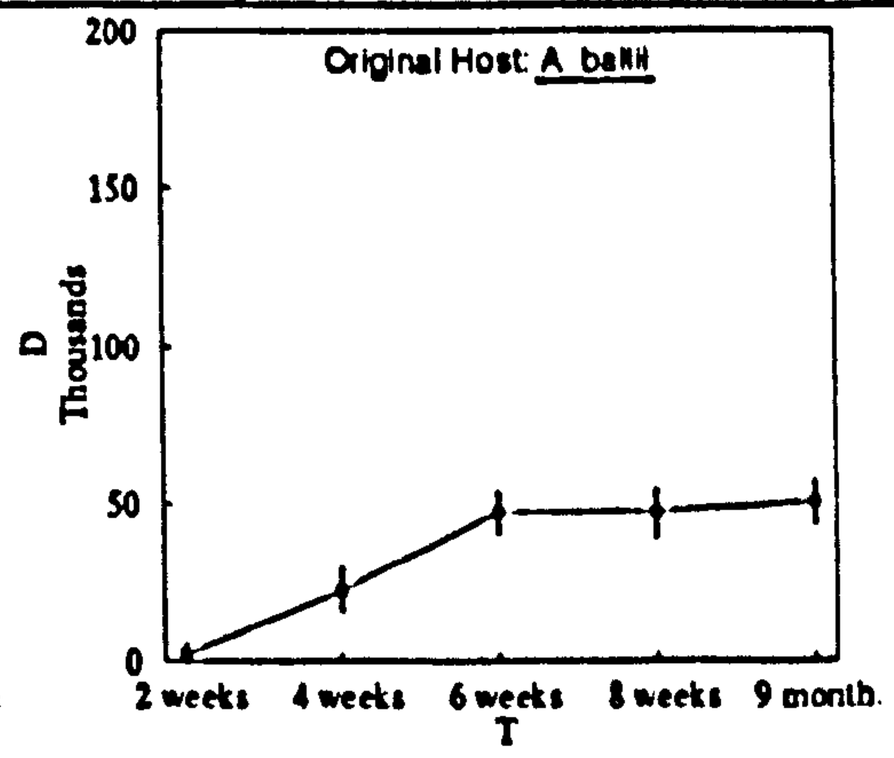


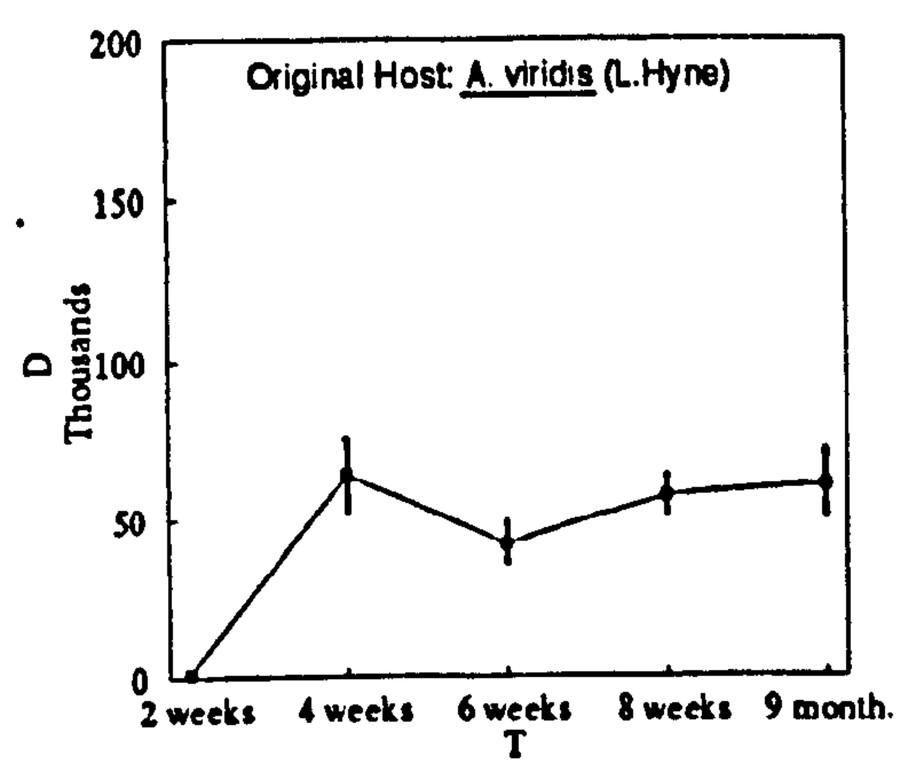
Axes Key:
T = Time
B = Biomass of symbionts endocytosed and persisting (pg cell carbon /mm oral disc ^{2})

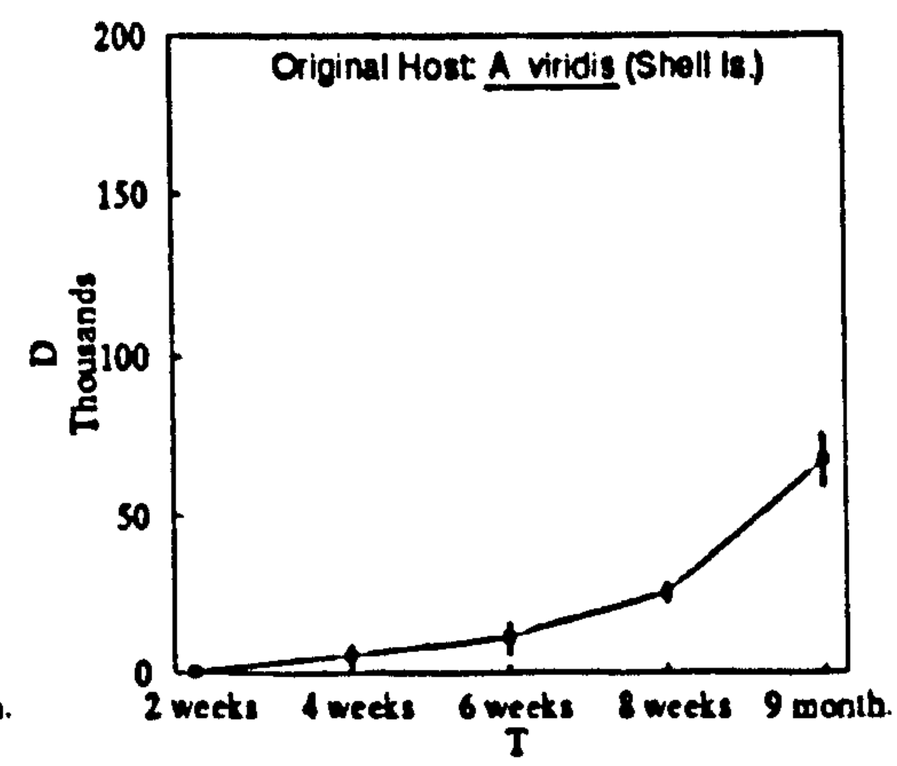
Graphs 4.16–4.22: Densities of 'homologous' and 'heterologous' symbionts over 9 months following endocytosis by aposymbiotic C. pedunculatus.

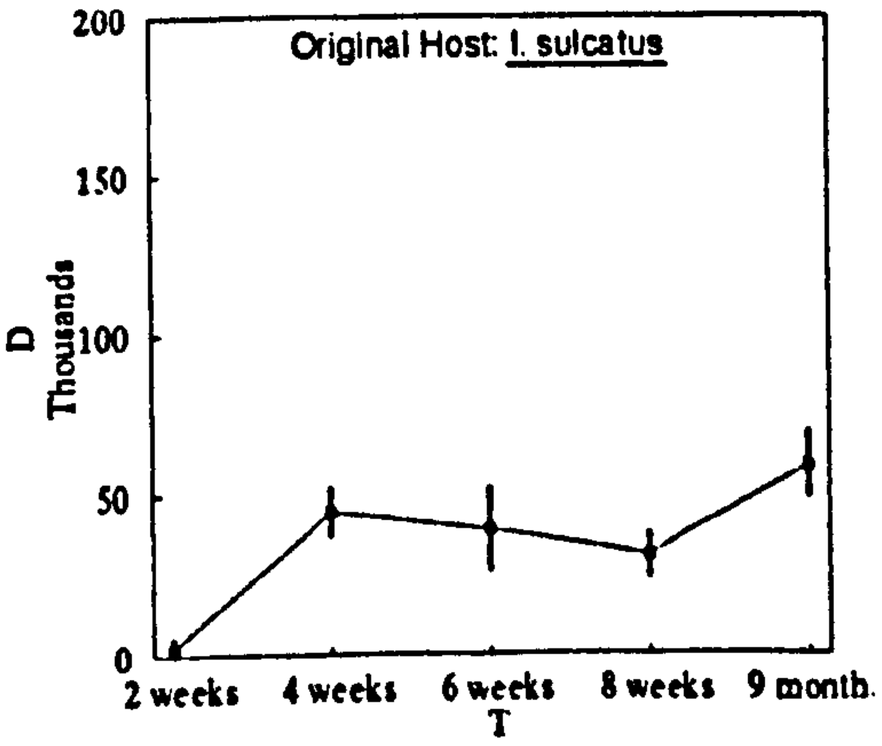
(N=5, except N=4 for A.ballii and I. sulcatus symbionts after 9 months) (+/– S.E.).

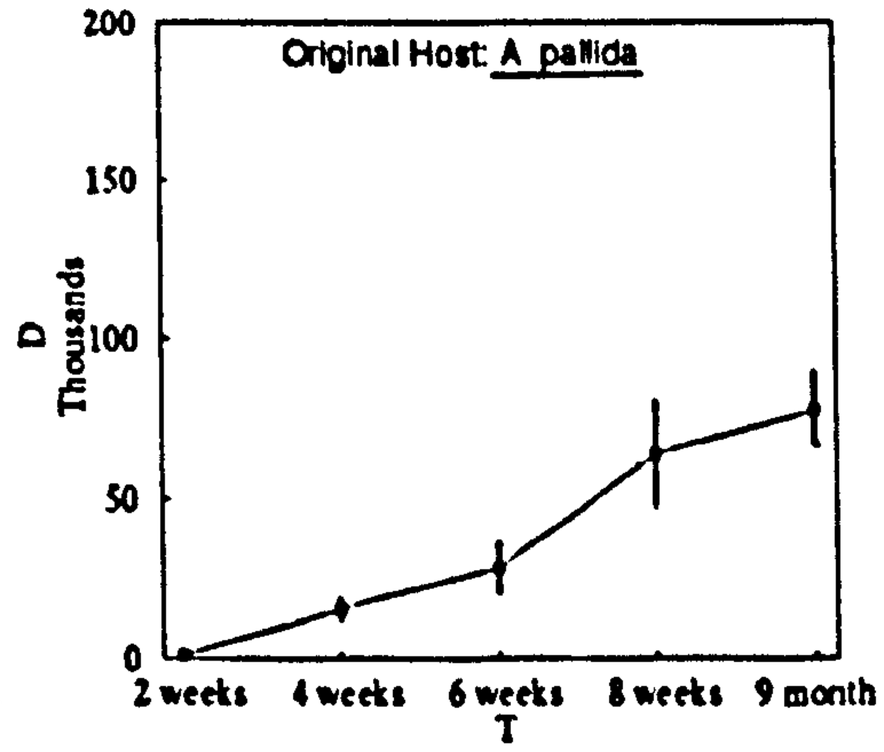


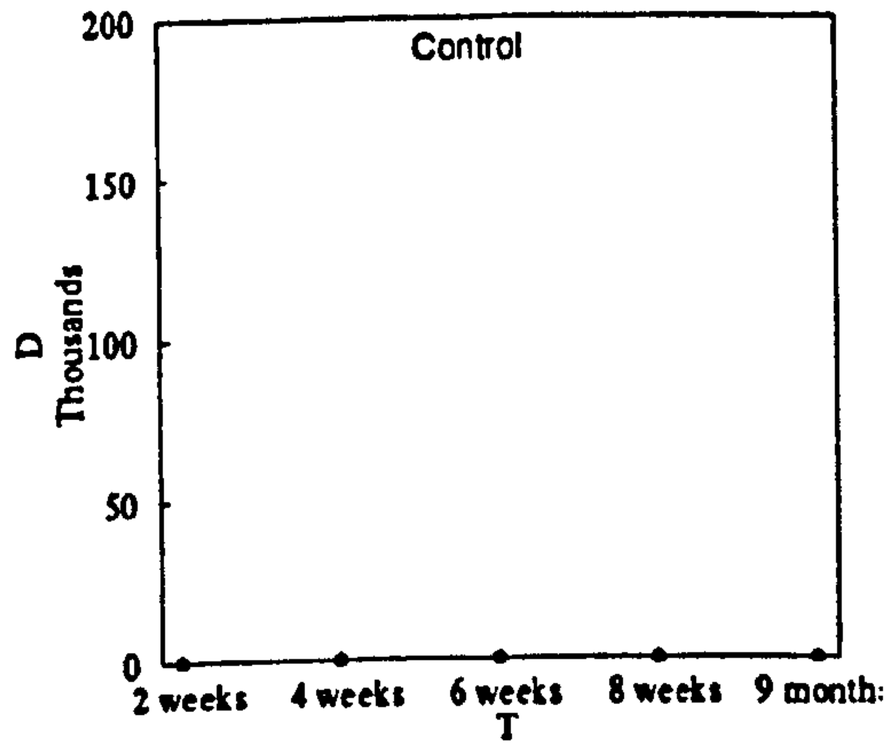








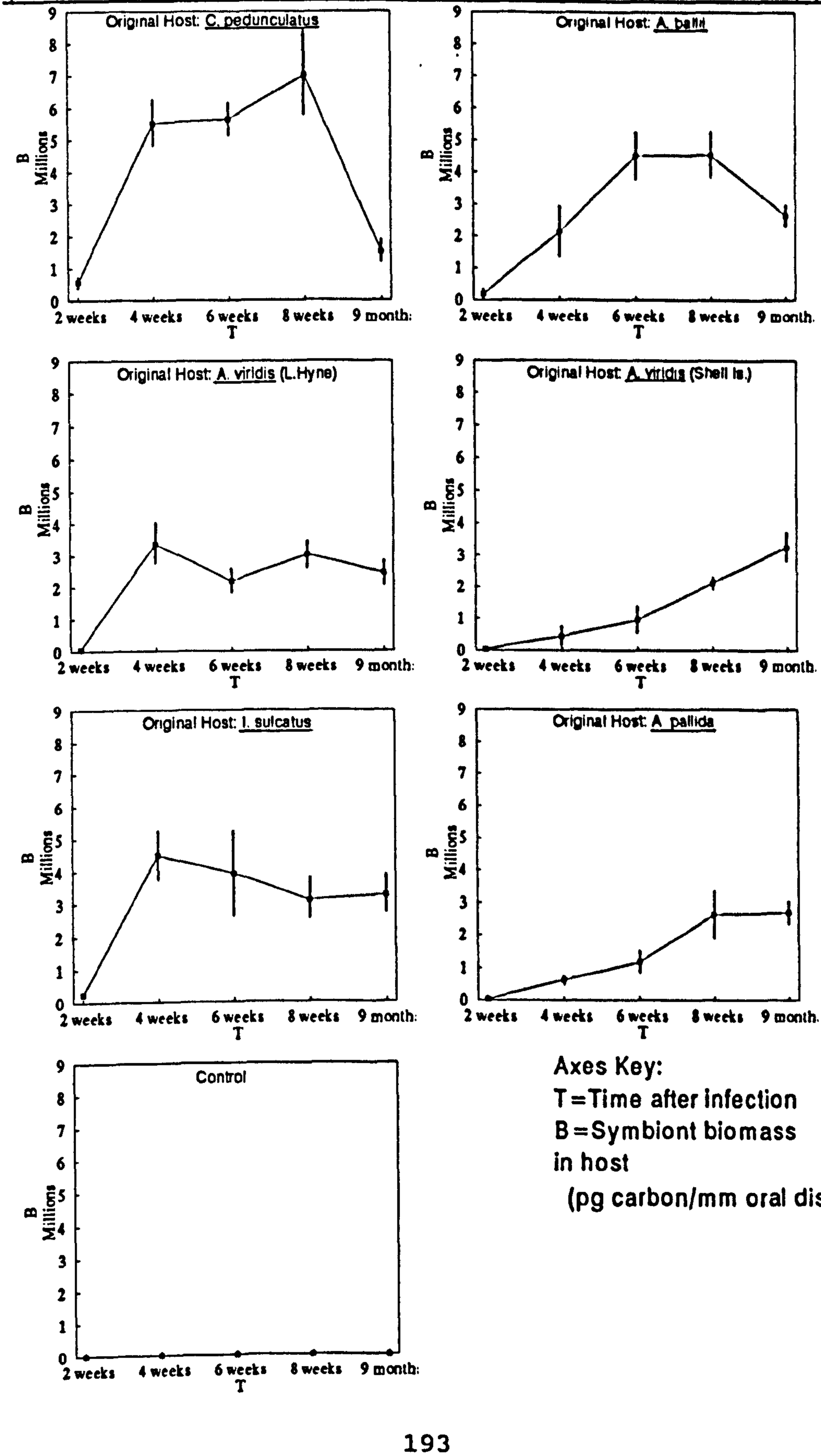




Axes Key:
T=Time after infection
D=Symbiont density in host
(cells/mm oral disc ^{2})

Graphs 4.23 – 4.29: Biomass of 'homologous' and 'heterologous' symbionts over 9 months following endocytosis by aposymbiotic C. pedunculatus.

(N = 5, except N = 4 for A. ballii and I. sulcatus symbionts after 9 months) (+/– S.E.).



Axes Key:
T = Time after infection
B = Symbiont biomass in host
(pg carbon/mm oral disc ^{2})

(f)Long term persistence of 'homologous' and 'heterologous' symbionts

The population densities of 'homologous' and 'heterologous' symbionts infected into C. pedunculatus after 2, 4, 6 and 8 weeks and after 9 months, which are given in appendix 8, table 8 (p.500), are illustrated in graphs 4.16 - 4.22 (p.192).

The precise pattern of events occurring between 4 days and 2 weeks, with respect to changes in the reinfected symbiont density, could not be ascertained from these results. But the apparently unchanged densities of some symbiont populations between 4 days and 2 weeks suggested that the symbiont population densities had either stabilized after 4 days or had decreased further after 4 days and then increased. The lower density of some of the symbiont 'strains' after 2 weeks than after 4 days indicated the continued decline of the symbiont density over this period.

2 weeks after reinfection significant differences were evident between the densities of the different symbiont populations ($H=23.71$, $P<0.0001$). Multiple comparisons revealed that 'homologous' symbionts were persisting at a significantly higher density than Symbiodinium cells originally isolated from A. viridis (Lough Hyne) and A. viridis (Shell Is.), and that Symbiodinium sp. isolated from I. sulcatus was also present at a significantly higher density than symbionts from A. viridis (Shell Island) (appendix 8, table 9 (p.501)).

The symbiont population density increased dramatically between 2 and 4 weeks, except in the case of algae originally from A. viridis (Shell Island). The symbiont density of 'heterologous' Symbiodinium sp. from A. viridis (Shell Island) was significantly lower than that of symbionts isolated from C. pedunculatus and A. viridis (Lough Hyne), and was approaching being significantly lower than the density of Symbiodinium cells from I. sulcatus. The density of 'homologous' symbionts was also significantly higher than that of 'heterologous' symbionts from A. pallida and approaching being significantly higher than that of symbionts originally from A. ballii after 4 weeks (appendix 8, table 10 (p.501)).

6 weeks after reinfection, significant differences between the densities of different 'strains' of Symbiodinium were again evident ($F=15.88$, $P<0.0001$). Tukey's Test revealed that the density of the 'homologous' symbionts was significantly greater than that of A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Is.), I. sulcatus and A. pallida; the density of symbionts originally from A. viridis (Shell Island) was now observed to have reached the same level as all the other 'heterologous' symbiont populations (appendix 8, table 11 (p.502)).

8 weeks after reinfection significant differences between symbiont densities were still present ($H=18.49$, $P=0.003$). But due to a large standard error (graphs 4.16-4.22 (p.192)), the density of 'homologous' symbionts was now only

significantly greater than the density of Symbiodinium sp. originally isolated from A. viridis (Shell Island) and I. sulcatus (appendix 8, table 12 (p.502)).

Thus, the density of symbionts isolated from A. viridis (Shell Island) was significantly lower than the density of 'homologous' symbionts throughout the 8 week period. But surprisingly, after the symbioses had been reestablished for 9 months the population densities of all the symbiont 'strains' were not significantly different (F=1.85, P=0.145). From graphs 4.16-4.22 (p.192) it can be seen that the population of 'homologous' symbionts was reduced by approximately 60 % between 8 weeks and 9 months, whereas the population originally from A. viridis (Shell Island) had continued to increase over the 7 month period. The average 'homologous' symbiont density after 9 months (45809.6 +/- 6398 cells/mm oral disc^2) was not significantly different to the average density of symbionts in permanently symbiotic C. pedunculatus of 2-3 mm oral disc diameter (68864.7 +/- 19288 cells/mm oral disc^2) (T=1.13, P=0.32). The populations of symbionts originally from A. ballii, A. viridis (Lough Hyne) and I. sulcatus appeared to have stabilized after 4-6 weeks and the population of symbionts from A. pallida appeared to have stabilized after 8 weeks. Throughout the 9 month period, uninfected aposymbiotic C. pedunculatus controls remained symbiont-free.

When long term persistence was investigated with respect to symbiont biomass, significant differences between the

success of different 'strains' of symbionts were evident at 2 weeks (H=24.93, P<0.0001), 4 weeks (H=22.23, P=0.001), 6 weeks (H=20.47, P=0.001), 8 weeks (H=14.94, P=0.011) and 9 months (F=3.07, P=0.030). The population biomasses over this period are given in appendix 8, table 13 (p.503). These biomasses are illustrated in graphs 4.23-4.29 (p.193).

After 2 weeks the biomass of 'homologous' symbionts was greater than that of symbionts from A. viridis (Shell Island). However, unlike the situation when differences were investigated with respect to symbiont density, a significantly larger biomass of 'homologous' symbionts than Symbiodinium cells originally from A. pallida and a significantly larger biomass of symbionts from I. sulcatus than symbionts from A. viridis (Shell Island) were present after 2 weeks (appendix 8, table 14 (p.504)).

These same significant differences were evident 4 weeks after reinfection (appendix 8, table 15 (p.504)).

After 6 weeks, there was no biomass difference between the populations of symbionts from I. sulcatus and A. viridis (Shell Island), although the biomass of the 'homologous' symbiont population was significantly greater than the biomass of the population of symbionts originally from A. viridis (Shell Is.) and A. pallida (appendix 8, table 16 (p.505)).

After 8 weeks, the biomass of 'homologous' symbionts was only significantly greater than the biomass of symbionts from A. viridis (Shell Island) (appendix 8, table 17

(p.505)).

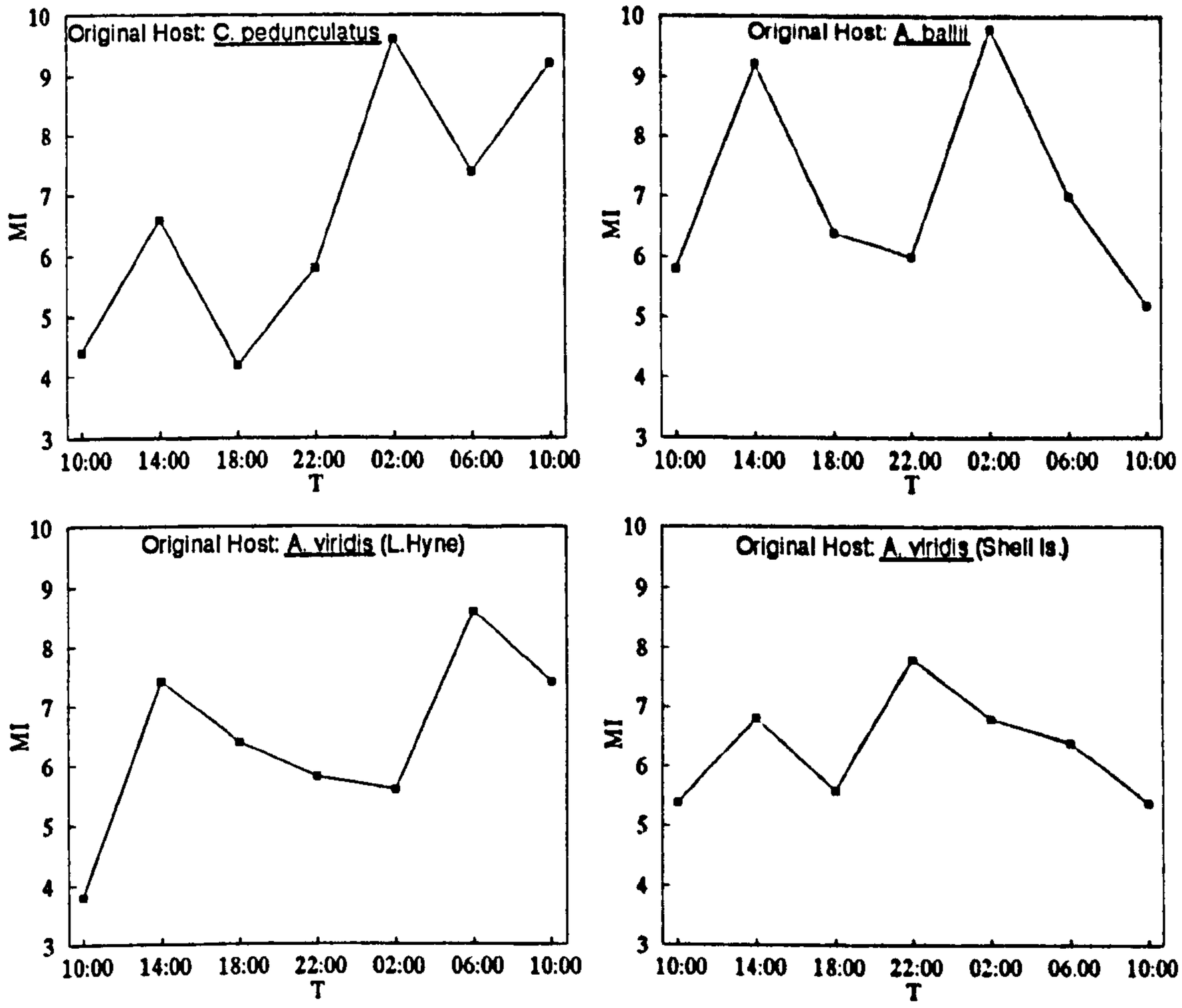
After 9 months, one-way ANOVA showed a significant difference between the biomasses of the different symbiont populations (F=3.07, P=0.030) (using the reevaluated average cell carbon contents given in table 4.2 (p.200)). After 9 months, the biomass of the 'homologous' symbiont population was significantly lower than that of the populations of the symbionts originally from A. viridis (Shell Is.) and I. sulcatus (appendix 8, table 18 (p.506)).

(g)Mitotic index (MI) of 'homologous' and 'heterologous' symbionts three weeks and nine months following reinfection

The mitotic index (MI) of the different 'strains' of symbionts after 9 months in the reestablished symbioses are shown in table 4.3 (p.200), together with the specific growth rate (μ) and doubling time (Dt). The diel division cycles are shown in graphs 4.30-4.35 (p.199), from which it can be seen that the symbionts maintained the asynchronous division pattern observed in their original host species.

The average MI of all the algal populations of the reinfected hosts, after population stability appeared to have been reached, was observed to be significantly greater than the MI when in the 'adult' host species from which the symbionts were originally isolated. This applied to symbionts from C. pedunculatus (T=6.00, P=0.0010), A. ballii (T=8.25, P=0.0002), A. viridis (Lough Hyne) (T=8.23, P=0.0002), A. viridis (Shell Is.) (T=14.80, P<0.0001), I. sulcatus (T=17.89, P<0.0001) and A. pallida (T=13.99,

Graphs 4.30 – 4.35: Diel division cycles of 'homologous' and 'heterologous' symbiont populations in reinfected C. pedunculatus after 9 months. (N=1).



Axes Key: T=Time (Hours), MI=Mitotic index (% symbionts dividing in a count of 500 cells)

TABLE 4.2 AVERAGE CELL DIMENSIONS AND CARBON CONTENTS OF DIFFERENT 'STRAINS' OF SYMBIONTS IN C. PEDUNCULATUS 9 MONTHS AFTER REINFECTION.

Original host species of symbiont	Average symbiont diameter +/- S.E. (µm) (N=100)	Average cell carbon content (pg)
Cp	7.18 +/- 0.08	33.18
Ab	8.58 +/- 0.12	52.71
AvLH	7.72 +/- 0.09	40.06
AvSI	8.27 +/- 0.12	47.90
Is	8.77 +/- 0.12	55.79
Ap	7.31 +/- 0.09	34.76

TABLE 4.3 MI, CELL SPECIFIC GROWTH RATES AND DOUBLING TIMES OF DIFFERENT 'STRAINS' OF SYMBIONTS AFTER BEING ESTABLISHED IN C. PEDUNCULATUS FOR 9 MONTHS.

Original host species of symbiont	Mitotic Index (MI) +/- S.E. (%) (N=7)	Cell specific growth rate (µ) (day^{-1})	Doubling Time (Dt) (Days)
Cp	6.7 +/- 0.8	0.142	4.9
Ab	7.1 +/- 0.7	0.149	4.7
AvLH	6.4 +/- 0.6	0.136	5.1
AvSI	6.3 +/- 0.3	0.134	5.2
Is	7.2 +/- 0.3	0.152	4.6
Ap	7.0 +/- 0.4	0.148	4.7

P<0.0001). The MI of the symbiont population of the permanently symbiotic juvenile C. pedunculatus was 1.9 +/- 0.2 % and was significantly lower than the MI in the 'homologous' reinfected population (T=5.79, P=0.0012).

All the different 'strains' of Symbiodinium did not exhibit a significantly different MI 9 months after the establishment of a symbiosis with C. pedunculatus (F=0.42, P=0.830). After 3 weeks in C. pedunculatus the diel division cycle of symbionts originally from A. viridis (Shell Island) was asynchronous (appendix 8, graph 2 (p.507)) with an average MI of 3.7 +/- 0.35 %, and the average MI of the asynchronously dividing 'homologous' symbionts (appendix 8, graph 1 (p.507)) was 6.2 +/- 0.43 %. The MI of the symbionts from A. viridis (Shell Island) was significantly lower than the MI of the 'homologous' symbionts (T=4.44, P=0.001). Whilst the MI of the 'homologous' symbionts was not significantly different between 3 weeks and 9 months after reinfection (T=0.59, P=0.57), the MI of symbionts originally from A. viridis (Shell Island) was significantly higher after 9 months than after 3 weeks (T=5.30, P=0.0002).

(h)Non-dividing cell dimensions of symbionts 9 months after reinfection

The average diameter and cell carbon content (as calculated using equation 3.1 (p.123)) of 100 cells of each 'strain' of symbiont 9 months after being in symbiosis with C. pedunculatus are given in table 4.2 (p.200). The dimensions of these symbionts were in approximately the same order as

when they were in their original hosts, with the smallest size group consisting of symbionts from A. pallida and C. pedunculatus, the second group containing Symbiodinium sp. from A. viridis (Lough Hyne) and the third group consisting of symbionts from A. viridis (Shell Island), A. ballii and I. sulcatus (appendix 8, table 19 (p.506)). However, Symbiodinium cells originally from C. pedunculatus (Mann-Whitney Statistic W =13983.5, P<0.0001), A. ballii (W=14239.0, P<0.0001), A. viridis (Lough Hyne) (T=6.35, P<0.0001), A. viridis (Shell Is.) (T=12.09, P<0.0001), I. sulcatus (W=14148.0, P<0.0001) and A. pallida (W=11473.5, P=0.0004) were all significantly smaller after 9 months in the reestablished symbiosis than when in their original host species.

(i)Possibilities of vital staining of symbionts

Vital staining was not viable for labelling symbionts for reinfections. Only apparently dead or degenerating algae appeared stained due to the masking effect of the red chlorophyll auto-fluorescence of 'healthy' symbionts.

4.4 Discussion

(a)The uptake of 'homologous' and 'heterologous' symbionts.

The results of the symbiont uptake and short term persistence investigation suggest that the algal cells are endocytosed irrespective of the host species from which they were isolated and that the biomass of the individual symbionts has no discernible influence on the numbers of

symbionts phagocytosed. But why only a small fraction (approximately 0.5 %) of the available Symbiodinium cells were phagocytosed at saturation point by the host animals is unknown. Berner et al. (1993) observed a similar occurrence when inoculating aposymbiotic A. pulchella, suggesting that the number of available uptake sites was relatively low or that many of the algal cells in the inoculum were unsuitable for retention. Endocytosis of the different 'strains' of Symbiodinium, with the exception of the 'strain' from A. ballii, was similar. This finding suggests that, whilst surface recognition may limit the extent to which symbionts of A. ballii are endocytosed, the other symbiont 'strains' possess similar surface characteristics. Consequently, cell recognition may play little part in the discrimination of these different symbiont 'strains'. Surface recognition, which potentially may occur when symbionts are phagocytosed by engulfing extensions of the host plasmalemma (Fitt and Trench 1983c), has previously been implicated in the successful entry of a symbiont into a host. In the Hydra-Chlorella symbiosis, "F/F" strain Chlorella uptake was reduced by more than 50 % when the algae were treated with "anti-F/F" antiserum, indicating blockage of membrane-bound binding sites (Muscatine et al. 1975). However, Jolley and D.C. Smith (1980) found no evidence for specific recognition of symbionts at surface contact in the Hydra-Chlorella symbiosis. The extent to which different strains of

Chlorella were phagocytosed was not related to the capacity to eventually establish a symbiosis. Direct evidence for surface recognition in Symbiodinium-invertebrate symbioses is absent. As discussed in chapter 3, section 3.2.4 (g) (p.110), and shown in plate 3.6 C (p.93), the surface of coccoid Symbiodinium cells from temperate hosts and A. pallida possess a 'coat'. Using Symbiodinium cells from tropical hosts, this 'coat' has been shown to exhibit the characteristics of an acid polysaccharide or glycoprotein (Trench et al. 1981a; Colley and Trench 1983). Recent analysis of these macromolecules using SDS-polyacrylamide gel electrophoresis of solubilized cell wall fractions revealed glycoproteins and proteins of 13.5 to greater than 200 KDa molecular weight (Markell et al. 1992). These molecules may be involved in a ligand-receptor interaction, particularly in light of a mucopolysaccharide/glycoprotein 'coat' observed on the endodermal cells of marine invertebrates (Trench 1987). Cell recognition may also be enabled by interactions between electrically charged cell surfaces (Trench 1988), although Symbiodinium cell surfaces have all been found to be negatively charged (Colley and Trench 1983). Surface recognition between gymnodinioid dinoflagellates and host cells has been inferred experimentally from the inability of some 'heterologous' strains of symbiont to be endocytosed by the host: Cultured Symbiodinium spp. from C. gigantea, Z. sociatus and P. mammilosa were not endocytosed by aposymbiotic A. pallida

(Schoenberg and Trench 1980c), the gorgonian Pseudopterogorgia bipinnata could not be infected by Amphidinium sp. (Kinzie 1974), and the flatworm Amphiscolops sp. only phagocytosed A. klebsii or Amphidinium sp. when also exposed to A. carterae and Symbiodinium spp. (Taylor, D.L. 1971; Trench and Winsor 1987). However, it has been shown that uptake of freshly isolated symbionts is largely determined by the presence of residual host material. In particular, the uptake of freshly isolated 'homologous' symbionts by Cassiopeia sp. is greatly enhanced by the presence of host tissue, in comparison to the uptake of 'heterologous' symbionts (Trench 1981; Trench et al. 1981a; Colley and Trench 1983). The 'homologous' symbionts of C. pedunculatus in this study were endocytosed to the same degree as some of the 'heterologous' symbionts, suggesting that uptake of 'homologous' symbionts was not enhanced by the presence of 'self' tissue. The indistinguishable levels of endocytosis of all but one of the different 'strains' of symbiont in this investigation and the subsequent differences in symbiont persistence indicate that recognition of the symbionts of the British Anthozoa and A. pallida by C. pedunculatus at the sub-genera level predominantly occurs after endocytosis.

(b)The short term persistence of 'homologous' and 'heterologous' symbionts.

The observed reduction in symbiont population density and biomass following uptake has been observed previously. The

approximately 60 % decrease in symbiont density observed in reinfected C. pedunculatus over 4 days is similar to the 50 % to 100 % decrease over a period of 1 to 3 days reported by Colley and Trench (1983) in reinfected Cassiopeia sp.. These authors noted that the density of 'heterologous' symbionts declined more rapidly than the density of 'homologous' symbionts, indicating post-endocytotic discrimination. A similar situation was evident in the reinfected C. pedunculatus. Also, Jolley and D.C. Smith (1980) reported a "resorting phase" when aposymbiotic Hydra sp. were reinfected with different strains of Chlorella sp., resulting in a minimum symbiont population density after 2 to 6 days. The decline in symbiont density may be due to either expulsion or digestion. Expulsion, which has been observed in the laboratory (Steele 1975; pers. obs.), is the more favoured hypothesis. This is due to only 4 % of living symbiont-containing vacuoles being observed to fuse with ferritin-labelled lysosomes following phagocytosis by Cassiopeia sp.. In contrast, 70 % of moribund symbiont-containing vacuoles were observed to fuse with lysosomes (Fitt and Trench 1983a; Fitt and Trench 1983b; Colley and Trench 1985). Therefore, the 50 % decline in symbiont density cannot be explained by digestion alone. The rate of lysosome fusion with symbiont containing vacuoles in anthozoans is unknown. In addition, a protein extract of the anemone Phyllactis flosculifera has been shown to destroy a greater percentage of 'homologous' than 'heterologous'

algae, suggesting that anemones may not be able to exert much control over 'heterologous' symbiont populations through digestion (Steele and Goreau, N.I. 1977).

The reestablished symbiont populations in C. pedunculatus appeared to be stabilizing after 2-4 days, although the precise period after which the minimum density was achieved is unknown. Stabilization of the symbiont population in reinfected Cassiopeia sp. occurred over a comparable period of 3-14 days, after which dead and non-compatible symbiont strains did not persist (Trench et al. 1981a; Colley and Trench 1983).

(c)The long term persistence of 'homologous' and 'heterologous' symbionts.

Following the "resorting phase" all the 'homologous' and 'heterologous' symbiont populations in C. pedunculatus grew rapidly, reaching a maximum population density after approximately 4-6 weeks, depending on the host species from which the symbionts were isolated. This rapid population growth following "resorting" also seems to be characteristic of events leading to the repopulation of aposymbiotic animals by algal symbionts and has been described previously by Jolley and D.C. Smith (1980) and Colley and Trench (1983). The proliferation of symbionts in reinfected Cassiopeia sp. initially occurs in "amoebocytes" which migrate from the endoderm to the mesogloea (Colley and Trench 1985), however the cellular events occurring during the proliferation of symbionts in anthozoans have not been

reported. 'Homologous' symbionts attained the highest maximum density of all 'strains' after only 3-4 weeks, indicating that the 'homologous' symbionts were the most compatible with C. pedunculatus. Similarly, 'homologous' symbionts introduced into aposymbiotic Cassiopeia sp. scyphistomae reached their maximum population density after only 2 weeks compared to the 3-4 week period required by the most compatible 'heterologous' symbionts (Colley and Trench 1983), and 'homologous' symbionts in A. pallida attained their maximum density before 'heterologous' symbionts (Schoenberg and Trench 1980c). 'Homologous' symbionts in reinfected A. pulchella have also been shown to reach their maximum density after only 19 days (Berner et al. 1993), a similar figure to that measured here in C. pedunculatus. Symbionts from A. viridis (Shell Is.) were the least compatible symbionts with C. pedunculatus and were notably less 'successful' in establishing a symbiosis than symbionts from A. viridis (Lough Hyne). Symbiodinium cells from A. viridis (Lough Hyne), A. ballii, I. sulcatus and A. pallida were all equally successful at establishing a symbiosis with C. pedunculatus, even though different strains or species were indicated by their differing morphological and behavioural characteristics. The varying levels of success of the different 'strains' of symbionts establishing a symbiosis with C. pedunculatus, despite similar levels of endocytosis, suggest that host-symbiont recognition occurs intracellularly. The view that recognition occurs after

phagocytosis is widespread. Some 'strains' of cultured or freshly isolated 'heterologous' symbionts may not be phagocytosed by Cassiopeia sp. (Schoenberg and Trench 1980c; Trench et al. 1981a), however the same symbionts, when introduced into the host via Artemia, have been observed to persist for a few hours or days before being lost (Fitt 1984; Trench 1988). An identical pattern has also been reported with regard to Amphiscolops sp., which were completely Symbiodinium-free 72 hours after feeding with Symbiodinium-laden Artemia nauplii (Trench and Winsor 1987). Post-phagocytotic recognition of symbionts is also supported by the observed endocytosis of Symbiodinium cells by the non-symbiotic jellyfish Aurelia aurita and the subsequent inability of the algal population to persist (Colley and Trench 1983). But the mechanism by which post-phagocytotic recognition occurs is unknown, although the possibilities of ligand-receptor interactions between the surfaces of the host vacuolar membrane and the algae (Trench 1988), and the movement of released 'strain' specific macromolecules between symbiont and host (Markell et al. 1992; Markell and Trench 1993) have been suggested. As discussed in chapter 3, section 3.2.4 (d) (p.100), cultured Symbiodinium cells release macromolecules to their medium. Markell and Trench (1993) demonstrated that the composition of the exudate from 5 "species" of Symbiodinium differs, particularly with respect to uronic acid content. These authors also suggested that different "species" of Symbiodinium may release

different amounts of exudate, as indicated by the varying degrees to which the cells clump in culture. Markell and Trench (1993) suggested that the chemical composition and/or the amount of the exudate may act as a signal in host-symbiont recognition.

The symbiont densities observed after 9 months indicated that the symbiont populations had stabilized at their maximum levels. The importance of observing symbiont population growth over a period of months has been stressed by Muscatine et al. (1975), for the density of algae from Oculina diffusa reached a peak 2 weeks after introduction into A. pallida and then steadily declined, indicating that apparent early proliferation in the host does not necessarily result in a successful symbiosis.

The differing degrees of compatability between C. pedunculatus and Symbiodinium cells from different host species indicate that, if 'homologous' and 'heterologous' symbionts were to infect an aposymbiotic host simultaneously, the 'homologous' symbionts may 'out-compete' the 'heterologous' symbionts. However, the possibilty of coexisting symbiont 'strains' under such circumstances cannot be discounted. If 'heterologous' symbionts are phagocytosed by symbiotic C. pedunculatus in the field it is unlikely that the 'heterologous' cells persist in competition with the 'homologous' population. Competition between 'strains' of Symbiodinium awaits investigation. Displacement of a 'heterologous' symbiont by 'homologous'

symbionts reinfected into a host at a later date has been demonstrated using the flatworm Convoluta roscoffensis, the 'heterologous' alga Prasinocladus marinus and the 'homologous' alga Platymonas convolutae (Provasoli et al. 1968). Infection of an already symbiotic host by 'heterologous' algae has been investigated in Hydra sp. by labelling the 'heterologous' algae with 'Calcofluor White'. The presence of an established symbiont population was found to prevent the avoidance of lysosomal degradation by 'heterologous' algae and the transportation of reinfected algae to the cell base of the endodermal cells (McAuley and Smith, D.C. 1982). The co-occurrence of taxonomically distinct algal symbionts in aquatic invertebrates is rare. The only reported examples are the co-existence of Symbiodinium and Amphidinium cells in Haplodiscus sp., and the occasional co-existence of Symbiodinium and Chlorella cells in A. elegantissima (Trench and Winsor 1987) and Anthopleura xanthogrammica (O'Brien 1980). The apparent genetic uniformity of Symbiodinium populations in different host species (Schoenberg and Trench 1980a; Rowan and Powers 1991b) may, in part, be due to the more rapid perpetuation of 'homologous' symbionts in the reinfected host and their competitive advantage over 'heterologous' symbionts. This point will be discussed further in chapter 6.

The observation of an approximately 50 % decline in the population density of 'homologous' Symbiodinium cells in the reinfected C. pedunculatus between 8 weeks and 9 months is

unparalleled. The reasons for this decline are unknown, but one hypothesis is that the aposymbiotic animals may have partially lost the capability to control the growth of the symbiont population during the 3 year dark period. Therefore, if 'homologous' symbionts are the most compatible 'strain' they may proliferate at a faster rate than the 'heterologous' 'strains' and therefore need to be 'brought under control' to prevent overgrowth of the host as the ability to regulate the symbiont population is regained. This hypothesis is supported by the fact that the symbiont density in non dark treated C. pedunculatus was comparable to that observed 9 months after reinfection.

(d)The mitotic index (MI) and cell dimensions of the symbionts during the reestablishment of the symbioses.

The reduction in the 'homologous' symbiont population between 8 weeks and 9 months was irrespective of the symbiont mitotic index (MI). 'Homologous' symbionts divided at the same rate both 3 weeks and 9 months after the establishment of the symbiosis. 'Homologous' symbionts divided more rapidly during the early stages of symbiosis reestablishment than symbionts from A. viridis (Shell Is.), suggesting that the slower rate of 'heterologous' symbiont proliferation was in part due to the lower MI. A higher expulsion rate or a reduced resistance to host digestion may also result in the observed differences. Notably, however, the MI of symbionts originating from A. viridis (Shell Is.) significantly increased from 3.7 to 6.3 % over the 9 month

period. The mechanism causing the increased division rate is unknown, but may be the result of adaptation by the symbionts to their new intracellular environment or by an adjustment of the host control of symbiont growth. Adjustment by the partners to the immediate situation was also suggested by the gradual convergence of the 'homologous' and 'heterologous' symbiont populations. Whether 'heterologous' symbionts freshly isolated from reinfected C. pedunculatus after the symbiosis has been established for several months can subsequently establish a symbiosis more rapidly with aposymbiotic C. pedunculatus awaits investigation. But the existence of 'heterologous' Symbiodinium cells in A. pallida for 2 months did not enhance the growth of the same symbionts when they were isolated and reinfected back into A. pallida, indicating clear symbiont strain or species specific differences (Schoenberg and Trench 1980c). The rate at which a 'heterologous' symbiont strain or species proliferates in an aposymbiotic host that has previously been in symbiosis with this same 'heterologous' strain or species is also an interesting question for future research. The unchanged MI of the 'homologous' symbionts and the increased MI of the 'heterologous' symbionts between 3 weeks and 9 months after reinfection of C. pedunculatus is in contrast to the finding of Berner et al. (1993). These authors, using 'homologously' reinfected A. pulchella and in vivo auto-fluorescence, demonstrated a decrease in the symbiont specific growth rate

(μ) of 0.4/day to 0.02/day as the symbiont population approached saturation.

All the 'strains' of Symbiodinium possessed a significantly higher MI in the reinfected animals, even after 9 months, than in their original adult hosts. It is uncertain whether this difference reflects loss of host control over symbiont division or host ontogenetic influences on symbiont division. Ontogenetic influences on the MI have been reported with regard to the symbionts of juvenile A. ballii (this work), juveniles of the tropical sea anemone Aulactinia stelloides, where the MI of the symbionts of juvenile hosts was twice as great as the MI of symbionts in adult hosts (Smith, G.J. 1986), and the jellyfish Mastigias sp., in which the symbiont MI was slightly lower in larger than in smaller medusae (Muscatine et al. 1986). However, the symbiont MI was significantly greater in the reinfected C. pedunculatus than in similarly sized, permanently symbiotic C. pedunculatus with the same densities of symbionts, suggesting some loss of host control over symbiont growth. This apparent loss of control indicates that aposymbiotic C. pedunculatus are physiologically different from symbiotic individuals, a point which should always be noted when using aposymbiotic anthozoans for experimental purposes.

The significantly smaller diameters of all the 'strains' of Symbiodinium after 9 months in symbiosis with C. pedunculatus reflects the higher MI, which results in algal

cells dividing at an earlier stage in their growth. This finding is in agreement with the situation in free-living phytoplankton and amongst the symbionts of Caribbean reef corals (Wilkerson et al.1988). In contrast, Symbiodinium sp. from Mastigias sp. and the sea slug Pteraeolidia ianthina simultaneously decreases in size and MI (Hoegh-Guldberg et al. 1986; Muscatine et al. 1986), and Symbiodinium sp. from A. stelloides does not change in size with changing division rates (Smith, G.J. 1986).

The implications of the higher MI in the reinfected animals than in the original host, the similar MI of each symbiont 'strain' after 9 months in symbiosis with C. pedunculatus and the decreased symbiont size with regard to the carbon budget of the symbiosis will be discussed in chapter 5.

(e)The influence of symbiont cell biomass on the endocytosis and persistence of 'homologous' and 'heterologous' symbionts.

Symbiont cells of significantly different biomasses were endocytosed to the same degree by C. pedunculatus. This finding suggests that the biomass of individual symbionts is not involved in selective endocytosis by this host species. Likewise, Trench and Winsor (1987) concluded that cell biomass played little part in selective endocytosis, demonstrating that the uptake of Amphidinium sp. by Amphiscolops sp. was not determined by the size of the algal cell. This flatworm was found to endocytose large Amphidinium cells, but not equivalently sized clumps of

Symbiodinium spp. or a similarly sized (22 x 35 μm), unidentified species of amphidinioid dinoflagellate.

The reinfected hosts did not appear to manipulate the densities of the persisting symbionts according to their cell biomasses. In contrast, Trench and Winsor (1987) measured the co-existing Amphidinium-Symbiodinium population in the flatworm Haplodiscus sp. to be maintained at a constant ratio of 13 Symbiodinium : 1 Amphidinium. This ratio ensures that the population biomasses of these differently sized algal cells are equal. Thus, Haplodiscus sp. appears to control the population densities of its symbionts on an algal cell biomass dependent basis. In the reinfected C. pedunculatus the 'homologous' symbiont populations had both the highest cell densities and biomasses over the first 8 weeks, clearly indicating host-symbiont specificity.

The fact that the 'homologous' symbionts persisted at a much higher biomass than the 'heterologous' symbionts over the first 8 weeks also suggested that the lower densities of the larger 'heterologous' symbionts were not due to the limits of the available host cell space. The concept of a symbiont population being limited by the volume of the host cell has been proposed by F.J.R. Taylor and Harrison (1983), who considered the animal membrane as the outer limit of the cytocosm.

(f)General conclusion.

The results presented here suggest that the host-symbiont relationship in C. pedunculatus is specific. But rather than C. pedunculatus exhibiting a "unique affinity" for its 'homologous' symbionts (Trench et al. 1981a), this anthozoan could associate with dissimilar 'heterologous' symbionts. Consequently, the definition of specificity given by Trench et al. (1981a) is not accurate in this case. D.C. Smith and Douglas (1987) provided a more precise definition of specificity. These authors stated that specificity "refers to the degree of taxonomic difference between acceptable partners, and may vary from very low (where an organism can associate with members of more than one class or even phylum) to high or very high (where only a single species or 'strain' is acceptable)". According to this scheme, whilst C. pedunculatus is probably highly/very highly specific in the field, in the laboratory C. pedunculatus is at most moderately specific, forming a symbiosis with symbionts of the same genus. But again, this definition fails to take account of the variable degrees to which different 'strains' or species within a genus associate with a particular host species. Trench et al.'s (1981a) definition of specificity should therefore be modified, referring to a greater affinity for one entity than another by an organism. Alternatively, if D.C. Smith and Douglas's (1987) definition is revised, then a moderately specific host (eg. C. pedunculatus) could be classed as being highly/very highly

specific, where a single species or 'strain' is the most acceptable.

The finding that specificity exists between C. pedunculatus and its symbionts raises the question of to what extent 'homologous' and 'heterologous' symbionts can supplement the carbon budget of the host and enhance host 'fitness'. This question is addressed in the following chapter. Thus, inferences can be made regarding the potential for recombined symbioses to propagate in the field.

4.5 Summary

(i)Using all of the available host species, the production of aposymbiotic Anthozoa was attempted. Temperature shock followed by dark treatment was employed successfully obtaining predominantly symbiont-free A. ballii and A. viridis. However, the most successful method of obtaining large numbers of aposymbiotic hosts was dark treatment of juvenile C. pedunculatus (Lough Hyne) directly extracted from the adult. C. pedunculatus was therefore used as the experimental host animal.

(ii)Aposymbiotic C. pedunculatus could be reinfected by being in the presence of symbiotic C. pedunculatus, indicating possible cross-infection in the field.

(iii)Aposymbiotic C. pedunculatus were reinfected with freshly isolated 'homologous' and 'heterologous' symbionts from C. pedunculatus, A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Is.), I. sulcatus and A. pallida.

(iv)The density (symbiont cells/mm oral disc^2) and biomass (pg symbiont carbon/mm oral disc^2) of the symbionts endocytosed and persisting in the host were measured 4 hours, 2 days, 4 days, 2 weeks, 4 weeks, 6 weeks, 8 weeks and 9 months after reinfection.

(v)All the symbiont 'strains', with the exception of Symbiodinium cells from A. ballii, were endocytosed to the same degree. This finding suggests that, whilst surface recognition may limit the uptake of symbionts from A. ballii, the other symbiont 'strains' possess similar surface characteristics. Consequently, surface recognition may play little part in the discrimination of different 'strains' by C. pedunculatus.

(vi)The reinfected symbiont population densities declined rapidly over the 2 day period following inoculation, but between 2 and 4 weeks after the establishment of the symbioses the symbiont population density increased greatly. The densities of the symbiont populations appeared to reach a stable maximum after approximately 4-6 weeks depending on the host species from which the symbiont was originally isolated.

(vii)Despite the densities of endocytosed symbionts not being significantly different, the symbiont populations persisted to different extents and proliferated at different rates. This indicated the presence of different strains or species of Symbiodinium and the involvement of post-endocytotic recognition in symbiont discrimination by the

host. 'Homologous' symbionts were the most 'successful' in establishing a symbiosis, indicating specificity between C. pedunculatus and its symbionts. Symbiodinium sp. from A. viridis (Shell Is.) was the least 'successful' in establishing a symbiosis with C. pedunculatus.

(viii)The differing rates of symbiosis establishment between Symbiodinium sp. cells from C. pedunculatus and A. viridis (Shell Is.) were reflected in their mitotic index (MI), with the 'homologous' symbionts dividing at a faster rate 3 weeks after the initiation of the symbioses.

(viii)After 9 months in symbiosis with C. pedunculatus, the symbiont population densities and biomasses, and the sizes and MI of the symbionts, did not significantly vary between the Symbiodinium sp. originally isolated from different host species. Therefore it is suggested that, whilst specificity for 'homologous' symbionts was exhibited by C. pedunculatus, over a prolonged period of time other 'strains' of symbionts and/or the reinfected hosts slowly adjust to the new situation. This enabled different symbiont 'strains' to persist at the same level. This view is supported by the increase in MI of symbionts from A. viridis (Shell Is.) between 3 weeks and 9 months in symbiosis with C. pedunculatus.

(ix)The population of 'homologous' symbionts decreased by approximately 50 % between 2 months and 9 months after the initiation of the symbiosis. The final density of symbionts was similar to that measured in similarly sized C.

pedunculatus that had not been rendered aposymbiotic. An hypothesis explaining this observation is that during the first 2 months after reinfection the host had not been able to control algal population growth to the extent observed in permanently symbiotic hosts. The control mechanism may have been lost during the 3 year period in darkness.

(x)The similar densities of differently sized cells endocytosed suggested that the symbiont cell biomass was not used to recognise 'homologous' and 'heterologous' symbionts. Also the observed 'heterologous' symbiont population biomasses were far below the values attained by the 'homologous' symbionts in C. pedunculatus. This suggested that the host did not manipulate the symbiont densities according to their biomasses and that the different symbiont densities were not the result of a limited host cell volume.

(xi)No suitable method of labelling symbiont cells for use in multiple symbiont 'strain' reinfections of a host was found.

CHAPTER 5

THE TRANSLOCATION OF FIXED CARBON FROM SYMBIONT TO HOST AND THE CONTRIBUTION OF THE TRANSLOCATED CARBON TO THE RESPIRATORY CARBON BUDGET OF THE HOST.

5.1 Introduction

This chapter aims to establish whether symbiosis specificity is reflected by the productivity of the symbionts, their competence to translocate photosynthate to the host, and the percentage contribution of this translocate to the daily respiratory carbon budget of the host (CZAR).

Four specific objectives, using both 'homologous' and 'heterologous' combinations of symbionts and hosts, were identified. These were: (a) Measurement of the net daily algal photosynthesis and hence the carbon available for translocation; (b) estimation of the percentage of photosynthetically fixed carbon translocated to the host using 3 comparable methods; (c) measurement of the daily animal respiration in carbon equivalents; and (d) calculation and comparison of the total CZAR, and the CZAR/symbiont cell/mg association protein and the CZAR/mg symbiont carbon/mg association protein (in the reestablished symbioses only).

Primary production by symbiotic dinoflagellates has been explored extensively (eg. Wethey and Porter 1976; Crossland and Barnes 1977; Chalker et al. 1983; Porter et al. 1984; Fisher et al. 1985). The productivity of Symbiodinium cells

may be 3 times greater than the primary production by phytoplankton in coral reef communities (Scott and Jitts 1977), and in certain localities the temperate A. elegantissima may be as productive as intertidal seaweed populations (Fitt et al. 1982).

The flow of photosynthetically fixed carbon and its energy equivalent through symbioses has been much studied, resulting in the compilation of integrated carbon and energy budgets for several species of coral, sea anemone and tridacnid clam (Muscatine et al. 1983; Muscatine et al. 1984; Spencer Davies 1984; Hunter 1984; Tytler and Spencer Davies 1984; Edmunds and Spencer Davies 1986; Stambler and Dubinsky 1987; Klumpp et al. 1992).

Carbon, which is probably photosynthetically fixed by the C_3 pathway (Streamer et al. 1993), is initially used in algal respiration and growth. The remainder of the carbon is then available for translocation to the host (Muscatine et al. 1983; Spencer Davies 1984; Stambler and Dubinsky 1987).

Translocation of fixed carbon from Symbiodinium cells to a sea anemone host was first shown directly using radioisotopic labelling in the A. elegantissima symbiosis (Muscatine and Hand 1958). Since this work, in vitro investigations have been employed to calculate the percentage of photosynthate translocated to the host (Muscatine 1967; Trench 1971a; Muscatine et al. 1984; Sutton and Hoegh-Guldberg 1990) and investigate the composition of this translocate (Muscatine 1967; Trench 1971b; Trench 1974;

Schlichter et al. 1983). These investigations have relied on the release of compounds by Symbiodinium cells in response to a host homogenate. In contrast to the response of Chlorella sp. to Hydra sp. homogenate (Smith, D.C. et al. 1969; Cernichiari et al. 1969), a "host factor" present in anthozoan homogenates is thought to stimulate Symbiodinium cells to release photosynthate in vitro (Muscatine 1967; Trench 1971b; Muscatine et al. 1972). A "host factor" has also been implicated in the inducement of excretion by the symbionts of the flatworm C. roscoffensis (Muscatine et al. 1974) and the saccoglossan mollusc Elysia viridis (Gallop 1974). Host homogenate from one host species stimulates photosynthate release by symbionts from other host species, although not always to the same extent (Muscatine 1967; Gallop 1974; Hinde 1988; Sutton and Hoegh-Guldberg 1990). Investigation of photosynthate release by symbionts of the same host species in response to homogenates of different host species could therefore indicate whether the "host factor" is species specific. Measuring photosynthate translocation by symbionts of different host species in response to a homogenate of the same host species could reveal whether the symbionts possess the same properties. Any differences in percentage release might enable host recognition of symbionts upon endocytosis and influence the flux of carbon to the host during the establishment of recombined symbioses.

Translocated carbon may be used in host growth (Spencer

Davies 1984; Muscatine et al. 1985), reproduction (Hunter 1984; Edmunds and Spencer Davies 1986; Rinkevich 1989) or respiration (Spencer Davies 1980; Muscatine et al. 1981; Muscatine et al. 1984; Hoegh-Guldberg et al. 1986), or be released (Cooksey and Cooksey 1972; Crossland et al. 1980b; Muscatine et al. 1984; Spencer Davies 1984).

The extent to which the translocated carbon supports animal respiration has been extensively investigated. Muscatine et al. (1981) introduced the CZAR ("Contribution of Zooxanthellae to Animal Respiration") model to quantify the importance of symbiont photosynthesis to the daily respiratory requirements of the corals P. damicornis and Fungia scutaria where:

$$\text{CZAR} = \frac{\text{Pz(net)}.T}{\text{Ra}} \quad \text{(Equation 5.1)}$$

where Pz(net) = net daily photosynthetic carbon fixation by the symbionts, Ra = carbon respired daily by the host and T = percentage of fixed carbon translocated to the host.

Muscatine et al. (1983) again discussed the calculation of CZAR in detail but modified the method of estimating the percentage translocation. Instead of in vivo radioisotopic labelling, these authors estimated the percentage of fixed carbon translocated to the host by assuming that all the photosynthetically fixed carbon not used in symbiont growth and respiration was translocated. The CZAR model has been

applied to symbioses between Symbiodinium spp. and A. elegantissima (Fitt et al. 1982; Shick and Dykens 1984; Zamer and Shick 1987), the zoanthids Z. sociatus and P. variabilis (Steen and Muscatine 1984), S. pistillata (McCloskey and Muscatine 1984), A. viridis (Stambler and Dubinsky 1987), the nudibranch P. ianthina (Hoegh-Guldberg et al. 1986) and Tridacna gigas (Klumpp et al. 1992).

From equation 5.1 (p.225) it is obvious that changes in the photosynthetic carbon fixation, respiration and translocation within a symbiotic association will be reflected in the CZAR.

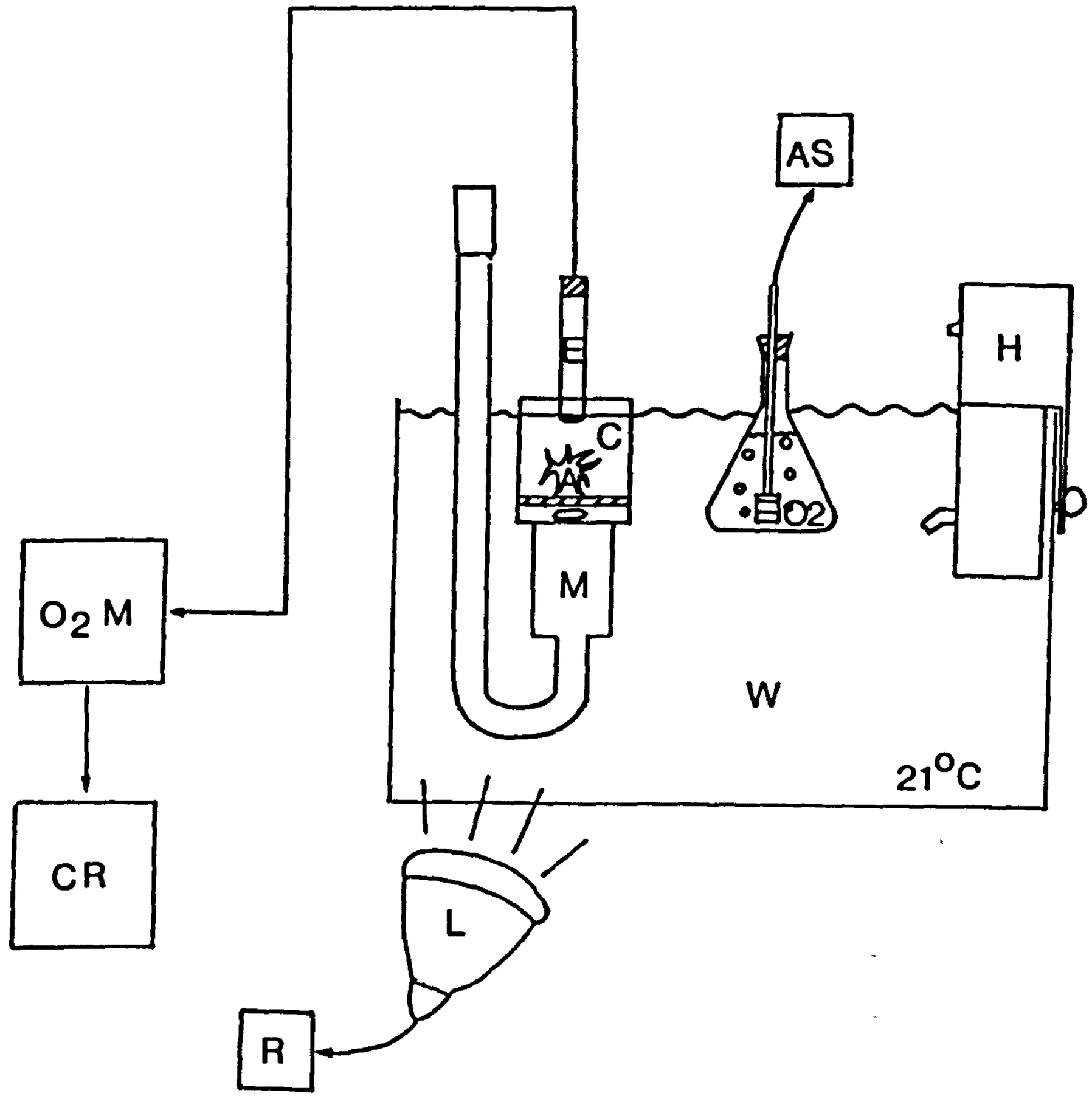
Anthozoan-dinoflagellate symbiosis specificity has not previously been investigated with regard to the photosynthetic fixation of carbon by the symbionts and its flux through the symbiosis. The potential for different values of the CZAR in 'homologous' and 'heterologous' symbioses is great: 'Strains' of Symbiodinium may possess different intrinsic photosynthetic and respiratory rates, use variable amounts of carbon in cell growth, or translocate differing percentages of their photosynthetically fixed carbon in response to the host cell environment in which they are residing. The compatability of symbiont cells with a host may result in varying symbiont population densities which ultimately effect the total fixed carbon available to the host (Smith, G.J. and Muscatine 1986; Hoegh-Guldberg et al. 1986). Symbiont density may also directly influence light-saturated photosynthetic rates/

symbiont cell (Smith, G.J. 1986), the algal mitotic index (MI), and hence the percentage of photosynthate translocated to the host (Smith, G.J. 1986; Hoegh-Guldberg et al. 1986). This investigation therefore attempted to compare the photosynthetic, repiratory and translocatory 'performance' of 'homologous' and 'heterologous' symbionts when in their original host species and after reinfection into C. pedunculatus, and to relate the CZAR in these reinfected polyps to the differences in symbiont-host compatability discussed in chapter 4. Inferences can also be made from the CZAR regarding the carbon available for host growth and reproduction. Translocated carbon is essential for the strobilation of Cassiopeia sp. (Trench et al. 1981a; Colley and Trench 1983; Fitt 1984), the survival and growth of juvenile Tridacna squamosa (Fitt and Trench 1981), and the metamorphosis of planulae from the octocoral Heteroxenia fuscescens (Benayahu et al. 1989). If host-symbiont specificity is reflected in the CZAR, then this specificity may also determine the extent to which 'novel' symbioses can grow and propagate.

5.2 Materials and Methods

(a)Preparation of symbiotic Anthozoa for carbon budget work

With the aim of eventually estimating the CZAR, photosynthetic and respiratory data were collected for 'natural' symbioses involving C. pedunculatus (Lough Hyne), A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Island), I. sulcatus and A. pallida. All these anthozoans had been maintained at 21 ^{o}C and 80 $\mu E/m^2/s$ (on a 12 hour light:dark cycle) for at least 8 months. In addition, I. sulcatus polyps were severed at their base using a razor blade and placed in a sea water filled polystyrene container to heal for 2 weeks, as suggested by Steen and Muscatine (1984). The approximate oral disc diameters of these anthozoans were: C. pedunculatus 2 cm, A. ballii 3.5-4 cm, A. viridis (Lough Hyne and Shell Island) 3.5-4 cm, I. sulcatus 2mm and A. pallida 7.5-10 mm. The aim of measuring photosynthetic and respiratory rates, percentage translocation and the CZAR in these 'natural' symbioses was to compare the performance of the symbionts in their original hosts to when established in a 'heterologous' host. The reestablished symbioses between symbionts from each of the 'natural' symbioses above and aposymbiotic C. pedunculatus were initiated 9 months prior to experimental use, as described in chapter 4, section 4.2 (h) (pp.180-181). Five replicates of each different reinfection were performed, although in the case of the reinfections with



Key for fig. 5.1:

A = Anthozoan

AS = Air supply

C = Respirometry chamber

CR = Chart recorder

E = Oxygen electrode

H = Heater used for circulating water

L = Lamp

M = Magnetic stirrer

O_2 = air equilibrated sea water

O_2M = Oxygen meter

R = Rheostat

W = Water bath

Figure 5.1: Diagram of the respirometry apparatus.

symbionts from A. ballii and I. sulcatus only 4 of the replicates survived until the initiation of the investigation. These anthozoans had been maintained under identical conditions to the 'natural' symbioses. The oral disc diameter of the reinfected C. pedunculatus was approximately 2-3 mm upon reinfection. All experimental animals were not fed for 3 days prior to use.

(b)Measurement of net symbiosis photosynthesis, respiration and gross photosynthesis.

The apparatus used to determine oxygen evolution and consumption is shown in figure 5.1 (p.229). The apparatus consisted of a transparent cylindrical perspex respirometry cell of volume 235 ml, 82.2 ml or 57.6 ml, or a clear glass respirometry cell of 7.4 ml or 5.0 ml volume. The respirometry chamber used depended on the size of the anthozoan being investigated. To prevent interference by bacterial respiration during experimental determinations, the respirometry chamber was washed for 1 hour prior to use in 'chloros' (sodium hypochlorite) (Hays Chemical Distribution Lmtd.) and thoroughly rinsed in autoclaved FSW. The respirometry chambers possessed a polythene covered magnetic 'flea' spin bar which was overlaid by a protective perforated false floor and a layer of nylon mesh. A second piece of mesh was placed over this to reduce water flow if less disturbance to the animal was required. All the mesh was held firmly in place using plasticine. The respirometry

chamber was placed on a magnetic stirrer with adjustable speed, which provided a homogeneous distribution of dissolved oxygen, and placed in a glass sided water bath at 21 ^{o}C in a temperature controlled room. The respirometry chamber possessed a perspex lid with a tapered aperture into which a Radiometer E5046 oxygen electrode (Strathkelvin Instruments) was inserted. The oxygen electrode contained electrolyte solution (Strathkelvin Instruments) and was covered by a polypropylene membrane (Strathkelvin Instruments) which was replaced at least weekly. The oxygen electrode was connected to an oxygen meter which was linked to a 'Graphic 1000' chart recorder (Lloyds Instruments). The chart recorder was set at a sensitivity of 100 mV and a paper speed of 1 mm/min. The entire apparatus was calibrated to 0 % and 100 % oxygen daily before use. 0 % calibration was achieved by placing the electrode in sea water which had been saturated with sodium dithionite. Subsequently, the electrode membrane was washed thoroughly with distilled water. 100 % calibration was achieved by placing the electrode in a flask containing air-equilibrated FSW until the O_2 meter reading stabilized. The air-equilibrated sea water was prepared by bubbling air overnight through a flask of FSW held in the water bath at 21 ^{o}C. The flask containing the water was bunged to prevent evaporation.

Prior to photosynthetic and respirometry measurements, a control was run to ensure that the respirometry chamber, sea

water and electrode were free of significant bacterial contamination. The respirometry cell was filled with UV treated FSW which had been equilibrated in air to 21 ^{o}C and, ensuring no air bubbles were trapped in the chamber, sealed with the lid using silicone grease. The light in the temperature controlled room was then switched off giving complete darkness and the chart recorder turned on for 1 hour to determine if any microbial respiration was occurring. If considerable respiration was detected the equipment was resterilized, but if respiration was considered minimal the rate of respiration was later subtracted from the experimental rates measured.

Before being placed in the respirometry chamber, all Anthozoa were cleaned of debris by washing in FSW. I. sulcatus polyps were cleaned using a seeker and a binocular microscope to remove sand particles attached to the column; the thin piece of slate to which many of the I. sulcatus polyps were still attached was trimmed with a scalpel but not removed from the base of the column. The volume of each polyp of adult C. pedunculatus, A. ballii and A. viridis was then estimated using a measuring cylinder. The volumes of A. pallida, I. sulcatus and reinfected C. pedunculatus were assumed to be negligible compared to the volume of the chamber in which they were placed.

The experimental anthozoan was then settled in the unsealed respirometry chamber, which contained 21 ^{o}C equilibrated UV treated FSW. This chamber was placed on the magnetic stirrer

and illuminated from the side at 40 $\mu E/m^2/s$ with a Thorn PAR 38 150 W sealed beam reflector lamp controlled by a rheostat. The irradiance was measured using a QSL-100 irradiance meter (Biospherical Instruments Inc.). The animal was settled for 5-6 hours or until it appeared stationary. Because the diffusive boundary layer around anthozoans differs in thickness depending upon the size and morphology of the animal (Shick 1990), the magnetic stirrer was adjusted to give the maximum current speed (and hence the thinnest boundary layer) at which the animal did not appear stressed. This current speed was not adjusted until the next animal was placed in the chamber. Thus, the rates of photosynthesis and respiration that were measured were the maxima for each individual anthozoan. Following settlement, 90 % of the water in the chamber was replaced with fresh, air and temperature equilibrated, UV treated FSW. The chamber was then sealed and the respiration rate was measured in darkness for approximately 1-2 hours, or until a constant rate of oxygen consumption was measured on the chart recorder. The oxygen tension was not allowed to fall below 50 % saturation (Spencer Davies 1984; Tytler and Spencer Davies 1984). Using the irradiance meter, the PAR lamp was then switched on and the net photosynthetic oxygen production rate measured at 40, 80, 120, 160, 200, 240, 320 and 400 $\mu E/m^2/s$, or higher irradiances (in multiples of 40 $\mu E/m^2/ s$), until photosynthesis was saturated. In addition, net photosynthetic production by I. sulcatus was measured at

10 $\mu E/m^2/s$. This was because, unlike the other Anthozoa, I. sulcatus had exceeded its compensation point at 40 $\mu E/m^2/s$. Photosynthetic rates at each irradiance were recorded until a constant rate had been recorded for 15-20 minutes. 5 replicate irradiance sequences were performed for C. pedunculatus, A. ballii, A. viridis (Lough Hyne and Shell Is.) and A. pallida, using a different individual for each set of measurements. Attempts to settle individual I. sulcatus and reinfected C. pedunculatus polyps in the 5.0 and 7.4 ml respirometry chambers were unsuccessful. Under these conditions these hosts rarely opened and occasionally produced copious amounts of mucus, indicating stress. Therefore, 10 polyps of I. sulcatus or 4/5 polyps of reinfected C. pedunculatus were used in the 57.6 ml chamber simultaneously. 3 replicate series using 10 different polyps on each occasion were run for I. sulcatus. Because only 4 or 5 replicate reinfected C. pedunculatus were available, the same polyps were subjected to 3 replicate irradiance series. Once the respiration and photosynthetic rates of the I. sulcatus polyps had been measured, the pieces of rock to which the animals had been attached were removed with a razor blade and placed alone in the respirometry chamber and their contribution to the recorded respiration rate estimated. This value was then subtracted from the measured respiration rate of the I. sulcatus polyps plus rock.

All Anthozoa used in respirometry work were saved in clearly labelled 100 ml polystyrene containers for carbon standing

stock and protein analysis within the following 48 hours.

The rates of respiratory oxygen consumption and photosynthetic oxygen production were calculated from the resultant chart recordings using values of temperature=21 ^{o}C and salinity=34 ^{o}/oo, the volume of sea water in which the animal was placed (ie. the original chamber volume - the volume of the anthozoan), the gradients of the chart recordings and the scale representing 0-100 % O_2 saturation. All these values were incorporated into a computer program available at the School of Ocean Sciences, Menai Bridge which calculated the rates of O_2 consumption and production in ml O_2/hour (c/o Dr. A.B. Yule).

The resultant rates of photosynthetic O_2 production represent the net change in oxygen tension within the sealed chamber. The gross photosynthetic production at each irradiance was calculated by adding the dark respiration rate to the net change in O_2 due to photosynthesis according to the equation:

$$\mathbf{P\ (gross) = P(net) + Rs} \quad \text{(equation 5.2)}$$

where P(gross) = gross photosynthetic production by symbionts, P(net) = net photosynthetic production by intact association and Rs = respiration of intact symbiosis.

It was assumed that no photorespiration, the consumption of O_2 and the production of CO_2 during the metabolism of phosphoglycolate in the C_3 fixation pathway, was occurring.

It was then necessary to standardize the gross photosynthetic rates to the total protein content of the association.

(c)Protein determination

Protein determination was performed using the method of Lowry et al. (1951), which is believed to be more accurate in determining the protein contents of Cnidaria than the Biuret or Bradford methods (Zamer et al. 1989). The method used was adapted by Dr. W. K. Fitt (R. Day, University of the West Indies, pers. comm.).

The following solutions were prepared: 1N NaOH, 2 % Na_2CO_3, 1 % $CuSO_4.5H_2O$ and 2 % Na tartrate. All solutions were prepared in 'miliQ' (0.2 µm) filtered distilled water. 'Reagent B' was then made up just prior to use by mixing 0.5 ml 1 % $CuSO_4.5H_2O$ and 0.5 ml 2 % Na tartrate in 49 ml 2 % Na_2CO_3. 'Reagent E' was also prepared just before use by diluting 2 N Folin-Ciocalteu reagent (Sigma Chemical Co.) to 1 N with 'miliQ' filtered distilled water. A protein standard, using bovine serum albumen (BSA) (Sigma Chemical Co.) which had been stored frozen, was also made up using 1 mg BSA/ml 'miliQ' filtered distilled water. The BSA standard was stored in a refrigerator when not in use and new stock solution was prepared every month.

The anthozoan used in the respirometry work was homogenized in a hand held glass tissue grinder in a known volume of 'miliQ' filtered distilled water. Each anthozoan was

prepared separately, including each reinfected C. pedunculatus, with the exception of the 10 I. sulcatus polyps used in each replicate respirometry series which were homogenized together. The volume in which the anthozoan was homogenized depended on the size of the polyp: Adult C. pedunculatus, A. ballii and A. viridis were homogenized in 10 ml distilled water, I. sulcatus polyps were homogenized in 5 ml distilled water, A. pallida were homogenized in 3 ml distilled water and reinfected C. pedunculatus were homogenized in 2 ml distilled water.

A dilution series of the BSA standard was then prepared by pipetting 0, 20, 40, 60, 80 and 100 µl of BSA solution into 10 ml boiling tubes using a micropipette with a 100 µl pipette tip (Brand). The final volume in each tube was adjusted to 100 µl with 'miliQ' filtered distilled water. 2 replicate tubes were prepared for each different dilution and the tubes clearly labelled. Three replicate tubes of the anthozoan homogenate were then prepared. 40 fold dilutions of the homogenate of adult C. pedunculatus, A. ballii and A. viridis were made to ensure that the final protein concentrations were comparable to those on the standard curve and that the concentrations would be below approximately 60 mg/ml where the standard curve begins to deviate from linearity. 100 µl of the homogenate was then pipetted into each of the 10 ml boiling tubes using a new pipette tip for each different homogenate.

0.3 ml of 1N NaOH was added to each standard and sample

using a 1 ml syringe and sterile hypodermic needle. The tubes were then placed in a rack in a boiling water bath for 10 minutes to solubilize the protein. Zamer et al. (1989) recommended that the protein content of the samples would be most accurately determined by adding deionized water to the standards rather than NaOH, but solubilizing low concentration samples in NaOH. In contrast to the findings of Zamer et al. (1989), appendix 9 (pp.508-512) shows that under the conditions used here, deionized water did not solubilize the BSA standard as successfully as boiling in NaOH. Thus, the solubilization of protein in NaOH was continued.

Following the 10 minute protein extraction period, the boiling tubes were cooled by placing them under running cold tap water. 3.0 ml of 'reagent B' was then added to each tube with a sterile 10 ml syringe, the tube contents were mixed immediately and the tubes left to stand for 10 minutes at room temperature. Over this period 'reagent B' complexes with the protein peptide bonds. Next, 0.3 ml of 'reagent E' was added to each tube, the contents mixed again and the tubes left for a further 30 minutes at room temperature. During this time the colour of the standard and sample solutions developed, giving a blue colouration proportional to the protein at concentrations below approximately 60-70 mg/ml. The absorbance of each standard and sample was then measured by placing the solutions in a nitric acid (HNO_3) washed 4 ml silica cuvette and reading the absorbances at

750 nm (to avoid interference from chlorophylls) on a Shimadzu UV-1201 UV-vis spectrophotometer. The standards and samples were all blanked against the 0 % BSA standard. A standard curve was then constructed, averaging the 2 replicates of each standard dilution. The 3 replicate values of the samples were averaged and their protein contents determined from the standard curve. The total protein content of the polyp(s) was calculated by multiplying the value read from the standard curve by the dilution factor of the sample and multiplying the resultant value by the volume in which the polyp(s) was originally homogenized.

The protein content of the symbiont population was estimated indirectly using the method of Muscatine et al. (1983) and Hoegh-Guldberg et al. (1986). A 1 ml subsample of the homogenate used in the protein determination was taken and the number of symbionts present per ml, and hence in the entire association determined using a modified Fuchs Rosenthal type haemocytometer. Following this, the number of symbionts was converted into the algal standing stock of the association by multiplying by the average symbiont cell carbon content, which is given in tables 3.6 (p.133) and 4.3 (p.200). Total algal protein was then estimated from the total standing stock of carbon by using a C:N ratio of 6.1:1. The carbon standing stock was divided by the C:N ratio to obtain the nitrogen content and the protein content was calculated from this value by assuming algal protein to be N X 6.25.

The ratio of algal symbiont protein : total symbiosis protein could therefore be calculated.
The standing stock of algal carbon/mg symbiosis protein was also calculated by dividing the total carbon content of the symbiont population by the total symbiosis protein content.
In addition, the symbiont cell standing stock and the total protein content of 5 further individuals of each host species, except I. sulcatus, was estimated and a plot of biomass vs. symbiont number drawn to investigate the linearity of the relationship (appendix 10 (pp.513-519)).

(d)Calculation of gross photosynthesis at saturation, net algal photosynthesis and algal and anthozoan respiration.

The rates of gross photosynthesis and association respiration were standardized to the total protein contents of the symbioses. Despite the protein ratios of the reinfected symbioses being determined for each polyp used, the protein contents of each different reinfection were pooled for all standardizations of gross photosynthesis and symbiosis respiration. Therefore, the total protein biomass contributing to photosynthesis and respiration in the respirometry chamber was accounted for. **All photosynthetic and respiratory rates discussed hereafter refer to rates standardized to the protein content of the association, except where stated.** Photosynthesis vs. irradiance (P vs. I) curves were then fitted 'by eye' (Spencer Davies 1984) for each host species or different reinfection, using the

average gross photosynthetic rate at each irradiance. From these graphs, the approximate irradiance at which saturation of gross photosynthesis occurred was estimated by identifying the irradiance at which the photosynthetic rate ceased to increase. The gross photosynthetic rates at and above the saturation irradiance were then averaged for each replicate. The irradiance at which photosynthesis was 95 % saturated (I(0.95)) was calculated by identifying the intersect, Ik, of the slope of the initial linear portion of the P vs. I curve ('a') with the average gross photosynthetic production at saturation (P(gross)max.), and multiplying Ik by 1.832 (Chalker 1981). I(0.95) has been used as an estimate of the saturation irradiance (Chalker 1981; Chalker et al. 1983; Spencer Davies 1991) and was compared with the saturation irradiance measured 'by eye'. Some P vs. I curves did not appear as the expected hyperbolic tangent function shape, but rather as rectilinear. As applying the hyperbolic tangent function to these curves would have resulted in overestimates of saturation irradiance, the rectilinear equation, where P(gross)max. is attained at irradiances above Ik, was applied (Chalker 1981). The gradient of 'a' and the compensation point, where gross photosynthesis equalled symbiosis respiration, were also noted.

The net algal photosynthetic rate was then calculated from the average rate of gross photosynthesis at saturation of each replicate anthozoan.

The assumption was made that the respiration rates of the symbionts and the host were proportional to their biomass ratio, as determined by the Lowry protein test (Muscatine et al. 1981; Muscatine et al. 1983; Hoegh-Guldberg et al. 1986).

This relationship was summarised by the equations of Muscatine et al. (1981):

$$B = \frac{Ra}{Rs} \quad \text{(equ. 5.3)} \qquad \text{and} \qquad 1-B = \frac{Rz}{Rs} \quad \text{(equ. 5.4)}$$

where B = the proportion of the symbiosis biomass attributable to the host, Ra = animal respiration, Rz = symbiont respiration and Rs = total symbiosis respiration.

The respiration rates of the symbiont population and host were calculated from the respiration rate of the intact symbiosis, using the calculated protein ratios. The rate of net algal photosynthesis (Pz(net)) was then calculated by subtracting algal respiration (Rz) from gross photosynthesis (P(gross)) according to the equation:

$$Pz(net) = P(gross) - Rz \quad \text{(equ. 5.5)}$$

Animal respiration, algal respiration and net algal photosynthesis were calculated for each reinfected polyp, assuming each replicate symbiosis to respire and photosynthesize at the same rate.

In order to investigate their influence on the CZAR, the photosynthetic and respiration rates of the symbiont populations and hosts in the different symbioses were compared using Kruskal-Wallis and multiple comparison tests. However, the rates of net algal photosynthesis and animal respiration in the reestablished symbioses were not compared statistically, because of the method by which replicate values were obtained resulting in little variation in the data.

Net algal photosynthesis was also standardized to symbiont density (cells/mg protein) and the association biomass standardized carbon standing stock (pg symbiont carbon/mg protein), giving the cell and biomass specific rates respectively. This enabled the photosynthetic capacity of different 'strains' of Symbiodinium when in C. pedunculatus and when in the original host species to be investigated. Standardized rates of net algal photosynthesis were compared between symbionts in 'natural' and/or reestablished symbioses using ANOVA, Scheffe's test or Student's t-test, again at the 5 % level.

(e)Conversion of photosynthetic oxygen production and respiratory oxygen consumption into carbon equivalents

To incorporate the photosynthetic and respiratory values into the computation of the CZAR, it was necessary to convert the oxygen values (ml O_2/h/mg association protein) into carbon units (mg C/h/mg association protein) using the equations of McCloskey et al. (1978) or the corrected equations of Parsons et al. (1984). The relevant equations are as follows:

(i) **ml O_2/l = 11.2 x mg-at O_2/l** (equ. 5.6)

(ii) **mg O2/l = 16.0 x mg-at O_2/l** (equ. 5.7)

(iii) **mg O_2 consumed per unit time x 12/32 x RQ = mg C used per unit time** (equ.5.8)

(iv) **mg C photosynthesized per unit time = (mg O_2 produced per unit time/ PQ) x 12/32** (equ. 5.9)

These equations can be summarised by :

(v) **mg C = 0.535 x ml O_2 [divided by PQ or x RQ]** (equ. 5.10)

Where PQ = the molecular photosynthetic quotient, + O_2/ - CO_2, which was assumed to be 1.1 and RQ = the molecular respiratory quotient, + CO_2/ - O_2, which was assumed to be

0.8 (Muscatine 1990).

(f)Estimates of the percentage of fixed carbon translocated to the host in 'heterologous' and 'homologous' symbioses.

3 methods of measuring the percentage translocation were employed:

(i)In vivo ^{14}C fixation and translocation

Firstly ^{14}C was used to label fixed carbon in vivo. A stock solution of ^{14}C labelled sodium bicarbonate ($NaH^{14}CO_3$) (Sigma Chemical Co.) was prepared by dissolving the $NaH^{14}CO_3$ in 'miliQ' filtered distilled water to give a final concentration of 10 µCi/ml. The stock solution was stored frozen in glass minivials which had been washed in both chromic acid and 10 % 'Decon' detergent to remove contamination (both particulate and radioactive).

The incubation apparatus was set up on a designated ^{14}C work bench. This apparatus consisted of a glass sided water bath set at 21 ^{o}C which was permanently covered in black plastic sheeting on 3 sides and across the top; the fourth side was left uncovered for light incubations but was also covered in sheeting when dark incubations were being performed. A rack to hold the incubation flasks or tubes was suspended just behind the glass window of the uncovered side of the water bath. The water bath was illuminated from the side with a Thorn 150 W PAR 38 sealed beam reflector lamp. No rheostat was available for this lamp, therefore the illumination (as determined using the QSL-100 irradiance meter) was changed by adjusting the distance of the lamp from the water bath.

Individual anthozoans were cleaned of debris before use and were settled at 80 $\mu E/m^2/s$ for 3 hours in 50 ml UV treated filtered sea water (FSW) in 100 ml Duran Schott transparent conical flasks, except in the case of I. sulcatus where 5 polyps were settled in each flask. The polyps were the same size to those used in the respirometry work. 3 replicate flasks for each host species were prepared for dark controls, except with respect to the reinfected C. pedunculatus where 3 replicates of the 'homologous' reinfection also acted as dark controls for all of the 'heterologous' reinfections. 5 replicate flasks of each host species were prepared for light incubations, except in the case of those reinfected C. pedunculatus where only 4 replicates were available and I. sulcatus where 3 replicate flasks were used. 50 % of the sea water was then decanted and replaced with fresh FSW to prevent stress due to the build up of waste material. Following this, the flasks were placed in the water bath and illuminated at their photosynthetic saturation irradiance, or were enclosed in the dark if a control was being performed. The dark control prevented photosynthetic carbon fixation but measured bacterial fixation and non-photosynthetic fixation by the symbionts and animal tissue. Ensuring that any control flasks were maintained in the dark, 5 μCi of $NaH^{14}CO_3$ (ie. 0.5 ml ^{14}C stock solution) was then pipetted into each flask using an Eppendorf micropipette. The flasks were immediately bunged with non-adsorbent cotton wool and the ^{14}C labelled

sea water mixed. The Anthozoa were subsequently incubated for 3 hours under constant conditions. The incubation period had been found not to influence the percentage translocation in A. viridis (Shell Is.), which were treated in an identical manner to all the experimental anthozoans except that 3 replicate animals were incubated for 1, 2, 3, 4 and 5 hours. Following the incubation period, the flasks were removed and placed in a sealed black photographic bag to prevent further carbon fixation and translocation; translocation only occurs during (and possibly for a short time after) a period of photosynthesis (Hoegh-Guldberg 1981, in Hinde 1988; Black and Burris 1983; Muscatine et al. 1984). All further procedures, using equipment decontaminated with 'Decon', were carried out in dim light. The FSW was decanted from each flask into marked, 'Decon' washed polystyrene containers. The anthozoans were washed in a further 50 ml of unlabelled FSW and this water was added to the 50 ml of labelled water already decanted, giving a final volume of 100 ml. The polyps were then homogenized in a glass tissue grinder. Adult C. pedunculatus, A. ballii and A. viridis were homogenized in 5 ml FSW, I. sulcatus and A. pallida were homogenized in 2 ml FSW and reinfected C. pedunculatus were homogenized in 1 ml FSW. The homogenates were poured into 10 ml plastic centrifuge tubes and centrifuged at x1200 r.p.m. for 10 minutes. The resultant supernatants were pipetted into labelled polystyrene containers using a new pasteur pipette for each different

sample. The algal pellets were then washed in 0.1 % sodium dodecyl sulphate (SDS) (Sigma Chemical Co.) in FSW to strip host material from around the algal cells at x1200 r.p.m. for a total of 10 minutes, shaking the tube after 5 minutes to free more host material. The supernatant was then added to the host material already extracted. The algal pellet was washed a final time in FSW at x3000 r.p.m. for 5 minutes and the supernatant again pooled with the other host homogenate. The volumes of sea water used in these 2 washes depended on the host species being analysed. Both washes were 10 ml with respect to adult C. pedunculatus, A. ballii and A. viridis, 1 ml with respect to I. sulcatus and A. pallida, and 0.5 ml for reinfected C. pedunculatus. The volumes of the pooled animal homogenates were made up to precisely 30 ml for C. pedunculatus, A. ballii and A. viridis, 5 ml for I. sulcatus and A. pallida, and 3 ml for reinfected C. pedunculatus using FSW. The algal pellet from C. pedunculatus, A. ballii and A. viridis was resuspended in 5 ml FSW, and the pellets from I. sulcatus, A. pallida and reinfected C. pedunculatus were resuspended in 2 ml FSW. Using a specimen of A. viridis (Shell Island) of oral disc diameter 4 cm, the effectiveness of this isolation procedure was checked by observing the host and algal fractions under the light microscope. A haemocytometer count revealed that there were only 16,680 symbiont cells per total sample of host homogenate following this procedure, just 0.05 % of the number present in the original homogenate. Host

contamination of the symbiont fraction was not quantified, but the absence of algal clumping suggested that the attempted removal of host debris had been largely successful.

The samples were then prepared for radioactivity counting. Using an Eppendorf micropipette with a new tip for each sample, 0.5 ml sample was pipetted into a clean glass screw topped scintillation vial. 3 replicates of individual samples were made once in every second series of incubations to ensure preparatory procedures were giving consistent results. 3.0 ml 20 : 1 methanol : glacial acetic acid (BDH) (Dr. C. M. Woods, UCNW, Bangor, pers. comm.) was then pipetted into each scintillation vial to drive off any unfixed bicarbonate and the vials placed open topped in a fume hood overnight. The efficiency of acidification had previously been tested, showing that only 0.01 % unfixed bicarbonate remained after acidification (appendix 12, table 1 (p.532)). 10 ml 'Aquasol' universal liquid scintillation cocktail (NEN Research Products, DuPont) was then pipetted into each scintillation vial, ensuring that the 'Aquasol' was not exposed to fluorescent lighting leading to erroneous measurements of the disintegrations per minute (d.p.m.) when scintillation counting. The capped scintillation vials were shaken vigorously and the absence of 'micelles', which indicate incomplete mixing, was ensured. Keeping all the vials in a black plastic photographic bag, the vials were transported to a Hewlett-Packard Canberra Tricarb - 1900 CA

TABLE 5.1 CONSTRUCTION OF A CHANNELS RATIO CALIBRATION CURVE

'Aquasol' (cm^3)	Xylene (cm^3)	Chloroform (cm^3)	^{14}C-Hexadecane (d.p.m.)
8.0	2.0	-	8720
8.0	1.8	0.2	8720
8.0	1.6	0.4	8720
8.0	1.3	0.7	8720
8.0	1.0	1.0	8720
8.0	0.5	1.5	8720
8.0	0.25	1.75	8720
8.0	-	2.0	8720

liquid scintillation counter (LSC).

A ^{14}C quench curve had been previously programmed into the LSC using the Channels Ratio method. The Channels Ratio method is based on the downward shift of the pulse height spectrum of the radionuclide in the presence of a quencher. The extent of the shift is related to the counting efficiency of the LSC system (Peng 1977). 4 steps were involved in the Channels Ratio method. Firstly, 2 channels were selected to count different portions of the pulse height spectrum. Secondly, a series of quenched standards of known d.p.m., which was prepared as shown in table 5.1 (p.250) (using chloroform as the quencher and xylene to adjust the volumes), was counted on the LSC. Thus, the channels ratio (the Quench Indicating Parameter (QIP)) and the counts per minute (c.p.m.) of the standards were obtained. Thirdly, the efficiency was calculated by:

$$\text{Efficiency} = \frac{\text{c.p.m.}}{\text{d.p.m.}} \times 100 \quad \text{(equ. 5.11)}$$

Finally, the efficiency of the first channel was plotted against the QIP and the plot stored in the LSC memory for future use. All samples were subsequently counted for 5 minutes and were referred to this plot to convert the c.p.m. of the sample into d.p.m. via the QIP. The QIP of all samples was also checked to ensure that the efficiency was not less than 40 %, the lower limit for satisfactory degrees

of quenching.

Next, the d.p.m. of the samples measured were adjusted to the volumes of the original algal, host and sea water fractions. The d.p.m. of the 3 different fractions following incubation in the dark were averaged and subtracted from each replicate value of the same fraction of the corresponding host species following incubation in the light. The control corrected d.p.m. of each individual were then pooled and the percentage of the total d.p.m. present in the algal, host and sea water fractions determined. The percentage translocation was calculated by adding the percentage of the total d.p.m. measured in the sea water (assumed to be fixed carbon released from the host as mucus or dissolved organic carbon (DOC)) to the percentage of the total d.p.m. measured in the host fraction.

Percentage translocation was compared between host species and between reinfections using Student's t-test, ANOVA and Scheffe's test as appropriate.

(ii)<u>In vitro ^{14}C fixation and translocation</u>

In response to the results obtained from the <u>in vivo</u> ^{14}C incubations of reinfected <u>C. pedunculatus</u>, it was attempted to replicate the response of the symbionts immediately following endocytosis by already symbiotic <u>C. pedunculatus</u>. Translocation by the symbiont cells upon endocytosis by aposymbiotic <u>C. pedunculatus</u> could not be simulated due to the limited supply of aposymbiotic host tissue. Photosynthate release by <u>Symbiodinium</u> cells from <u>C.</u>

TABLE 5.2 COMBINATIONS OF SYMBIONTS AND HOMOGENATES (FROM NAMED HOST SPECIES) USED FOR IN VITRO ^{14}C INCUBATIONS (combinations used marked with an asterisk).

SYMBIONTS	HOMOGENATES										
Host sp	Cp	Cp Nt	Ab	Is	Ap	Av LH	Av SI	Av TB	Av LS	Av WH	Av Bm
Cp	*	*	*	*	*	*	*				
Cp Nt	*	*									
Ab	*		*								
Is	*			*							
Ap	*				*						
Av LH	*					*	*	*	*	*	*
Av SI	*					*	*				
Av TB						*		*			
Av LS						*			*		
Av WH						*				*	
Av Bm						*					*

pedunculatus and A. viridis from different geographical locations in response to conspecific host homogenates from different locations, and photosynthate release by C. pedunculatus (Lough Hyne) symbionts in response to homogenates of other host species were also investigated. Thus, it could be inferred whether specificity exists in these symbioses with respect to the stimulation of photosynthate release. The combinations of host homogenates and symbiont pellets incubated together are shown in table 5.2 (p.253).

The homogenate and algal fractions were prepared as follows. 9.5 ml FSW was made up to 10 ml with oral disc material (where C. pedunculatus fractions were required) or tentacles excised from 3 individual polyps; all the anthozoans used had previously been cleaned of debris and squeezed to remove air or water. The 10 ml of anthozoan material + FSW was then homogenized in a glass tissue grinder and the resultant homogenate centrifuged once at x 3000 r.p.m. for 10 minutes to obtain a predominantly symbiont free supernatant. The supernatant was filtered through a piece of 5 μm mesh plankton net using a 10 ml syringe to remove any remaining algal cells and large pieces of host tissue. Finally, the host homogenate was adjusted to a volume of 10 ml with FSW and used within an hour. When a sample of host material prepared this way was observed under the light microscope on the x 25 objective it was seen to be free of algal contamination. 0.125 ml wet packed Symbiodinium cells was

obtained by washing the algal pellet remaining after preparation of host homogenate 2 times at x1200 r.p.m. for 5 minutes. No SDS was used in the preparation procedure in case of detrimental effects upon the permeability of the symbiont cell membranes leading to erroneous estimates of the percentage translocation. To remove contaminating host tissue the algae were resuspended in 10 ml FSW and filtered through 15 μm plankton mesh using a 10 ml syringe. The filtered algae were washed a final time at x 3000 r.p.m. and the supernatant decanted. The volume of the final wet packed symbiont pellet was adjusted to 0.125 ml and the pellet was resuspended in 8 ml FSW. 1 ml of algal suspension was then pipetted into each plastic centrifuge tube to be used in the incubations. These tubes were all centrifuged once at x 3000 r.p.m. for 2 minutes and the supernatants discarded. 2 ml of the host homogenate was then pipetted into each tube and shaken vigorously to resuspend the algal pellet. The tubes were placed in the water bath at 21 ^{o}C, enclosed in the dark if controls or illuminated at 400 $\mu E/m^2/s$ if light incubations and labelled with 5 μCi $NaH^{14}CO_3$ as before. The incubation periods lasted 3 hours, during which the tubes were shaken every 30 minutes to maintain the algal cells in suspension and prevent self-shading. Five replicates of each light incubation were performed. 3 dark control replicates, using 'homologous' C. pedunculatus incubations, acted as the controls for all incubations.

Despite the attempts to cleanse the algal material of host

debris, photosynthate release by algae in response to host contamination was quantified. This was performed by incubating 3 algal pellets from each host species in FSW, without any additional host homogenate. Three dark controls of this incubation were also carried out. The percentage release occurring in FSW may also reflect 'natural' excretion or release due to damage incurred during isolation.

Following incubation, the tubes were maintained in the dark or in dim light at all times and were centrifuged once at x 3000 r.p.m. for 5 minutes. All large pieces of host tissue had been removed by the filtering procedure, thereby minimizing the possibility of contamination of the algal cells when centrifuged at this speed. The supernatant was pipetted off into a polystyrene container and both the algal pellet and host homogenate/host-free sea water made up to 3 ml with FSW. The radioactivity of the algal and host/host-free sea water fractions was determined as for the <u>in vivo</u> incubations. The d.p.m. of the algal fraction of each light incubation was checked to ensure that cell lysis, which results in a cessation of photosynthesis (Sutton and Hoegh-Guldberg 1990), had not occurred. Once the absence of cell lysis had been established, the average d.p.m. of the dark control was subtracted from the average d.p.m. of the corresponding light incubated fraction, and the percentage translocation in each replicate calculated. The percentage translocation resulting from host contamination or 'natural'

release was then subtracted from the percentage translocation by the symbionts in the host homogenates, giving the percentage translocation due to stimulation by the added host homogenate only.

Photosynthate release was compared between the different incubations using Student's t-test, ANOVA and Tukey's test, or the Kruskal-Wallis test and multiple comparisons as appropriate.

(iii) 'Growth rate method' of estimating the percentage translocation of fixed carbon from symbiont to host.

As a comparison to the ^{14}C methods, which are believed to underestimate percentage translocation (Muscatine et al. 1983; Muscatine et al. 1984; Muscatine 1990), the percentage of photosynthate translocated to the host was estimated indirectly using the 'growth rate method' of Muscatine et al. (1983). Unlike short term ^{14}C incubations, the 'growth rate method' estimates translocation over 24 hours. The percentage translocation was calculated for each individual polyp used in the respirometry work, except for I. sulcatus where the percentage translocation represented the average of the 10 polyps used in each replicate irradiance series.

It was assumed that fixed carbon not used in respiration or growth by the algal symbionts was translocated to the host.

Therefore, the carbon specific growth rate (μc) was calculated where:

$$\mu c = \frac{\text{net increment of C added per day}}{C'} \quad \text{(equ. 5.12)}$$

Where C' = standing stock of algal carbon.

The net increment of carbon added per day was equal to the net algal photosynthetic production, which was calculated from the equation:

$$\mathbf{Pz(net) = P(gross)\ (12\ hours) - Rz\ (24\ hours)} \quad \text{(equ. 5.13)}$$

Where Pz(net) = net algal photosynthesis, P(gross) = gross photosynthesis and Rz = algal respiration. All these values had been standardized to the biomass of the association. Algal respiration over 24 hours was subtracted due to the 24 hourly nature of the translocation estimate. The standing stock of algal carbon had been estimated earlier and standardized to symbiosis biomass.

The cell specific growth rate (μ) of the algal symbionts *in situ* in the 'natural' symbioses (table 3.8 (p.154)) and in the reinfected symbioses (table 4.2 (p.200)) can be expressed as:

$$\mu = \frac{\text{number of new cells added per day}}{\text{number of cells in standing stock}} \quad \text{(equ. 5.14)}$$

Therefore, if the net carbon added daily is used in symbiont growth or otherwise translocated then:

$$\frac{\mu c - \mu}{\mu c} \times 100 = T \text{ (the percentage translocation)} \quad \text{(equ. 5.15)}$$

The percentage translocation was calculated using average estimates of the cell specific growth rate for each host species or different reinfection. Different values of net algal photosynthesis and algal carbon standing stock were used for each individual anthozoan in the case of the 'natural' symbioses. However, because gross photosynthesis and symbiosis respiration of all replicates of reinfected C. pedunculatus were measured simultaneously, the only varying figures used in the calculation of the carbon specific growth rate in the reinfected symbioses were the protein ratios of the symbionts : host, and hence algal respiration and the standing stock of algal carbon.

The percentage translocations estimated by the 'growth rate method' were compared between different 'natural' and reinfected symbioses, and with the percentage translocations measured by in vivo ^{14}C incubations, using Student's t-test, the Kruskal-Wallis test and multiple comparisons.

(g)Calculation of the CZAR in 'natural' and reestablished symbioses.

As stated in the introduction to this chapter, the contribution of the "zooxanthellae" to the daily respiratory carbon requirements of the host (CZAR) is calculated by the equation:

$$CZAR = \frac{Pz(net).\ T}{Ra} \times 100 \quad \text{(equ. 5.1)}$$

Where Pz(net) = the net algal photosynthetic carbon production at saturation over 12 hours (the light period during maintenance in the laboratory), Ra = animal respiratory carbon utilization over a 24 hour period (assuming Ra to be the same during light and dark periods) and T = the percentage of fixed carbon translocated. The value of T was either the average figure measured by the ^{14}C *in vivo* incubations, or the value specific to each polyp used in respirometry work as determined by the 'growth rate method'. Translocation was assumed to only occur during periods of photosynthesis (Hoegh-Guldberg 1981, in Hinde 1988; Black and Burris 1983; Muscatine *et al*. 1984). The CZAR was calculated for each individual anthozoan used in the respirometry investigations, with the exception of *I. sulcatus* where the CZAR was estimated for each group of 10 polyps used to measure photosynthetic production and respiration. The average CZAR of each 'natural' symbiosis

was compared using Kruskal-Wallis tests and multiple comparisons. However, only the CZAR calculated using the percentage translocation estimated by the 'growth rate method' could be compared between the reestablished symbioses. This was because the methodology by which the rate of net algal photosynthesis, the rate of animal respiration and the percentage translocation (using the ^{14}C method) were calculated for these symbioses resulted in little variation in the data. The CZAR values of the reestablished symbioses were then standardized to the algal number/mg association protein and the weight of symbiont carbon/mg association protein to investigate whether different 'strains' of symbionts are capable of contributing to the C. pedunculatus respiratory carbon budget to the same extent. The standardized CZAR values were compared using Student's t-test, the Kruskal-Wallis test and multiple comparisons, or ANOVA and Scheffe's test.

Finally, a 'Quattro' spreadsheet (Borland Inc.) was employed to model the influence of raised mitotic indices and symbiont respiration rates on the average CZAR of the 'natural' symbioses. Zamani and Brown (1992) claimed that counting the percentage of symbiont cells appearing as doublets may underestimate the mitotic index (MI) by 5 times, thus leading to overestimates of the percentage translocation by the 'growth rate method' and consequently overestimates of the CZAR. Therefore, the influence of a 5 fold increase in MI on the CZAR was estimated. The

relationship between the ratio of symbiont : host biomass and symbiont and host respiration rates also remains unproven and Muscatine (1990) suggested that the biomass model probably underestimates symbiont respiration. In vitro measurements of symbiont respiration have been shown to be up to 8 times higher than respiration rates predicted by the biomass model (McCloskey and Muscatine 1984; Muscatine 1990), so the influence of an 8 fold increase in the algal respiration rate was investigated. Finally, the effect of a simultaneous 5 fold increase in the MI and an 8 fold increase in the algal respiration rate on the CZAR was estimated.

(h)Diagrammatic representation of flux of photosynthetically fixed carbon from symbiont to host.

Flow diagrams showing the daily (24 hour) utilization of gross photosynthetically fixed carbon by the different carbon sinks in the intact symbioses were drawn. The percentage translocation obtained from the in vivo ^{14}C incubations and by the 'growth rate method' was incorporated into these diagrams. The carbon utilized in symbiont growth was estimated by subtraction of the carbon translocated and used in algal respiration from the gross photosynthetic production.

5.3 Results

(a)Measurement of gross photosynthesis vs. irradiance.

(i)Gross photosynthesis vs. irradiance in the 'natural' symbioses.

The average values of gross photosynthesis vs. irradiance for each 'natural' symbiosis are given in appendix 11, table 1 (p.521). These values are shown as photosynthesis (P) vs. irradiance (I) curves in graphs 5.1-5.6 (pp.265-267). The P vs. I curves also show the irradiance, Ik, at which the linear slope of the curve ('a') intersected the average gross photosynthetic rate at saturation (P(gross)max.). Ik, I(0.95), the saturation irradiance determined 'by eye', the slope 'a', the compensation irradiance and P (gross) max., as determined from the P vs. I curves, are given in table 5.3 (p.271). From table 5.3 it is evident that I(0.95) agreed with the saturation irradiances determined visually for the 'natural' symbioses, whose P vs. I curves all showed the characteristic shape of a hyperbolic tangent function curve (Chalker 1981).

From the graphs and tables it is evident that the I. sulcatus and A. pallida symbioses reached compensation point and photosynthetic saturation at low irradiances compared to the other 'natural' symbioses. In contrast, the symbionts of adult C. pedunculatus reached compensation point at a higher irradiance and did not saturate until approximately 571.6 $\mu E/m^2/s$, 2 to 3 times the saturation irradiance of the symbiont populations of the other anthozoan species.

(ii)Gross photosynthesis vs. irradiance in the reestablished symbioses.

The average rates of gross photosynthesis vs. irradiance for each reestablished symbiosis are shown in appendix 11, table 2 (p.522). P vs. I curves constructed from these results are given in graphs 5.7-5.12 (pp.268-270). Ik, I(0.95), the saturation irradiance determined 'by eye', the slope 'a', the compensation irradiance and the average P(gross)max. determined from graphs 5.7-5.12 are presented in table 5.4 (p.272).

In contrast to the situation with the 'natural' symbioses, I(0.95) clearly overestimated the saturation irradiance of the reestablished symbioses, with the exception of the curve for the 'homologous' symbiosis (graph 5.7 (p.268)). This was due to the rectilinear shape of these curves. But when the rectilinear assumption that saturation occurs at Ik (Chalker 1981) was applied, the saturation irradiances agreed with those determined 'by eye'.

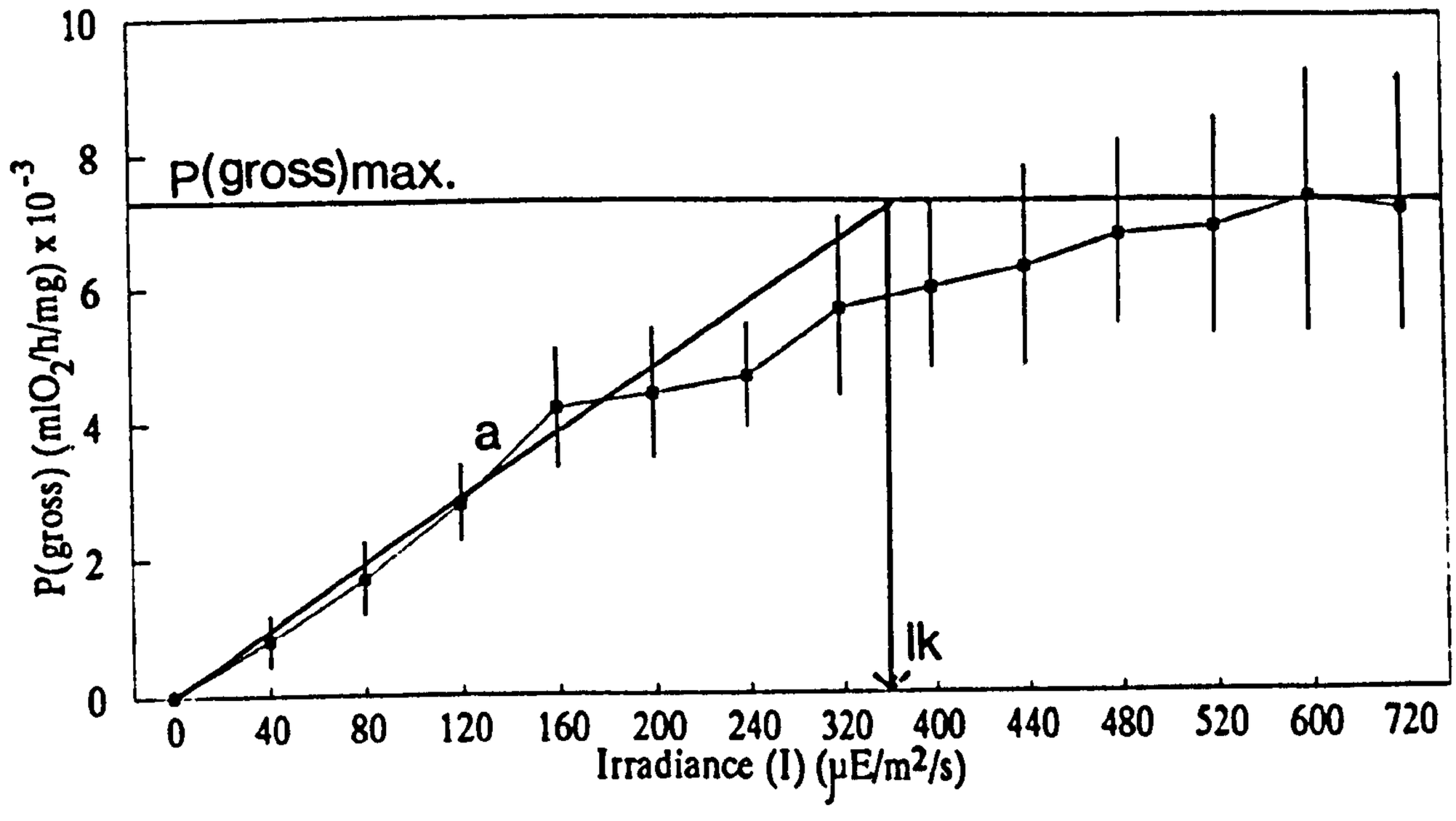
Of the reestablished symbioses, the 'homologous' associations saturated at a higher irradiance (168.5 $\mu E/m^2/s$) than the 'heterologous' symbioses, which saturated between 68 and 120 $\mu E/m^2/s$. There was little difference between the compensation points of the reestablished symbioses, which ranged from 28 to 64 $\mu E/m^2/s$.

Graphs 5.1 – 5.12: P vs I curves.

(P (gross) max.= gross photosynthesis at saturation, 'a' = slope of linear P vs I relationship and Ik = irradiance at intersect of 'a' and P(gross)max).

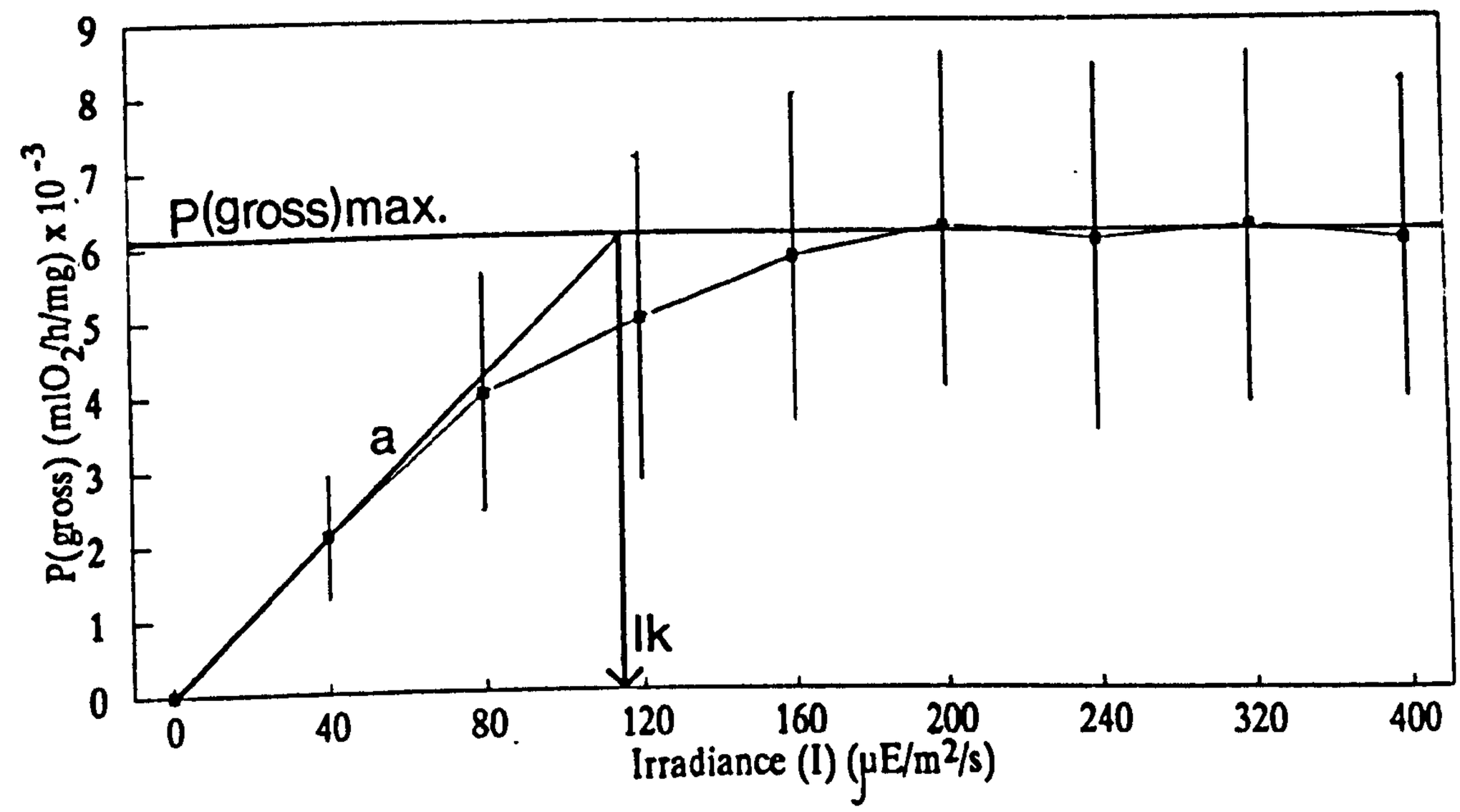
Graph 5.1: P vs.I curve for C. pedunculatus.

(N=5) (+/– S.E.).

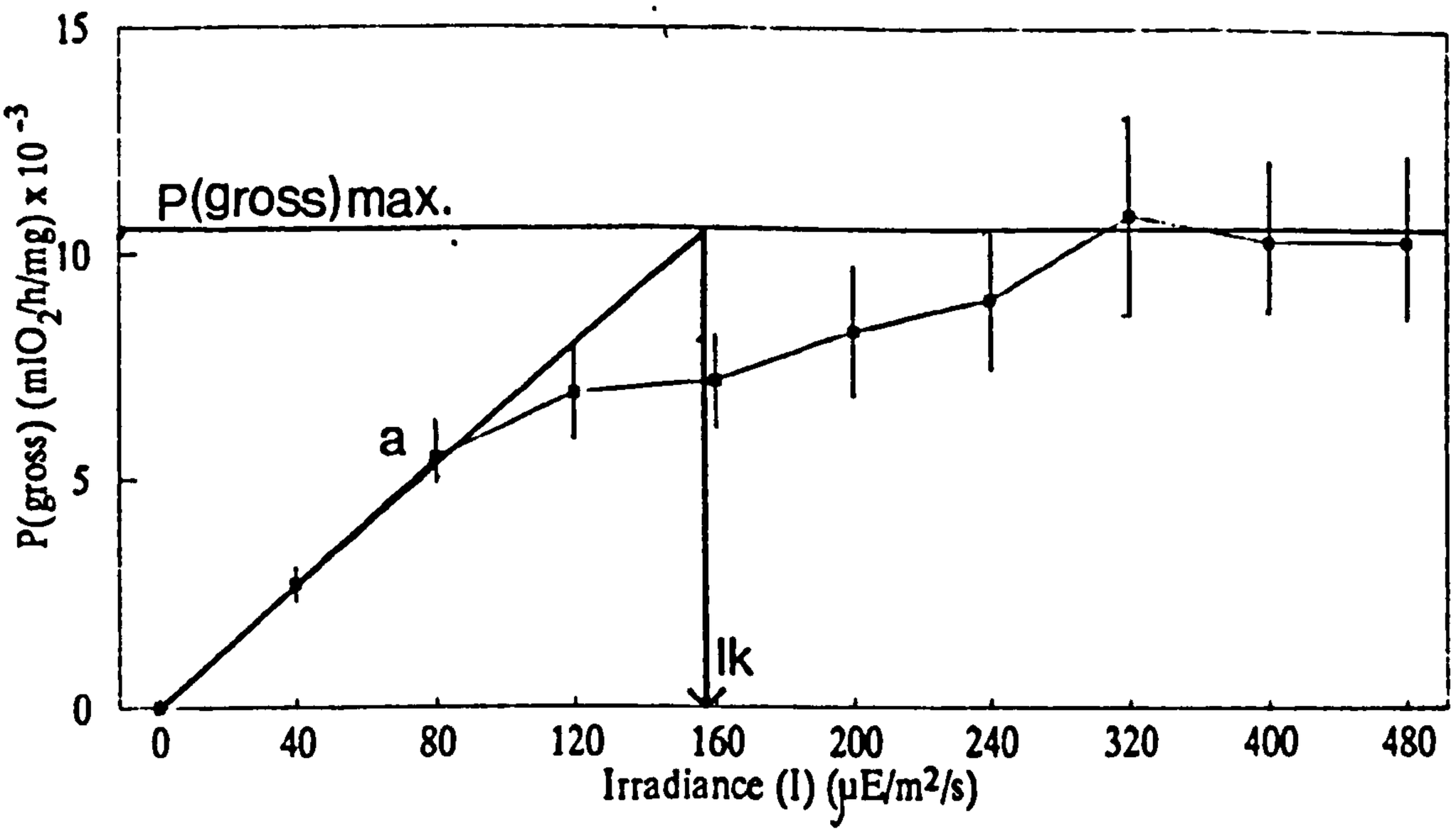


Graph 5.2: P vs.I curve for A. ballii.

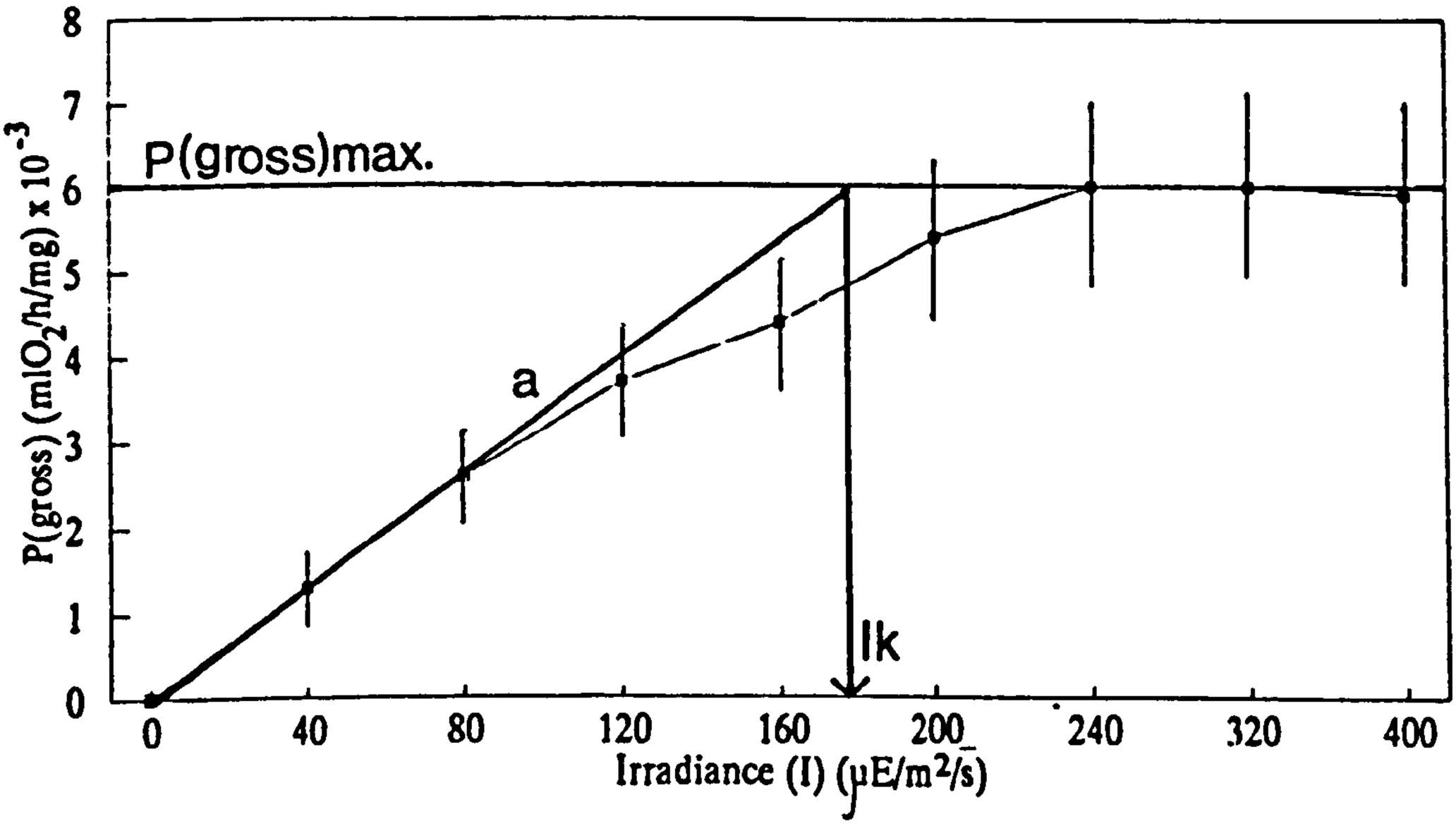
(N=5) (+/– S.E.).



Graph 5.3: P vs.I curve for A. viridis (Lough Hyne).
(N=5) (+/- S.E.).

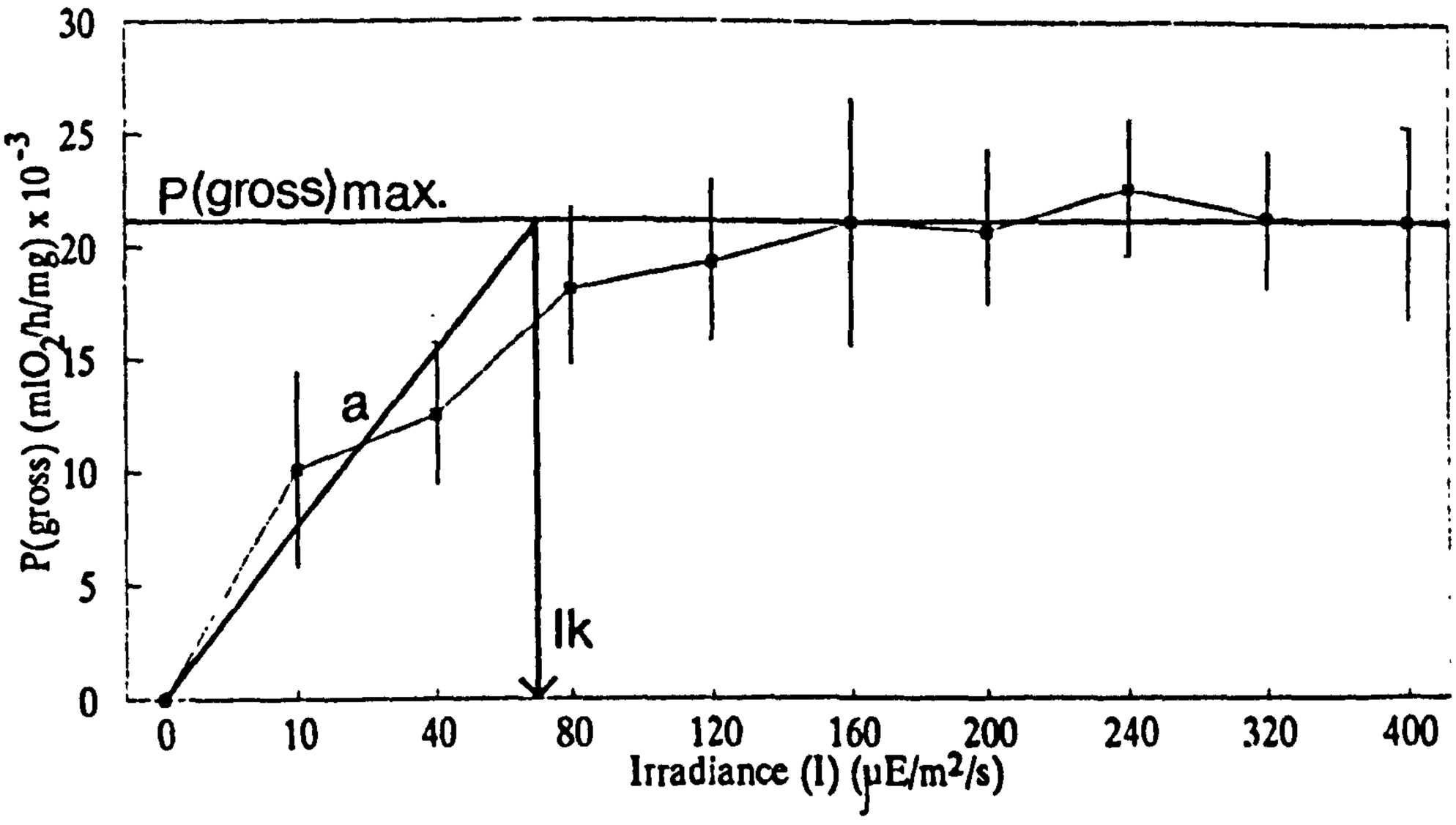


Graph 5.4: P vs.I curve for A. viridis (Shell Is.).
(N=5) (+/- S.E.).



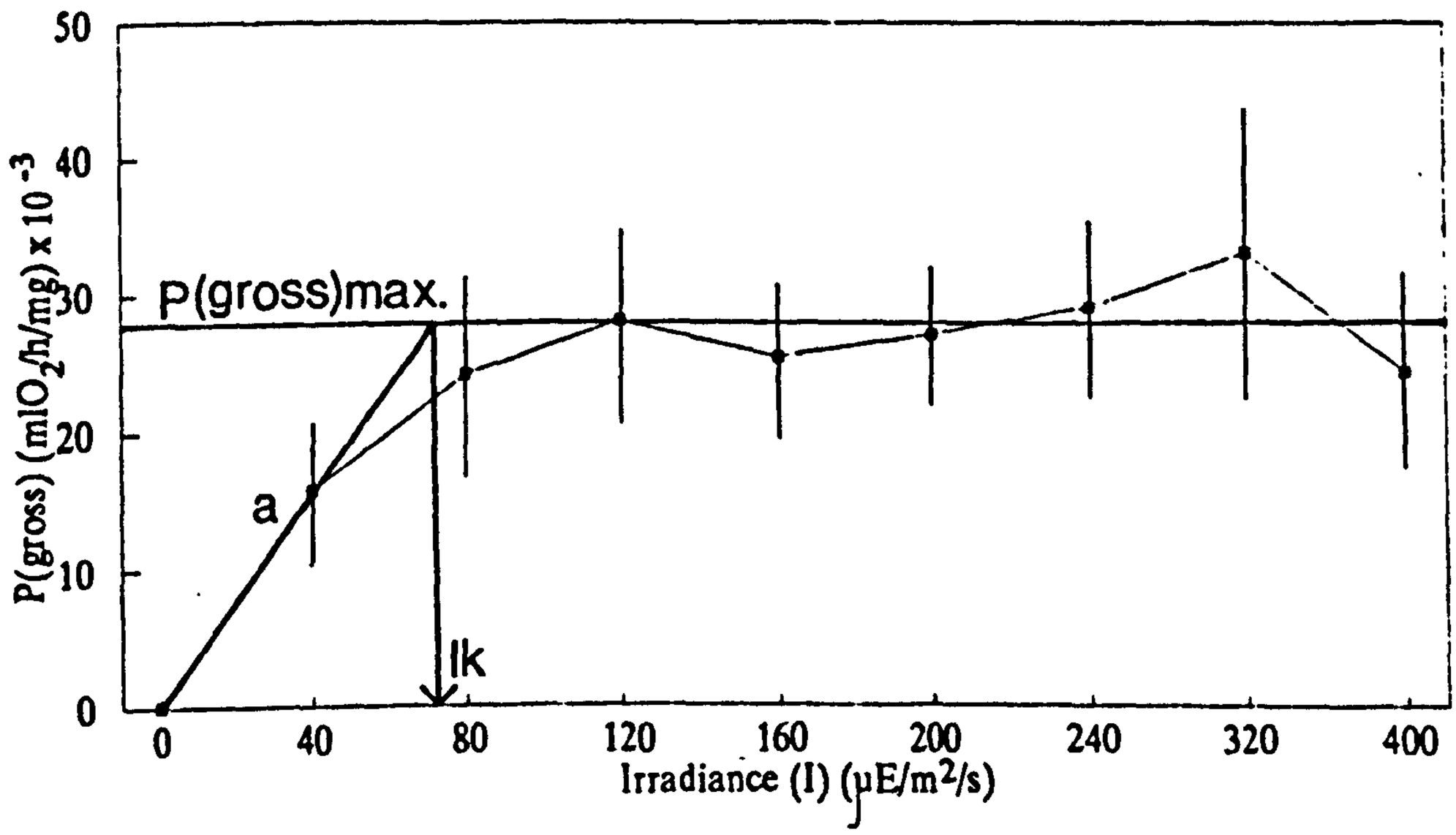
Graph 5.5: P vs.I curve for I. sulcatus.

(N=3) (+/- S.E.).



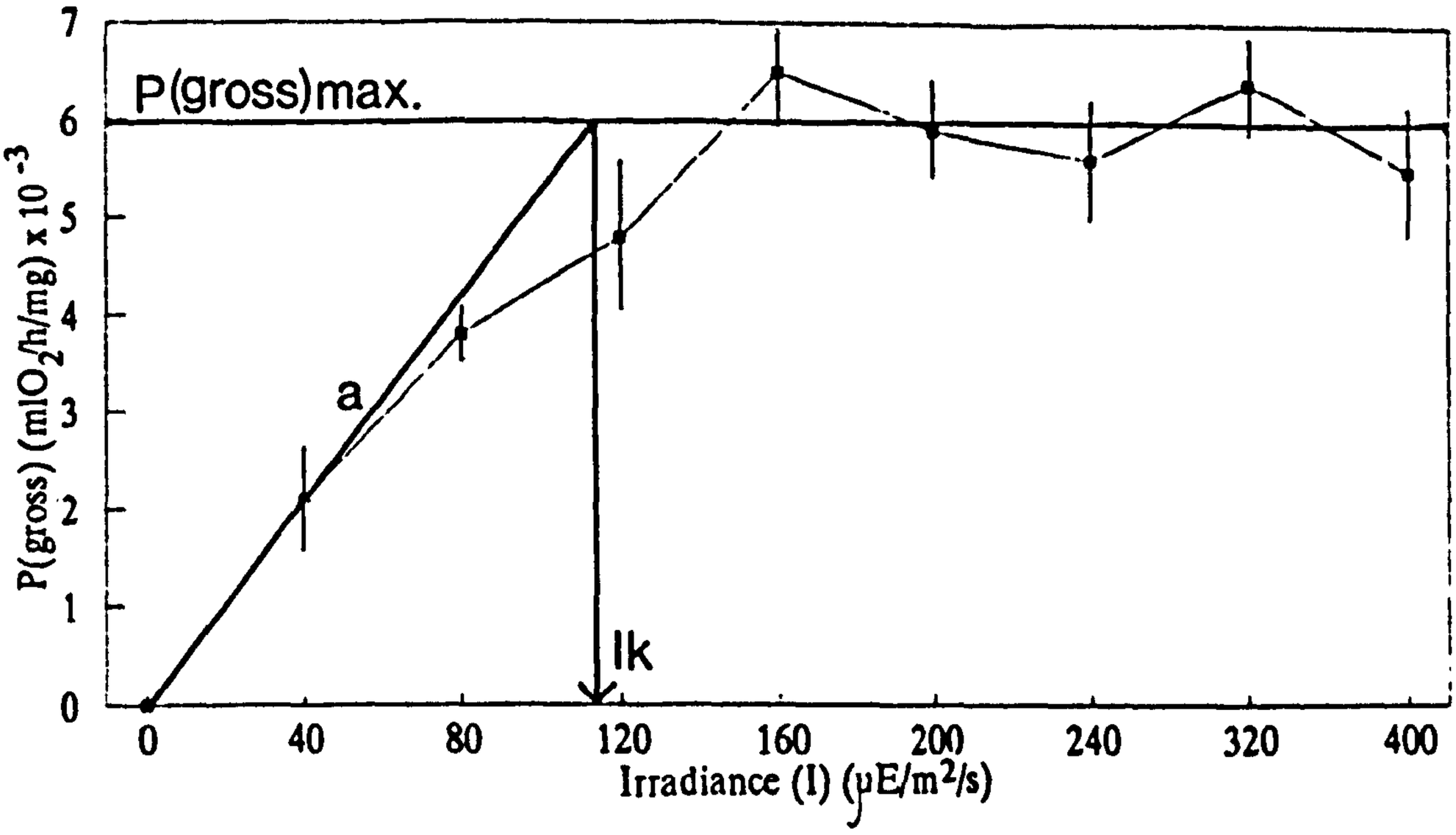
Graph 5.6: P vs.I curve for A. pallida.

(N=5) (+/- S.E.).



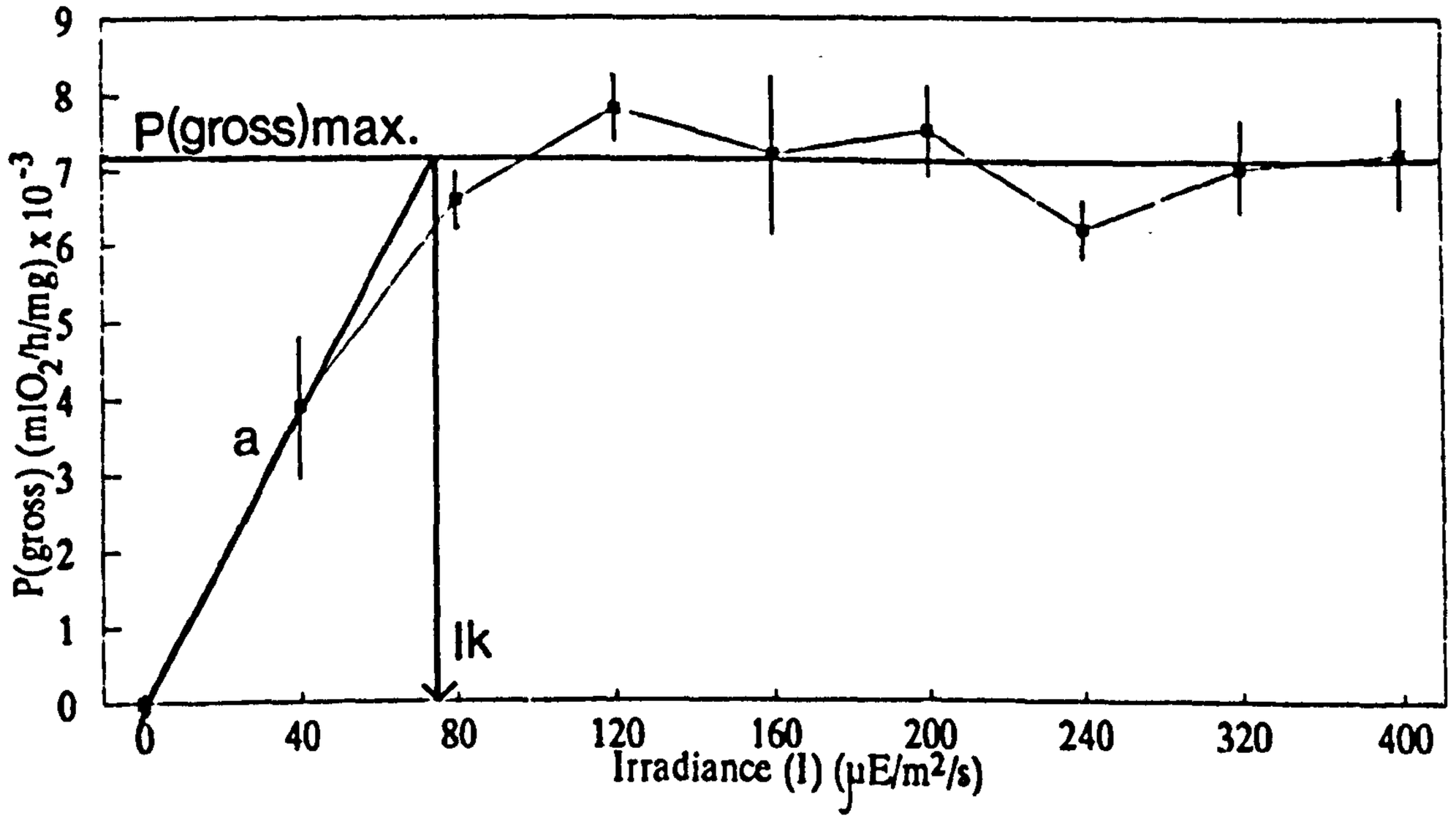
Graph 5.7: P vs.I curve for C. pedunculatus reinfected with symbionts from C. pedunculatus.

(N=3) (+/- S.E.).

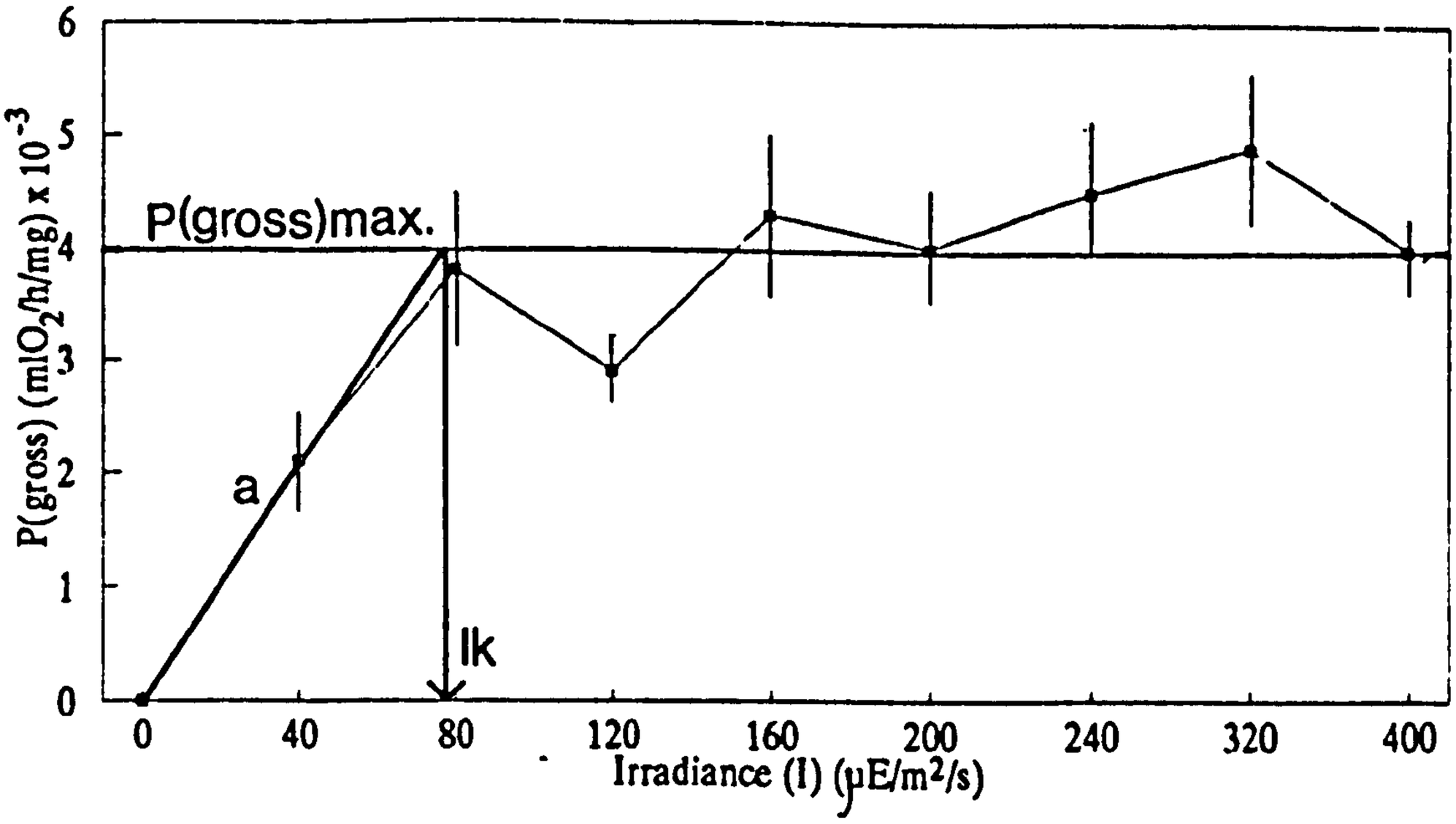


Graph 5.8: P vs.I curve for C. pedunculatus reinfected with symbionts from A. ballii.

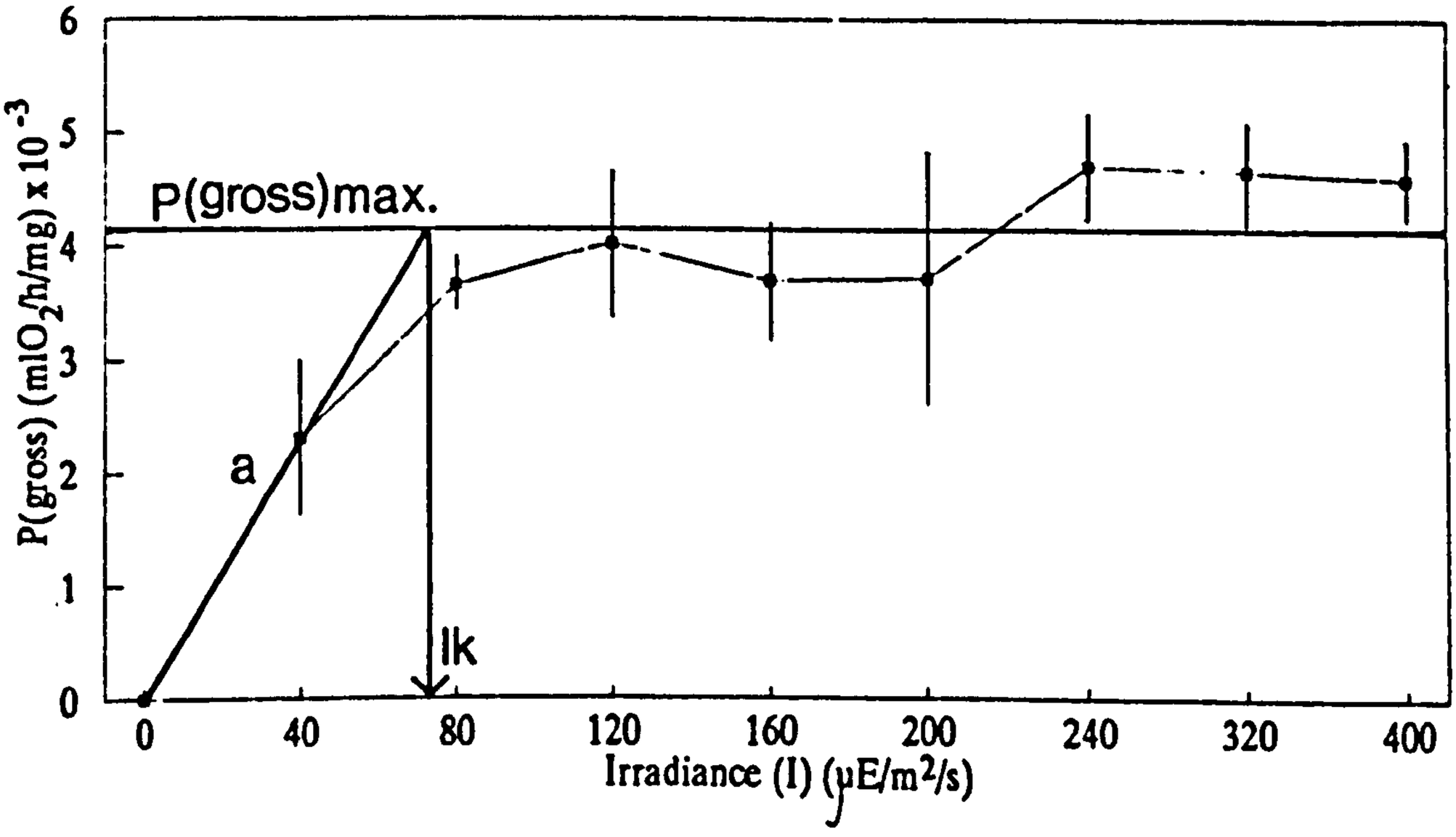
(N=3) (+/- S.E.).



Graph 5.9: P vs.I curve for C. pedunculatus reinfected with symbionts from A. viridis (Lough Hyne). (N=3) (+/- S.E.).

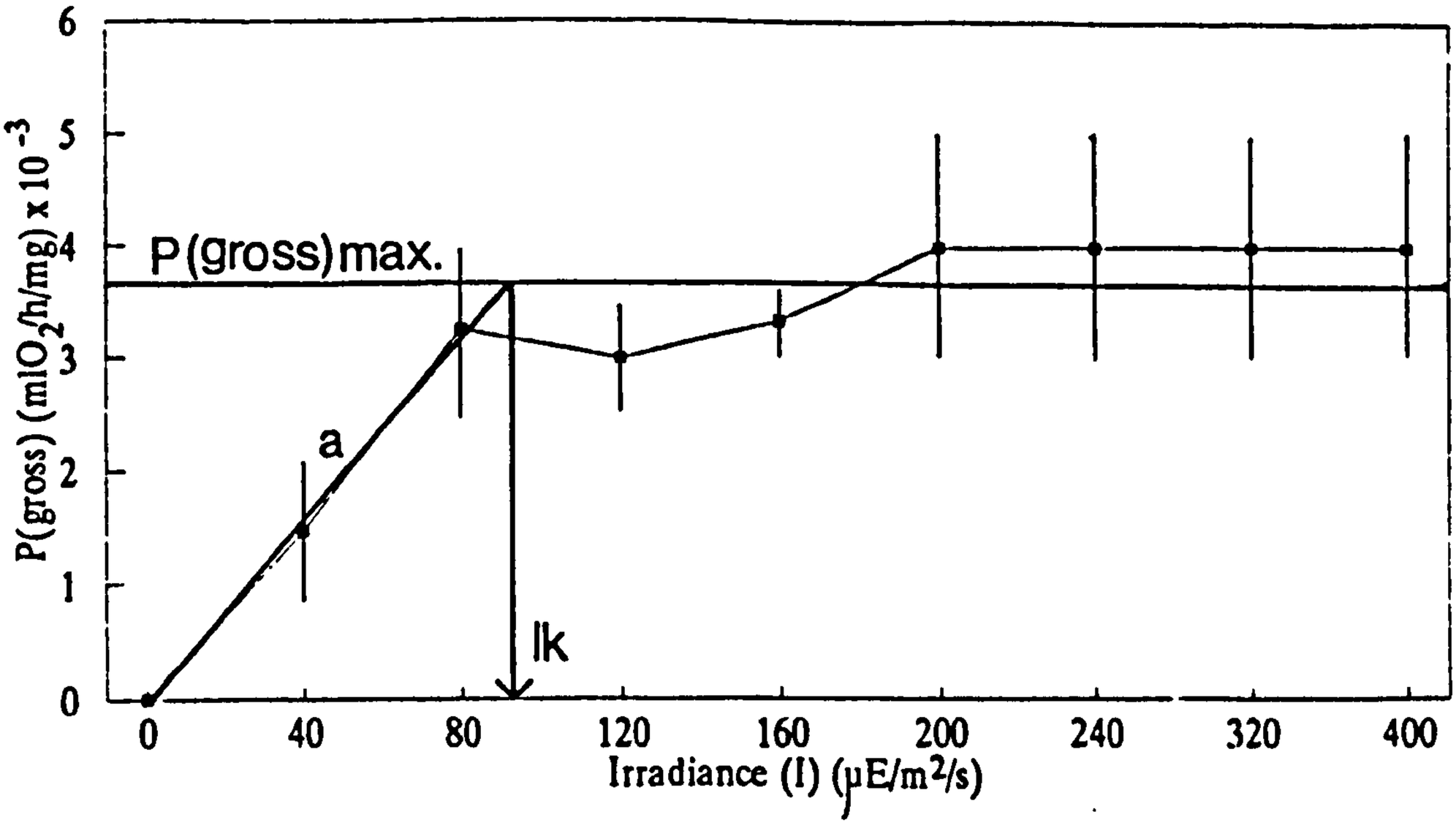


Graph 5.10: P vs.I curve for C. pedunculatus reinfected with symbionts from A. viridis (Shell Is.). (N=3) (+/- S.E.).



Graph 5.11: P vs.I curve for C. pedunculatus reinfected with symbionts from I. sulcatus.

(N=3) (+/- S.E.).



Graph 5.12: P vs.I curve for C. pedunculatus reinfected with symbionts from A. pallida.

(N=3) (+/- S.E.).

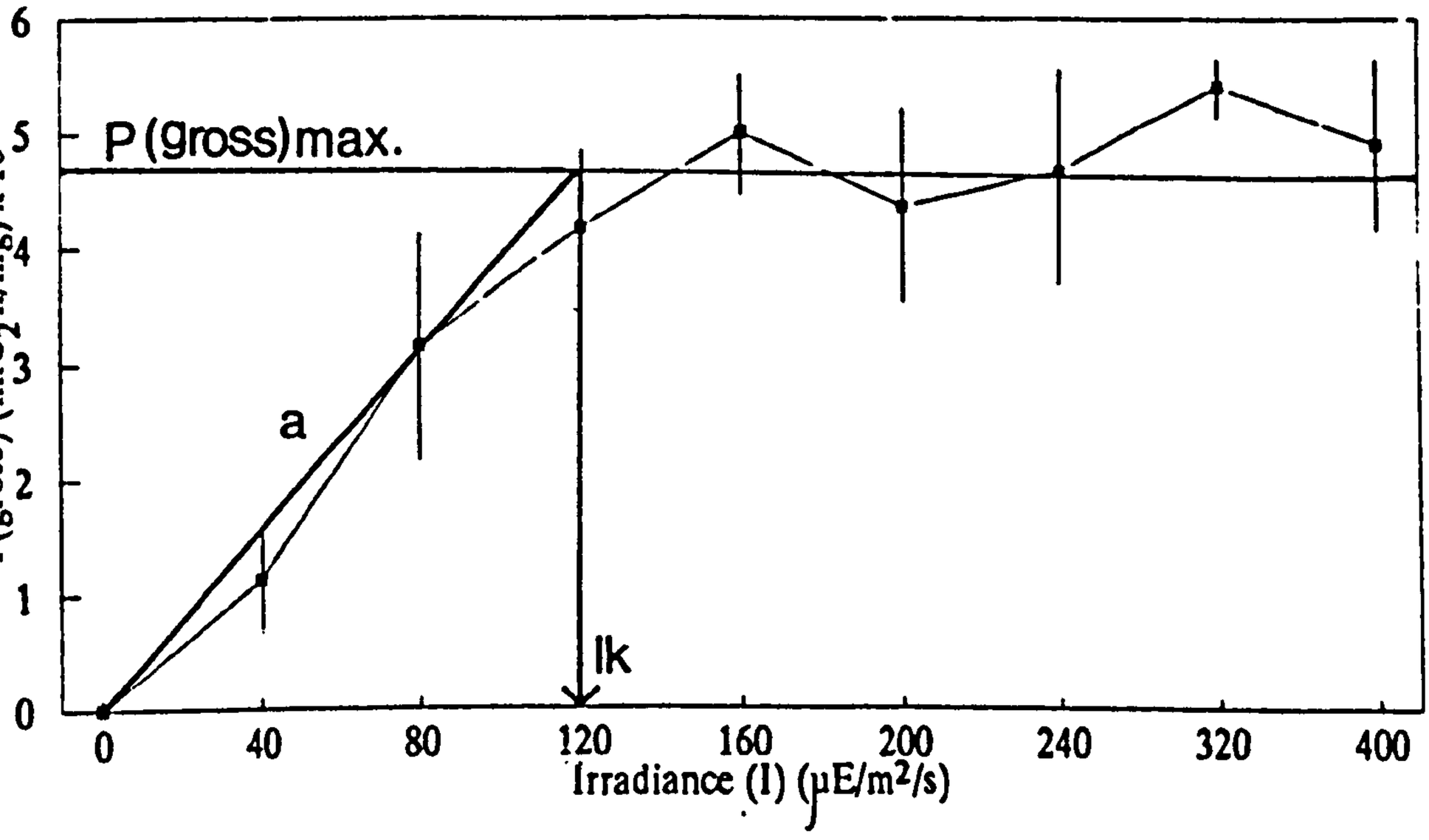


TABLE 5.3 Ik, I(0.95), THE SATURATION IRRADIANCE DETERMINED 'BY EYE', THE COMPENSATION IRRADIANCE, THE SLOPE OF THE LINEAR PORTION OF THE P VS. I CURVE ('a') AND P(gross)max. IN THE 'NATURAL' SYMBIOSES.

Host sp.	Ik (µE/ m^2/s)	I(0.95) (µE/ m^2/s)	saturation irradiance 'by eye' (µE/ m^2/s)	slope 'a' x 10^{-5} (mlO_2 /h/mg assoc. prot./ µE/m^2/ s)	compensation irradiance (µE/ m^2/s)	P(gross)max. +/- S.E. (mlO_2/ h/mg assoc. prot)
Cp (N=5)	320	586.2	600	2.21	180	0.0068 +/- 0.0019
Ab (N=5)	118	216.2	200	4.77	108	0.0061 +/- 0.0023
AvLH (N=5)	168	307.8	320	5.58	64	0.0105 +/- 0.0018
AvSI (N=5)	175	320.6	240	3.41	84	0.0060 +/- 0.0011
Is (N=3)	68	124.6	160	33.3	16	0.0214 +/- 0.0034
Ap (N=5)	65	119.1	120	107.1	24	0.0278 +/- 0.0066

TABLE 5.4 Ik, I(0.95), THE SATURATION IRRADIANCE DETERMINED 'BY EYE', THE SLOPE 'a', THE COMPENSATION IRRADIANCE AND P(gross)max. IN THE REESTABLISHED SYMBIOSES.

Cp + symbionts from named host sp.	Ik (µE/m^2/s)	I(0.95) (µE/m^2/s)	saturation irradiance 'by eye' (µE/m^2/s)	slope 'a' x 10^{-5} (mlO_2/h/mg assoc. prot./µE/m^2 s)	compensation irradiance (µE/m^2/s)	P(gross) max. +/- S.E. (mlO_2/h/mg assoc. prot.)
Cp (N=5)	92	168.5	160	5.21	56	0.0060 +/- 0.0003
Ab (N=4)	68	124.6	80	41.7	28	0.0071 +/- 0.0004
AvLH (N=5)	72	131.9	80	5.44	36	0.0041 +/- 0.0001
AvSI (N=5)	70	128.2	80	5.68	40	0.0041 +/- 0.0005
Is (N=4)	82	150.2	80	3.91	40	0.0037 +/- 0.0007
Ap (N=5)	120	219.8	120	3.91	64	0.0047 +/- 0.0006

(b)**Estimation of the ratio of algal symbiont : association protein, animal respiration, algal respiration and net algal photosynthesis at saturation**

The average total number of symbionts and weight of symbiont protein per anthozoan, the average weight of association protein and the average symbiont : association protein ratios for each host species or different reinfection are given in appendix 11, tables 3 and 4 (p.523). Average rates of animal respiration, algal respiration and net algal photosynthesis, calculated using the protein ratios for each different host species or reinfection, are shown in tables 5.5 (p.274) and 5.6 (p.275).

(i)Net algal photosynthesis in the 'natural' symbioses.

The net algal photosynthetic rate was significantly different between the 'natural' symbioses (H=18.33, P=0.003). The net photosynthetic rate of the symbiont population in A. pallida was significantly greater than that in A. ballii and A. viridis (Shell Is.), and was approaching being significantly greater than that in C. pedunculatus. The net photosynthetic rate of the symbiont population in I. sulcatus was approaching being significantly higher than that in A. ballii (appendix 11, table 5 (p.524)).

(ii)Animal respiration in the 'natural' symbioses.

Animal respiration was significantly different between different host species (H=15.12, P=0.010). But the only specific significant difference measured was the 4 fold

TABLE 5.5 THE SYMBIONT:SYMBIOSIS PROTEIN RATIO, SYMBIOSIS RESPIRATION (Rs), ANIMAL RESPIRATION (Ra), ALGAL RESPIRATION (Rz) AND NET ALGAL PHOTOSYNTHESIS (Pz(net)) IN THE 'NATURAL' SYMBIOSES'.

Host sp.	Algal: symbiosis protein ratio +/- S.E.	Rs (mlO_2/h/mg assoc. prot.) +/- S.E.	Ra (mlO_2/h/mg assoc. prot.) +/- S.E.	Rz (mlO_2/h/mg assoc. prot.) +/- S.E.	Pz(net) (mlO_2/h/mg assoc. prot.) +/- S.E.
Cp (N=5)	1:68.1 +/- 9.	0.00393 +/- 0.00064	0.00387 +/- 0.00063	0.00006 +/- 0.00001	0.00670 +/- 0.00193
Ab (N=5)	1:38.4 +/- 1.9	0.00472 +/- 0.00176	0.00459 +/- 0.00171	0.00013 +/- 0.00005	0.00595 +/- 0.00222
AvLH (N=5)	1:49.3 +/- 4.3	0.00387 +/- 0.00039	0.00379 +/- 0.00038	0.00008 +/- 0.00001	0.01040 +/- 0.00183
AvSI (N=5)	1:27.7 +/- 2.9	0.00262 +/- 0.00063	0.00253 +/- 0.00060	0.00009 +/- 0.00003	0.00585 +/- 0.00111
Is (N=3)	1:42.6 +/- 2.7	0.00651 +/- 0.00094	0.00636 +/- 0.00095	0.00015 +/- 0.00003	0.02123 +/- 0.00337
Ap (N=5)	1:39.0 +/- 3.0	0.01029 +/- 0.00062	0.01003 +/- 0.00061	0.00026 +/- 0.00002	0.02757 +/- 0.00656

TABLE 5.6 THE SYMBIONT:SYMBIOSIS PROTEIN RATIO, SYMBIOSIS RESPIRATION (Rs), ANIMAL RESPIRATION (Ra), ALGAL RESPIRATION (Rz) AND NET ALGAL PHOTOSYNTHESIS (Pz(net)) IN THE REESTABLISHED SYMBIOSES.

Cp + symbionts from named species	Algal : symbiosis protein ratio +/- S.E.	Rs (mlO_2/h/mg assoc. prot.)	Ra (mlO_2/h/mg assoc. prot.) +/- S.E.	Rz (mlO_2/h/mg assoc. prot.) +/- S.E.	Pz(net) (mlO_2/h/mg assoc. prot.) +/- S.E.
Cp (N=5)	1:74.4 +/- 2.2	0.00264	0.00260 +/- 0.00000	0.00004 +/- 0.00000	0.00594 +/- 0.00000
Ab (N=4)	1:44.0 +/- 6.1	0.00254	0.00248 +/- 0.00001	0.00006 +/- 0.00001	0.00701 +/- 0.00001
AvLH (N=5)	1:55.1 +/- 5.9	0.00198	0.00194 +/- 0.00000	0.00004 +/- 0.00000	0.00402 +/- 0.00000
AvSI (N=5)	1:45.6 +/- 6.8	0.00232	0.00227 +/- 0.00001	0.00005 +/- 0.00001	0.00407 +/- 0.00001
Is (N=4)	1:53.7 +/- 13.7	0.00192	0.00188 +/- 0.00001	0.00004 +/- 0.00001	0.00362 +/- 0.00001
Ap (N=5)	1:55.1 +/- 8.2	0.00251	0.00246 +/- 0.00001	0.00005 +/- 0.00001	0.00469 +/- 0.00001

higher rate of respiration of A. pallida than A. viridis (Shell Is.) (appendix 11, table 6 (p.524)). It was noted that the 2 highest host respiration rates measured belonged to A. pallida and I. sulcatus, the 2 species whose symbiont populations also showed the highest rates of net photosynthesis.

(iii)Gross and net photosynthesis by the symbionts in the reestablished symbioses.

Statistics were not performed on net algal photosynthesis and animal respiration in the reestablished symbioses for the reason given in section 5.2 (d) (p.243). But the net photosynthetic rate of the symbiont population in the 'homologous' association was 1.26 to 1.64 times higher than that of the symbiont populations in the 'heterologous' symbioses. This was with the exception of the net photosynthetic rate by the population of symbionts originally from A. ballii when in C. pedunculatus, which was 1.2 times greater than that by the 'homologous' symbiont population. However, saturated gross photosynthesis in the reestablished symbioses, which is approximate to net algal photosynthesis because the ratio of gross photosynthesis : algal respiration exceeded 100 : 1 in all symbioses, was compared statistically. The gross photosynthetic rate of the C. pedunculatus reinfected with 'homologous' symbionts and symbionts isolated from A. ballii was significantly greater than that of C. pedunculatus reinfected with symbionts from A. viridis (Lough Hyne and Shell Island)), I. sulcatus and

TABLE 5.7 THE SYMBIONT DENSITY AND CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN, AND THE CALCULATION OF THE CARBON SPECIFIC GROWTH RATE (μc) IN THE 'NATURAL' SYMBIOSES.

Host sp.	Symbiont density (cells/mg assoc. prot.) +/- S.E.	C'(mg symbiont C/mg assoc. prot.) +/- S.E.	Daily Pz (net)(mg C/24 h/ mg assoc. prot.) +/- S.E.	Carbon specific growth rate (μc) (day^{-1}) +/- S.E.
Cp (N=5)	298649 +/- 37728	0.0155 +/- 0.0020	0.0388 +/- 0.0112	2.59 +/- 0.61
Ab (N=5)	270150 +/- 13571	0.0257 +/- 0.0013	0.0341 +/- 0.0127	1.25 +/- 0.40
AvLH (N=5)	390091 +/- 36325	0.0204 +/- 0.0019	0.0603 +/- 0.0107	3.01 +/- 0.59
AvSI(N=5)	445277 +/- 117921	0.0370 +/- 0.0044	0.0336 +/- 0.0064	0.94 +/- 0.17
Is (N=3)	227998 +/- 14300	0.0232 +/- 0.0015	0.1232 +/- 0.0200	5.29 +/- 0.76
Ap (N=5)	624279 +/- 48220	0.0257 +/- 0.0020	0.1596 +/- 0.0383	6.70 +/- 2.16

TABLE 5.8 THE SYMBIONT DENSITY AND CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN, AND THE CALCULATION OF THE CARBON SPECIFIC GROWTH RATE (μc) IN THE REESTABLISHED SYMBIOSES.

Cp + symbionts from named host sp.	Symbiont density (cells/mg assoc. prot.) +/- S.E.	C' (mg symbiont carbon/mg assoc. prot.) +/- S.E.	Daily Pz (net) (mg C/24 h/ mg assoc. prot.) +/- S.E.	Carbon specific growth rate (μc) (day^{-1}) +/- S.E.
Cp (N=5)	396878 +/- 11661	0.0132 +/- 0.0004	0.0345 +/- 0.0000	2.63 +/- 0.08
Ab (N=4)	449262 +/- 68313	0.0237 +/- 0.0036	0.0406 +/- 0.0001	1.83 +/- 0.26
AvLH (N=5)	465388 +/- 56688	0.0186 +/- 0.0023	0.0233 +/- 0.0005	1.32 +/- 0.14
AvSI (N=5)	492699 +/- 82097	0.0236 +/- 0.0039	0.0235 +/- 0.0001	1.10 +/- 0.17
Is (N=4)	393336 +/- 90260	0.0219 +/- 0.0050	0.0209 +/- 0.0001	1.15 +/- 0.30
Ap (N=5)	564322 +/- 94075	0.0196 +/- 0.0033	0.0271 +/- 0.0001	1.53 +/- 0.23

A. pallida (appendix 11, table 7 (p.525)).

(iv)Respiration by the hosts in the reestablished symbioses.

As observed in the 'natural' symbioses, the 2 reestablished symbioses whose symbiont populations exhibited the highest net photosynthetic rates also showed the highest animal respiration rates. But the highest rate of animal respiration only differed from the lowest by a factor of 1.38.

(c)Calculation of the symbiont cell density, carbon standing stock and carbon specific growth rate.

(i)The symbiont densities and carbon standing stocks in the 'natural' symbioses.

The average number of symbionts and algal carbon standing stock per mg of association protein, and the calculation of the carbon specific growth rate for the 'natural' symbioses are shown in table 5.7 (p.277).

The symbiont density ($F=13.09$; $P<0.0001$) and carbon standing stock/mg association protein ($F=8.61$; $P<0.0001$) were significantly different between the 'natural' symbioses. A. pallida possessed a significantly greater density of symbionts than A. ballii, C. pedunculatus, A. viridis (Lough Hyne) and I. sulcatus (appendix 11, table 8 (p.526)). However, because of the significantly smaller size of symbionts from A. pallida, the algal carbon standing stock/mg association protein did not significantly differ between A. pallida and the other 'natural' symbioses. In contrast, A. viridis (Shell Is.) had a significantly higher

symbiont carbon standing stock/mg association protein than C. pedunculatus and A. viridis (Lough Hyne) (appendix 11, table 9 (p.527)).

(ii)The symbiont densities and carbon standing stocks in the reestablished symbioses.

The average number of symbionts and algal carbon standing stock per mg of association protein, and the calculation of the carbon specific growth rate for the reestablished symbioses are given in table 5.8 (p.277).

Neither the symbiont density ($F=0.81$, $P<0.81$) nor the algal carbon standing stock/mg association protein ($H=9.89$, $P=0.079$) differed significantly between the reestablished symbioses.

(d)**Standardization of the net algal photosynthetic rate to symbiont density and carbon content.**

(i)Net algal photosynthesis standardized to symbiont density and carbon standing stock in the 'natural' symbioses.

The net algal photosynthetic rates of the symbionts in the 'natural' symbioses, standardized to symbiont density (the cell specific rate) and carbon standing stock/mg association protein (the biomass specific rate), are shown in table 5.9 (p.283).

The cell specific rate of net algal photosynthesis was significantly different between 'natural' symbioses ($H=15.40$, $P=0.009$). The only specific difference was the significantly higher rate of net algal photosynthesis/cell

by Symbiodinium sp. in I. sulcatus than by the symbionts in A. viridis (Shell Is.) (appendix 11, table 10 (p.527)).

The biomass specific rate of net algal photosynthesis was also significantly different between different 'natural' symbioses ($H=19.63$, $P<0.05$). The rate of net algal photosynthesis/mg symbiont carbon was significantly greater by the symbionts of I. sulcatus than by the symbionts of A. viridis (Shell Is.), and significantly higher by the symbionts of A. pallida than those of A. ballii and A. viridis (Shell Is.) (appendix 11, table 11 (p.528)). These differences were assumed to reflect differences in the gross photosynthetic rate.

(ii)Net algal photosynthesis standardized to symbiont density and algal carbon standing stock in the reestablished symbioses.

The net algal photosynthetic rate in the reestablished symbioses standardized to symbiont density and the algal carbon standing stock/mg association protein is given in table 5.10 (p.283).

The cell specific rate of net algal photosynthesis varied significantly between different 'strains' of Symbiodinium when in C. pedunculatus ($F=4.20$, $P=0.008$). However, no specific significant differences were identified with Scheffe's test (appendix 11, table 12 (p.528)), although the rate of net algal photosynthesis/cell was approaching being significantly higher by the symbionts originally isolated from A. ballii than by the symbionts originally isolated

from A. viridis (Lough Hyne and Shell Island).

When net algal photosynthesis was standardized to the algal carbon standing stock/mg association protein, there were again significant differences between the symbiont 'strains' residing in C. pedunculatus ($F=8.70$, $P<0.0001$). The only significant differences were between the 'homologous' and 'heterologous' symbionts. The biomass specific rate of net algal photosynthesis was significantly greater by 'homologous' symbionts than by symbionts originally from A. viridis (Lough Hyne and Shell Island), I. sulcatus and A. pallida (appendix 11, table 13 (p.529)).

The net algal photosynthetic rate standardized to symbiont density and carbon standing stock/mg association protein is compared between when the symbionts were in reinfected C. pedunculatus and when they were residing in their original host species in graphs 5.13 and 5.14 (p.284).

The cell specific rate of net algal photosynthesis was significantly lower by symbionts from A. viridis (Lough Hyne) ($T=3.24$, $P=0.032$), I. sulcatus ($T=5.98$, $P=0.027$) and A. pallida (Mann-Whitney statistic=40.0, $P=0.012$) when in C. pedunculatus than when in their original host species. Likewise, the biomass specific rate of net algal photosynthesis was also significantly lower by Symbiodinium cells from A. viridis (Lough Hyne) ($T=2.80$, $P=0.049$), I. sulcatus ($T=5.06$, $P=0.037$) and A. pallida (Mann-Whitney statistic=40.0, $P=0.012$) when in symbiosis with C. pedunculatus. The cell and biomass specific rates of net

algal photosynthesis by the symbionts of C. pedunculatus, A. ballii or A. viridis (Shell Is.) did not vary significantly between when in the reestablished symbioses and when in their 'natural' host species (appendix 11, tables 14 (p.529) and 15 (p.530)).

TABLE 5.9 NET ALGAL PHOTOSYNTHESIS (Pz(net)) STANDARDIZED TO SYMBIONT DENSITY AND CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN IN THE 'NATURAL' SYMBIOSES

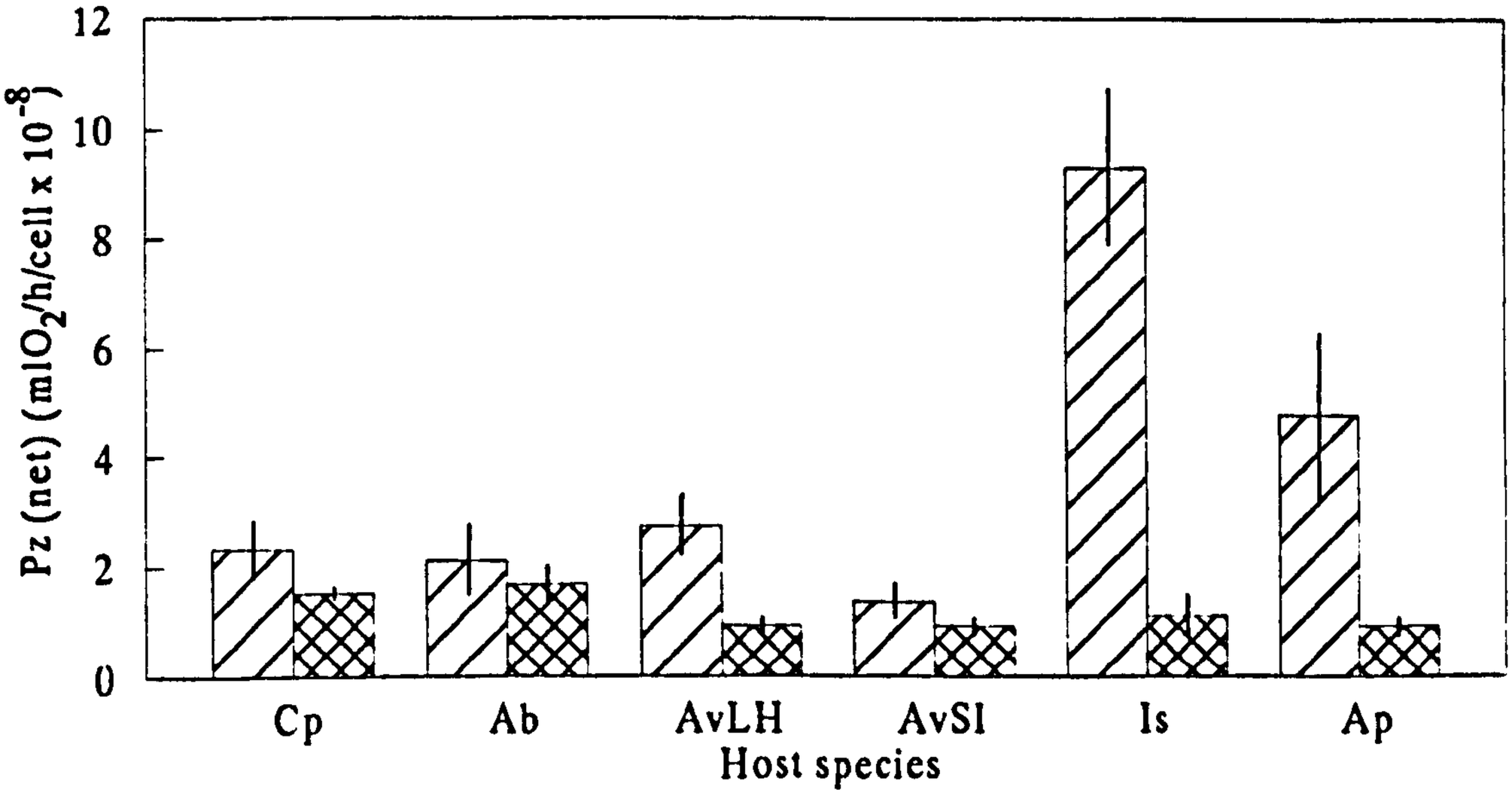
Host sp.	Pz(net) standardized to cell density ($mlO_2/h/cell \times 10^{-6}$) +/- S.E.	Pz(net) standardized to C'/mg assoc. prot. ($mlO_2/h/mg\ C$) +/- S.E.
Cp (N=5)	2.3123 +/- 0.5120	0.4175 +/- 0.0989
Ab (N=5)	2.0877 +/- 0.6740	0.2193 +/- 0.0708
AvLH (N=5)	2.7172 +/- 0.5190	0.5184 +/- 0.1010
AvSI (N=5)	1.3536 +/- 0.2400	0.1630 +/- 0.0289
Is (N=3)	9.2770 +/- 1.3400	0.9118 +/- 0.1310
Ap (N=5)	4.7616 +/- 1.5200	1.1560 +/- 0.3700

TABLE 5.10 NET ALGAL PHOTOSYNTHESIS (Pz(net)) STANDARDIZED TO SYMBIONT DENSITY AND CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN IN THE REESTABLISHED SYMBIOSES.

Cp + symbionts from named host sp.	Pz(net) standardized to cell density ($mlO_2/h/cell \times 10^{-6}$) +/- S.E.	Pz (net) standardized to C'/mg assoc. prot. ($mlO_2/h/mg\ C$) +/- S.E.
Cp (N=5)	1.5013 +/- 0.0440	0.4525 +/- 0.0133
Ab (N=4)	1.6649 +/- 0.234	0.3159 +/- 0.0444
AvLH (N=5)	0.9103 +/- 0.0986	0.2273 +/- 0.0246
AvSI (N=5)	0.9121 +/- 0.1368	0.1904 +/- 0.0286
Is (N=4)	1.1105 +/- 0.2850	0.1990 +/- 0.0511
Ap (N=5)	0.9209 +/- 0.1390	0.2649 +/- 0.0399

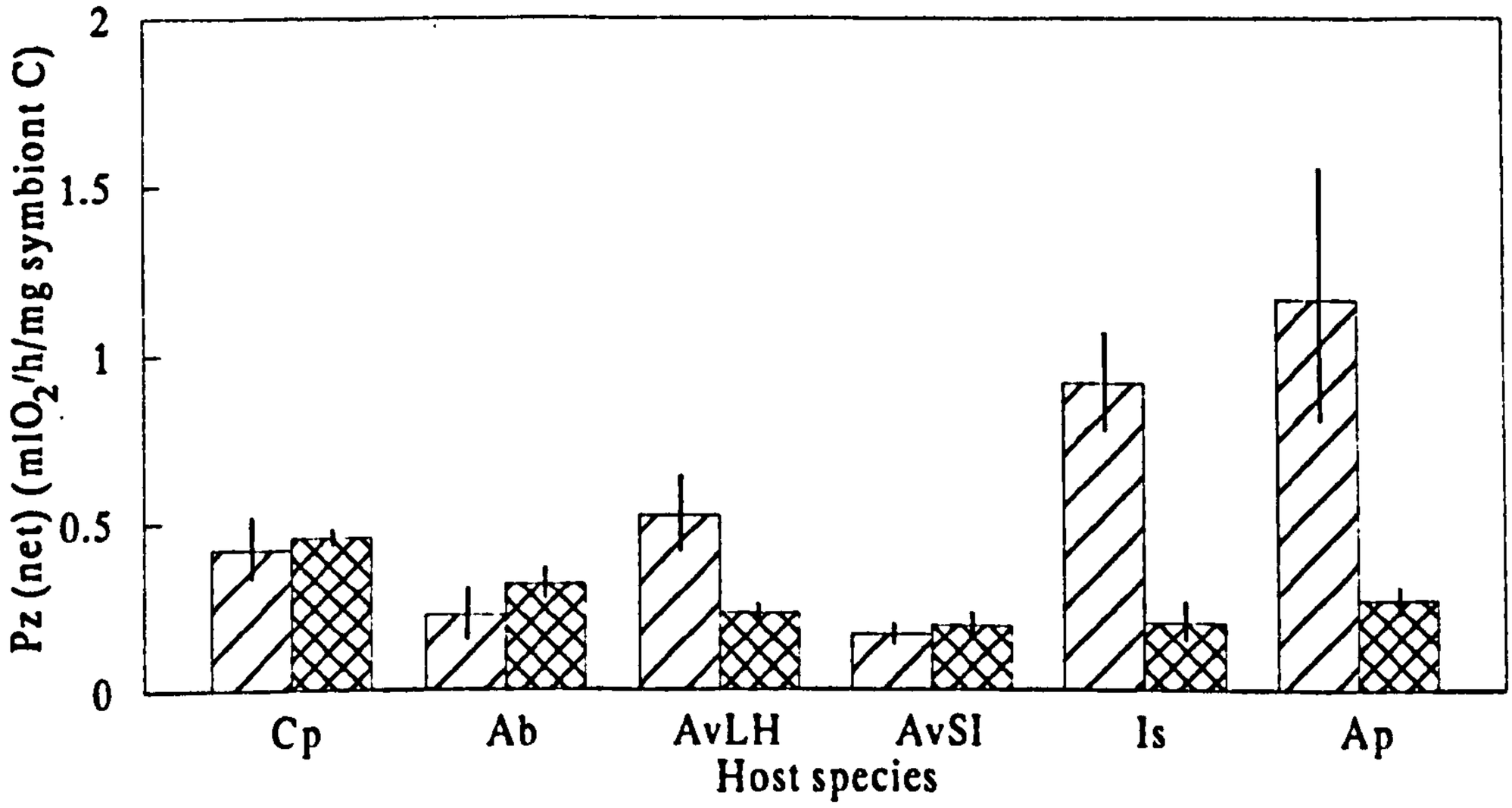
Graph 5.13: Net algal photosynthesis (Pz(net)) standardized to symbiont density in the 'natural' and reestablished symbioses.

(N = 5, except N = 3 for I. sulcatus and N = 4 for C. pedunculatus reinfected with symbionts from A. ballii and I. sulcatus) (+/− S.E.).



Graph 5.14: Net algal photosynthesis (Pz(net)) standardized to carbon standing stock (C') in 'natural and reestablished symbioses.

(N as in graph 5.13) (+/− S.E.).



(e)**Estimation of the percentage translocation of photosynthetically fixed carbon.**

(i)**In vivo ^{14}C incubations**

(1)The effect of the incubation period on the percentage translocation.

The average d.p.m. value of the symbiont, host and sea water fractions of the A. viridis (Shell Is.) dark control and experimental incubations investigating the influence of time on the percentage translocation are given in appendix 12, tables 2 and 3 (p.532). The corrected percentage translocation in these incubations is shown in appendix 12, table 4 (p.533). The percentage of photosynthate translocated did not differ significantly with incubation period ($F=1.10$, $P=0.399$).

(2)The in vivo percentage translocation in the 'natural' symbioses.

The average d.p.m. value of each fraction of the in vivo dark and light incubations of the 'natural' symbioses are shown in appendix 12, tables 5 (p.533) and 6 (p.534). The average percentages of fixed carbon retrieved from the symbiont, host and sea water fractions and the total percentage translocated in the 'natural' symbioses are listed in table 5.11 (p.287).

The percentage translocation measured using in vivo ^{14}C incubations showed significant differences between different host species ($F=8.27$, $P<0.0001$). The percentage of fixed carbon translocated in A. pallida was significantly lower

than in C. pedunculatus and I. sulcatus, and the percentage translocation was significantly lower in A. ballii than in I. sulcatus (appendix 12, table 8 (p.535)).

(3)The in vivo percentage translocation in the reestablished symbioses.

The average dark corrected d.p.m. values of the different fractions of the in vivo incubations of the reestablished symbioses are given in appendix 12, table 7 (p.534). The average percentages of fixed carbon retrieved from the symbiont, host and sea water fractions, and the percentage translocation in the reestablished symbioses are given in table 5.12 (p.287).

In contrast to the situation with the 'natural' symbioses, the percentages of ^{14}C labelled fixed carbon translocated in the reestablished symbioses exhibited no significant differences ($F=1.76$, $P=0.162$). This was because of the significantly greater percentage translocation by symbionts of A. pallida when residing in C. pedunculatus than when in their original host species ($T=-6.27$, $P=0.0004$); the percentage translocation by symbionts of A. ballii when in C. pedunculatus was approaching being significantly higher than when in the original host species ($T=-2.22$, $P=0.062$).

The percentage of photosynthate translocated by the Symbiodinium cells originally from C. pedunculatus ($T=-1.60$, $P=0.15$), A. viridis (Lough Hyne) ($T=-1.44$, $P=0.21$), A. viridis (Shell Is.) ($T=-2.20$, $P=0.092$) and I. sulcatus ($T = -0.58$, $P=0.59$) remained unchanged in the reestablished

TABLE 5.11 THE PERCENTAGE OF ^{14}C LABELLED FIXED CARBON TRANSLOCATED TO THE HOST (T) IN VIVO IN 'NATURAL SYMBIOSES.

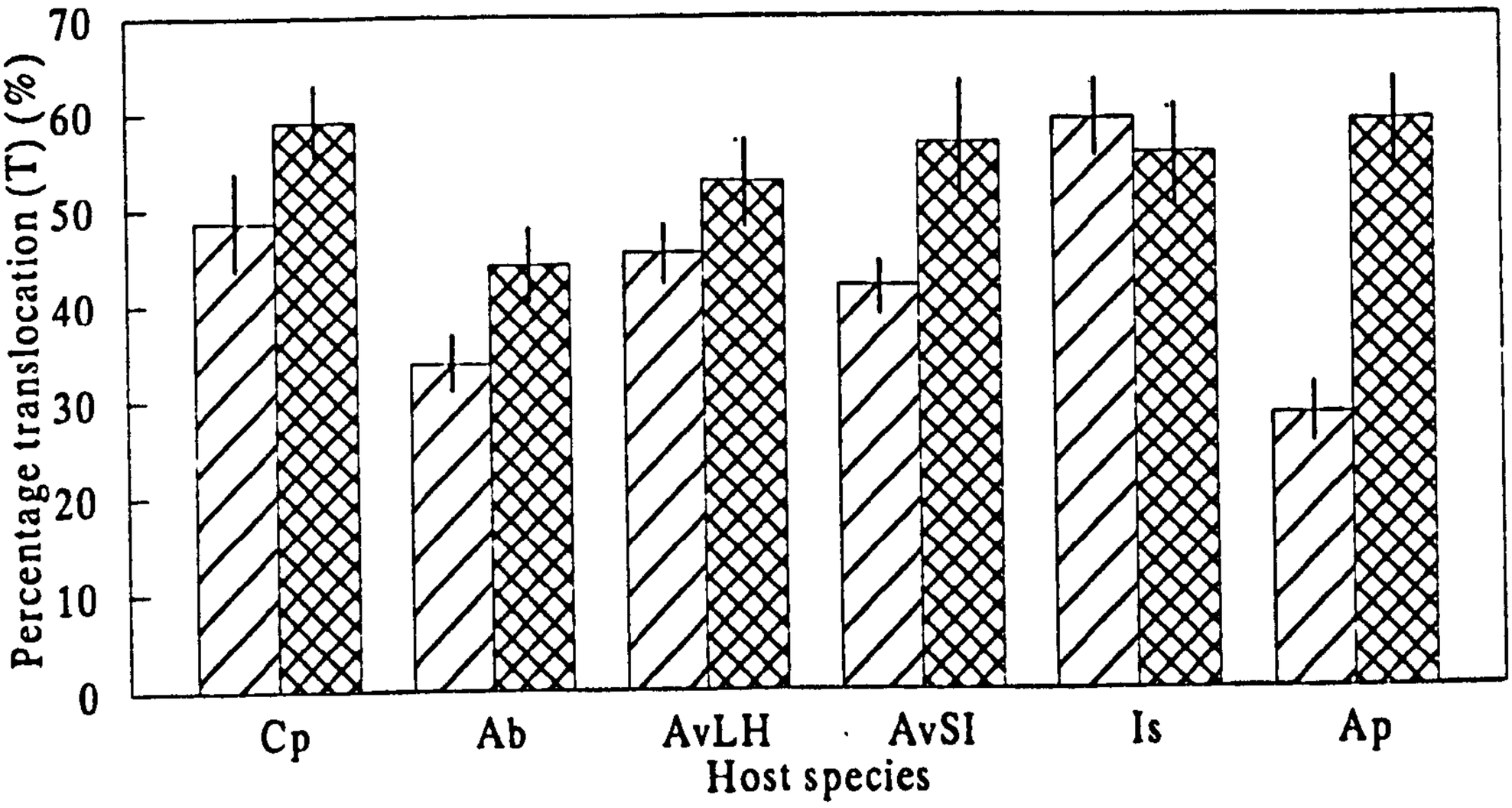
Host sp.	% in algal fraction +/- S.E.	% in host fraction +/- S.E.	% in sea water fraction +/- S.E.	% translo-cated to host (T) +/- S.E.
Cp (N=5)	51.4 +/- 5.4	48.0 +/- 4.9	0.6 +/- 0.6	48.6 +/- 5.4
Ab (N=5)	66.2 +/- 2.7	32.5 +/- 2.8	1.3 +/- 0.9	33.8 +/- 2.7
AvLH (N=5)	54.6 +/- 2.8	45.2 +/- 2.7	0.2 +/- 0.2	45.4 +/- 2.8
AvSI (N=5)	58.1 +/- 2.6	40.0 +/- 2.9	1.9 +/- 1.3	41.9 +/- 2.6
Is (N=3)	40.7 +/- 3.9	59.3 +/- 3.9	0.0 +/- 0.0	59.3 +/- 3.9
Ap (N=5)	71.6 +/- 3.0	23.4 +/- 3.0	5.0 +/- 3.0	28.4 +/- 3.0

TABLE 5.12 THE PERCENTAGE OF ^{14}C LABELLED FIXED CARBON TRANSLOCATED TO THE HOST (T) IN VIVO IN REESTABLISHED SYMBIOSES.

Cp + symbionts from named host sp.	% in algal fraction +/- S.E.	% in host fraction +/- S.E.	% in sea water fraction +/- S.E.	%transloca -ted to host +/- S.E.
Cp (N=5)	40.9 +/- 3.7	59.1 +/- 3.7	0.0 +/- 0.0	59.1 +/- 3.7
Ab (N=5)	56.0 +/- 3.7	44.0 +/- 3.7	0.0 +/- 0.0	44.0 +/- 3.7
AvLH (N=4)	47.2 +/- 4.3	52.7 +/- 4.3	0.1 +/- 0.1	52.8 +/- 4.3
AvSI (N=4)	43.1 +/- 6.3	56.9 +/- 6.3	0.0 +/- 0.0	56.9 +/- 6.3
Is (N=5)	44.2 +/- 4.7	55.8 +/- 4.7	0.0 +/- 0.0	55.8 +/- 4.7
Ap (N=5)	40.9 +/- 3.9	59.1 +/- 3.9	0.0 +/- 0.0	59.1 +/- 3.9

Graph 5.15: Percentage translocation (T) measured in vivo using ^{14}C in 'natural' and reestablished symbioses.

(N = 5, except N = 3 for I. sulcatus and N = 4 for reinfections with symbionts of A. viridis (Lough Hyne) and A. viridis (Shell Is.)) (+/− S.E.).



symbioses from when in the original symbioses. These comparisons are shown in graph 5.15 (p.288).

(ii)**In vitro ^{14}C incubations**

(1)Percentage translocation by symbionts in response to 'homologous' homogenates.

The average d.p.m. values of all the light incubations and controls investigating translocation in response to 'homologous' homogenates are given in appendix 12, table 9 (p.536). The percentage translocation due to contamination/natural leakage and the corrected percentage translocation for the 'homologous' incubations involving different host species are shown in table 5.13 (p.296).

When symbionts of the different host species were incubated with homogenates of the host species from which they were isolated they released significantly different percentages of photosynthate ($H=24.78$, $P<0.0001$). Symbiodinium cells from A. viridis (Shell Is.) released significantly less fixed carbon than symbionts from A. viridis (Lough Hyne) and C. pedunculatus (appendix 12, table 10 (p.537)).

When photosynthate release in the 'homologous' in vitro incubations was compared to translocation in the in vivo ^{14}C incubations, only release by symbionts of C. pedunculatus did not differ significantly ($T=-2.62$, $P=0.059$); symbionts isolated from A. ballii ($T=-6.42$, $P=0.0003$), A. viridis (Shell Is.) ($T=-15.76$, $P=0.0001$), I. sulcatus ($T=-9.67$, $P=0.0006$) and A. pallida ($T=-5.57$, $P=0.0051$) all released a significantly lower percentage of photosynthate in vitro.

In contrast, symbionts isolated from A. viridis (Lough Hyne) translocated a greater percentage of fixed carbon when stimulated in vitro than in vivo (T=8.45, P<0.0001).

(2)Percentage translocation by symbionts when incubated with 'heterologous' host homogenates from different host species.

The average d.p.m. values of the light incubations and controls of the 'heterologous' incubations investigating inter-host species "host factor" specificity are given in appendix 12, table 9 (p.536). The percentage translocation resulting from natural leakage/contamination and the corrected percentage translocation for these incubations are shown in table 5.13 (p.296). The results revealed that, whilst different 'strains' of Symbiodinium release similar percentages of fixed carbon to C. pedunculatus after 9 months in symbiosis, these same symbionts do not behave in an identical manner when they first contact C. pedunculatus homogenate (H=19.79; P=0.001). Symbionts isolated from C. pedunculatus released a significantly greater percentage of photosynthate when stimulated by C. pedunculatus homogenate than symbionts isolated from A. ballii, A. viridis (Shell Is.) and A. pallida (appendix 12, table 11 (p.537)). Although the percentage translocation by the symbionts isolated from A. ballii was not significantly different when in A. ballii homogenate or C. pedunculatus homogenate (T=-1.43, P=0.20). Symbionts from I. sulcatus also released indifferent percentages of photosynthate when in C. pedunculatus or I. sulcatus homogenates (T=0.34, P=0.75). In

contrast, Symbiodinium cells isolated from A. viridis (Lough Hyne) (T=-14.17, P<0.0001), A. viridis (Shell Is.) (T=4.66, P=0.0096) and A. pallida (T=4.03, P=0.0051) all released a lower percentage of photosynthate when incubated in C. pedunculatus homogenate than in their 'homologous' homogenate. Graph 5.16 (p.297) compares the percentage translocation by the algal cells in response to their 'homologous' homogenate and homogenate of C. pedunculatus. Symbiodinium cells isolated from C. pedunculatus translocated significantly different percentages of photosynthate when stimulated by 'homologous' and 'heterologous' homogenates (F=255.97, P<0.0001). 'Homologous' incubations released a significantly larger percentage of photosynthate than when C. pedunculatus symbionts were incubated in homogenates of A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Is.), I. sulcatus and A. pallida. In fact, C. pedunculatus were only observed to be stimulated to release photosynthate by 'heterologous' homogenates of A. ballii and A. viridis (Lough Hyne), and release was more than 40 % greater in the 'homologous' incubations than in any of the 'heterologous' incubations. Homogenate of A. viridis (Lough Hyne), which was the second most successful homogenate at stimulating translocation by algae isolated from C. pedunculatus, stimulated a significantly greater percentage translocation than host homogenates of A. viridis (Shell Is.), I. sulcatus and A. pallida (appendix 12, table 12 (p.538)). Graph 5.17 (p.297)

compares the percentage translocation by algae isolated from C. pedunculatus when suspended in 'homologous' and 'heterologous' homogenates.

(3)Percentage translocation by symbionts when incubated with homogenates of conspecific hosts from different geographical locations.

The average d.p.m. of each fraction of the light incubations and controls investigating intra-host species "host factor" specificity are listed in appendix 12, table 13 (p.539). Table 5.14 (p.298) shows the percentage translocation by the symbionts of C. pedunculatus and A. viridis in response to 'homologous' homogenates and homogenates of these same host species but from different geographical locations.

Significant differences existed between the release of photosynthate by symbionts of C. pedunculatus from Lough Hyne and Netley when incubated in homogenates of C. pedunculatus from the 2 different locations ($F=7.78$, $P=0.002$). Symbionts isolated from C. pedunculatus from both locations released the same percentages of their photosynthate when stimulated by homogenates from the same location, with release being similar to that measured in vivo. However, algae from C. pedunculatus (Netley) translocated significantly less photosynthate when in a homogenate of C. pedunculatus from Lough Hyne than symbionts isolated from C. pedunculatus (Lough Hyne) did in the 'homologous' incubations. In contrast, the percentage of photosynthate translocated by symbionts isolated from C.

pedunculatus (Lough Hyne) was not reduced when incubated in C. pedunculatus (Netley) homogenate, and was significantly greater than by Symbiodinium sp. from C. pedunculatus (Netley) when incubated in C. pedunculatus (Lough Hyne) homogenate (appendix 12, table 14 (p.540)). The percentage of photosynthate translocated by the symbionts of C. pedunculatus from Lough Hyne and Netley when stimulated by homogenates of C. pedunculatus from these 2 locations is compared in graph 5.18 (p.299).

Differences between the stimulatory capacities of host homogenates and/or the abilities to release photosynthate by the symbionts of anthozoans from different geographical locations were more sharply defined with regard to A. viridis. When symbionts of A. viridis were incubated with their 'homologous' homogenates they released significantly different percentages of photosynthate ($H=26.69$, $P<0.0001$). In 'homologous' incubations, symbionts of A. viridis (Lough Hyne) released a significantly larger proportion of their photosynthate than symbionts of A. viridis from Shell Island, Loch Sween and Bembridge. The only other significant difference between the 'homologous' incubations was the greater percentage of photosynthate released by the symbionts of A. viridis (Trearddur Bay) than by the symbionts of A. viridis (Loch Sween) (appendix 12, table 15 (p.540)). Symbionts of A. viridis from Loch Sween, Shell Island, Bembridge and Weymouth all virtually ceased to release photosynthate in the presence of a 'homologous'

homogenate, and only Symbiodinium sp. from A. viridis (Lough Hyne) released a percentage of fixed carbon comparable to that measured in vivo in A. viridis.

When symbionts isolated from A. viridis from different locations were incubated in A. viridis (Lough Hyne) homogenate, only the symbionts of A. viridis (Shell Is.) and A. viridis (Trearddur Bay) behaved significantly differently to when in the 'homologous' incubations (appendix 12, table 16 (p.540)). Symbiodinium sp. isolated from A. viridis (Shell Is.) released a significantly larger percentage of photosynthate and symbionts of A. viridis (Trearddur Bay) translocated a significantly lower percentage of fixed carbon when in the presence of A. viridis (Lough Hyne) homogenate than when stimulated by their 'homologous' homogenates. The percentage translocation by symbionts of A. viridis from different locations in response to a 'homologous' homogenate and a homogenate of A. viridis (Lough Hyne) is compared in graph 5.19 (p.299).

Symbionts of A. viridis from different locations released significantly different percentages of photosynthate when suspended in homogenates of A. viridis (Lough Hyne) ($H=27.16$, $P<0.0001$). The percentage translocation in the 'homologous' A. viridis (Lough Hyne) incubation was significantly greater than when symbionts of A. viridis from Loch Sween and Bembridge were suspended in A. viridis (Lough Hyne) homogenate. The percentage of photosynthate released was also significantly higher by symbionts of A.

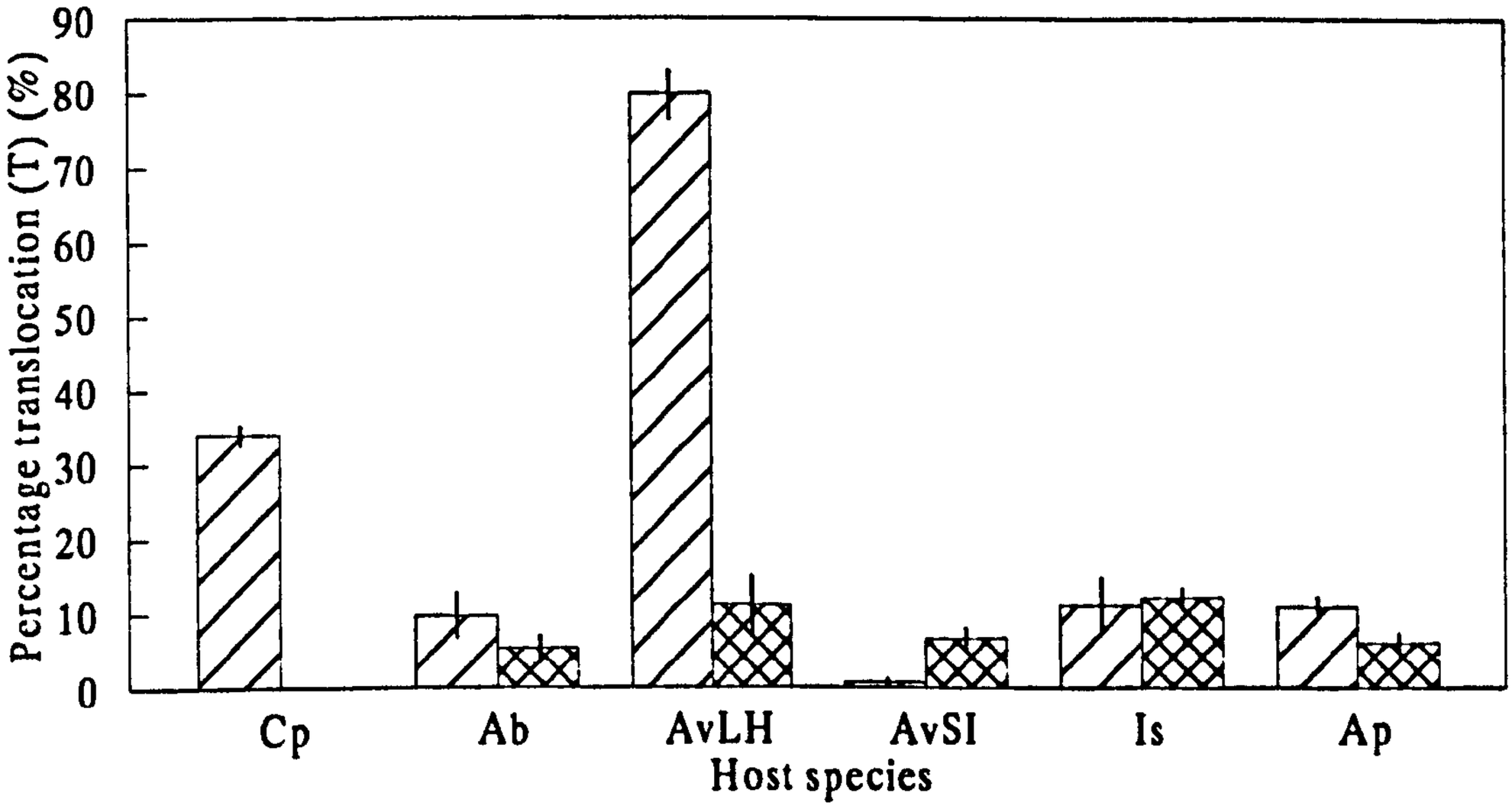
viridis from Weymouth than by symbionts of A. viridis from Loch Sween when both were incubated in A. viridis (Lough Hyne) homogenate (appendix 12, table 17 (p.541)).

Significant differences were also observed between the percentage of photosynthate released by symbionts of A. viridis (Lough Hyne) when stimulated by homogenates of A. viridis from different locations ($F=17.02$, $P<0.0001$). Symbiodinium cells from A. viridis (Lough Hyne) released a significantly greater percentage of photosynthate when in an 'homologous' incubation than when in the presence of homogenate of A. viridis from Bembridge, Weymouth and Shell Island. In addition, a significantly greater percentage of fixed carbon was translocated by symbionts isolated from A. viridis (Lough Hyne) when in A. viridis (Loch Sween) homogenate than when in A. viridis (Bembridge) homogenate. Also, symbionts of A. viridis (Lough Hyne) translocated a significantly higher percentage of photosynthate when incubated in A. viridis (Trearddur Bay) homogenate than when in homogenates of A. viridis from Bembridge, Weymouth Harbour and Shell Island (appendix 12, table 18 (p.541)). Of note, Symbiodinium sp. from A. viridis (Lough Hyne) released a significantly greater percentage of photosynthate in these 'heterologous' incubations than the symbionts of A. viridis from the other locations did when incubated in their 'homologous' homogenates (table 5.15 (p.300)). The percentage of photosynthate translocated by the symbionts of A. viridis (Lough Hyne), when suspended in 'homologous'

TABLE 5.13 THE PERCENTAGE OF ^{14}C LABELLED FIXED CARBON TRANSLOCATED WHEN SYMBIONTS WERE STIMULATED IN VITRO BY 'HOMOLOGOUS' AND 'HETEROLOGOUS' HOST HOMOGENATES OF DIFFERENT HOST SPECIES +/- S.E. (N=5, EXCEPT N=3 FOR SEA WATER CONTROLS) (ALL PERCENTAGES HAVE BEEN CORRECTED FOR SEA WATER CONTROLS).

SYMBIONTS	HOMOGENATE						
Host sp.	sea water	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	11.7 +/- 0.7	34.1 +/- 1.1	2.4 +/- 1.1	5.3 +/- 0.5	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0
Ab	20.1 +/- 0.3	5.3 +/- 1.4	9.6 +/- 2.7	-	-	-	-
AvLH	5.3 +/- 0.3	11.1 +/- 3.8	-	79.9 +/- 3.0	-	-	-
AvSI	6.3 +/- 0.6	6.5 +/- 1.1	-	-	0.8 +/- 0.3	-	-
Is	2.6 +/- 0.8	12.1 +/- 1.0	-	-	-	11.0 +/- 3.1	-
Ap	5.3 +/- 1.0	6.0 +/- 0.9	-	-	-	-	10.8 +/- 0.8

Graph 5.16: Percentage translocation (T) in in vitro 'homologous' incubations and when 'heterologous' symbionts were incubated in C. pedunculatus homogenate (N=5) (+/- S.E.).



Graph 5.17: Percentage translocation (T) when symbionts of C. pedunculatus were incubated in vitro with homogenates of different host species (N=5) (+/- S.E.).

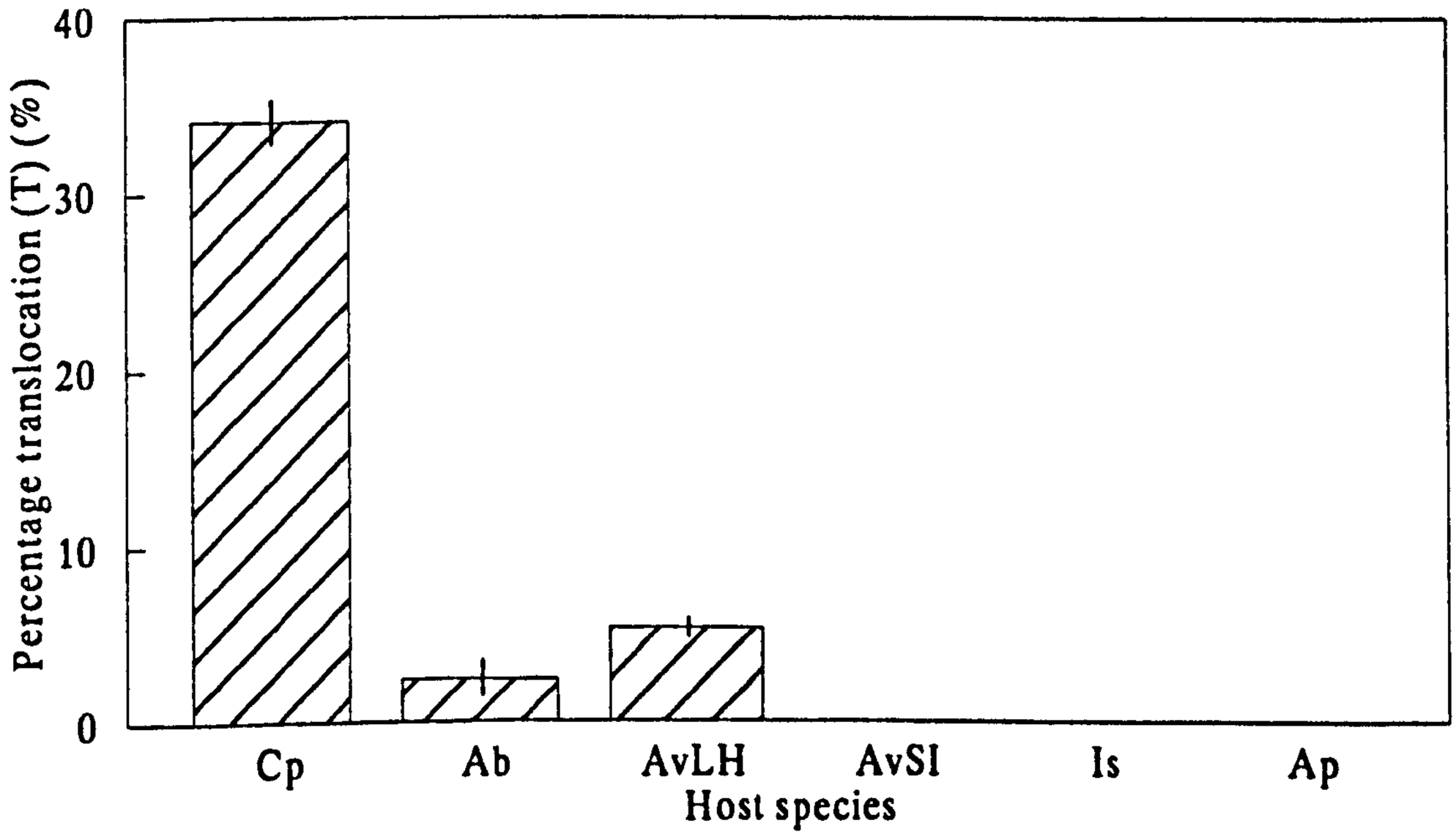
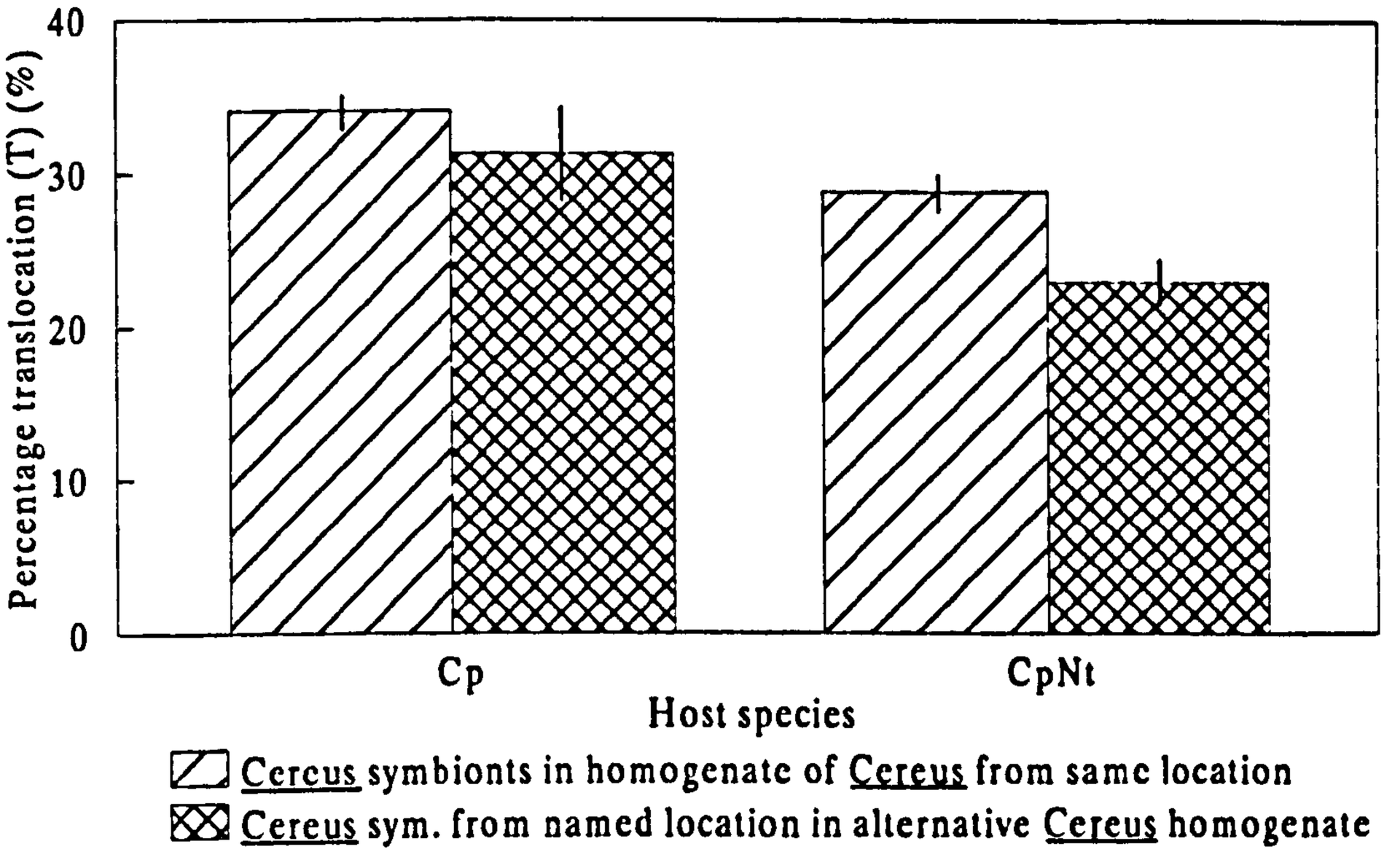


TABLE 5.14 THE PERCENTAGE OF ^{14}C LABELLED FIXED CARBON TRANSLOCATED BY SYMBIONTS, WHEN STIMULATED IN VITRO WITH HOMOGENATES OF THE SAME HOST SPECIES BUT FROM DIFFERENT GEOGRAPHICAL LOCATIONS (N=5, EXCEPT N=3 FOR SEA WATER CONTROLS)(ALL PERCENTAGES HAVE BEEN CORRECTED FOR SEA WATER CONTROLS).

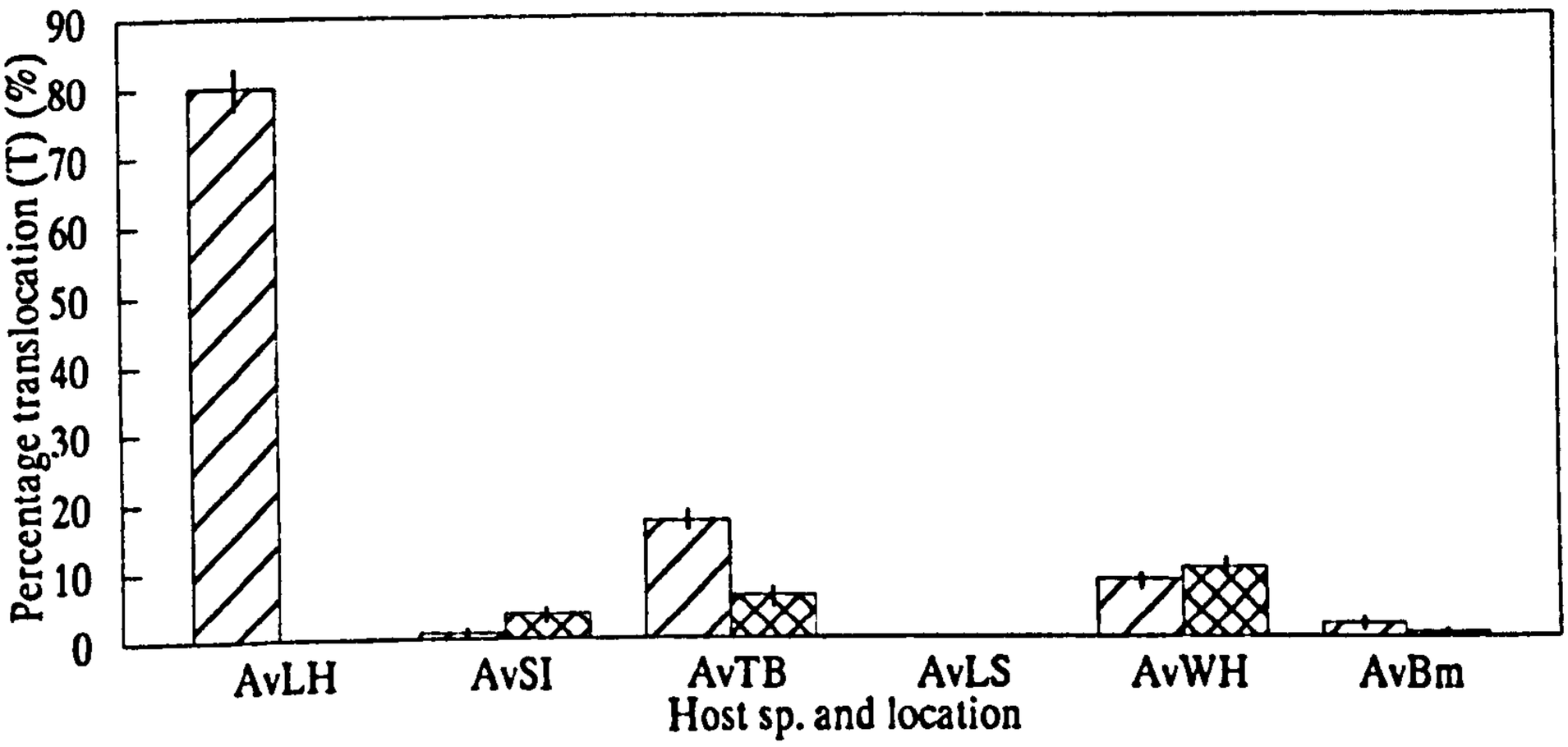
SYMBIONTS	HOMOGENATE									
Host sp.	sea wat -er	Cp	Cp Nt	Av LH	Av SI	Av TB	Av LS	Av WH	Av Bm
Cp	11.7 +/- 1.0	34 +/- 1.1	31.3 +/- 2.8	-	-	-	-	-	-
Cp Nt	7.9 +/- 1.5	22.9 +/- 1.3	28.8 +/- 1.1	-	-	-	-	-	-
Av LH	5.3 +/- 0.3	-	-	79.9 +/- 3.0	43.2 +/- 6.4	65.2 +/- 3.0	60.6 +/- 3.8	44.3 +/- 3.2	28 +/- 6.1
Av SI	6.3 +/- 0.6	-	-	3.7 +/- 0.8	0.8 +/- 0.3	-	-	-	-
Av TB	6.2 +/- 0.42	-	-	6.2 +/- 1.1	-	17 +/- 1.3	-	-	-
Av LS	7.5 +/- 1.0	-	-	0.0 +/- 0.0	-	-	0.0 +/- 0.0	-	-
Av WH	7.2 +/- 1.2	-	-	10 +/- 1.1	-	-	-	8.3 +/- 1.1	-
Av Bm	5.5 +/- 0.6	-	-	0.7 +/- 0.2	-	-	-	-	1.9 +/- 0.9

Graph 5.18: Percentage translocation (T) in vitro by symbionts of C. pedunculatus from Lough Hyne and Netley when incubated in homogenates of Cereus from both locations (N=5) (+/- S.E.).



Graph 5.19: Percentage translocation (T) in vitro when symbionts of A. viridis from different locations were incubated in homogenates of A. viridis from the same locations and from Lough Hyne.

(N=5) (+/- S.E.).



Graph 5.20: Percentage translocation (T) in vitro when symbionts of A. viridis (Lough Hyne) were incubated in homogenates of A. viridis from different locations (N=5) (+/- S.E.).

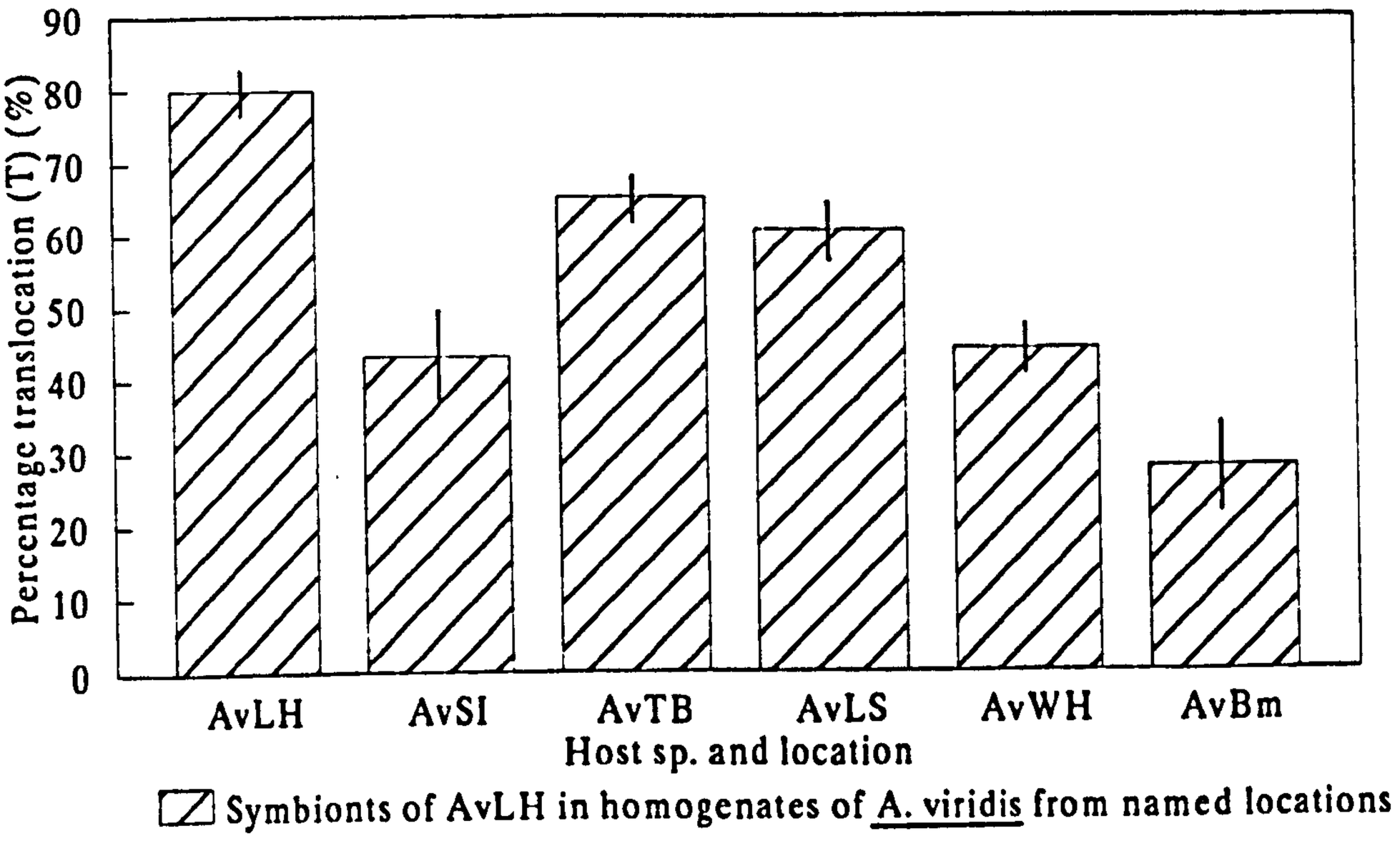


TABLE 5.15 RESULTS OF STUDENT'S T-TEST COMPARING THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF A.VIRIDIS (LOUGH HYNE) IN RESPONSE TO HOMOGENATES OF A. VIRIDIS FROM DIFFERENT LOCATIONS VS. THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF A. VIRIDIS FROM DIFFERENT LOCATIONS IN RESPONSE HOMOGENATES OF THEIR ORIGINAL HOSTS (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

AvLH symbionts in homogenate of A. viridis from named location vs. A. viridis from named location in own homogenate.	T statistic and probability
AvSI (N=5)	**T=6.58, P=0.0028 ***
AvTB (N=5)	**T=14.64, P<0.0001 ***
AvLS (N=5)	T cannot be calculated due to percentage translocation in 'homologous' incubation = 0 %
AvWH(N=5)	**T=10.66, P=0.0004 ***
AvBm (N=5)	**T=4.22, P=0.014 ***

homogenate and homogenates of A. viridis from other locations, is compared in graph 5.20 (p.300).

(iii)**Estimation of the percentage translocation using the 'growth rate method'.**

(1)The percentage translocation in the 'natural' symbioses.

The calculation of the percentage translocation in the 'natural' symbioses using the 'growth rate method' is shown in table 5.16 (p.303).

All the values of the percentage translocation estimated using the 'growth rate method' were substantially higher than the measurements of the percentage translocation made using ^{14}C *in vivo*.

Despite the percentage translocation approaching 100 % in all the 'natural' symbioses, significant differences existed between the percentages of photosynthate translocated in these associations ($H=20.17$, $P=0.001$), which are shown in detail in appendix 12, table 19 (p.542). A significantly greater percentage of photosynthate was estimated to be translocated in *A. pallida* than in *A. ballii* and *A. viridis* (Shell Is.). The very high percentage translocation in *A. pallida* estimated here was in contrast to this symbiosis being estimated to translocate the lowest percentage of photosynthate when *in vivo* ^{14}C labelling was used. Possible reasons for this discrepancy will be discussed later.

(2)The percentage translocation in the reestablished symbioses.

The calculation of the percentage translocations in the reestablished symbioses using the 'growth rate method' is shown in table 5.17 (p.303). The percentage translocations calculated with this method in the 'natural' and reestablished symbioses are compared in graph 5.21 (p.304). The percentage translocation in the reestablished symbioses estimated by the 'growth rate method' was, in contrast to the situation observed when *in vivo* ^{14}C labelling was used, significantly lower than when the symbionts were in their original host species (table 5.18 (p.304)). The percentage of photosynthate translocated in the reestablished symbioses was significantly different between the different reinfections ($H=15.34$, $P=0.009$). The percentage of carbon translocated by the symbionts in 'homologous' reestablished symbioses was significantly higher than the percentage translocation in the 'heterologous' symbioses involving symbionts from *A. viridis* (Shell Is.) and *I. sulcatus*; no significant differences existed between the percentage of photosynthate translocated in the other reestablished symbioses (appendix 12, table 20 (p.542)).

TABLE 5.16 CALCULATION OF THE PERCENTAGE TRANSLOCATION (T) IN THE 'NATURAL' SYMBIOSES USING THE 'GROWTH RATE METHOD'.

Host sp.	Cell specific growth rate (μ) (day^{-1})	Carbon specific growth rate (μc) +/- S.E. (day^{-1})	Percentage translocation (T) +/- S.E. (%)
Cp (N=5)	0.040	2.59 +/- 0.61	98.0 +/- 0.6
Ab (N=5)	0.031	1.25 +/- 0.40	96.5 +/- 1.1
AvLH (N=5)	0.032	3.01 +/- 0.59	98.7 +/- 0.3
AvSI (N=5)	0.025	0.94 +/- 0.17	96.9 +/- 0.6
Is (N=3)	0.025	5.29 +/- 0.76	99.5 +/- 0.1
Ap (N=5)	0.019	6.70 +/- 2.2	99.6 +/- 0.2

TABLE 5.17 CALCULATION OF THE PERCENTAGE TRANSLOCATION (T) IN THE REESTABLISHED SYMBIOSES USING THE 'GROWTH RATE METHOD'

Cp + symbionts from named host sp.	Cell specific growth rate (μ) (day^{-1})	Carbon specific growth rate (μc) (day^{-1}) +/- S.E.	Percentage translocation (T) (%) +/- S.E.
Cp (N=5)	0.142	2.63 +/- 0.08	94.6 +/- 0.2
Ab (N=4)	0.149	1.83 +/- 0.26	91.3 +/- 1.3
AvLH (N=5)	0.136	1.32 +/- 0.14	89.1 +/- 1.4
AvSI (N=5)	0.134	1.10 +/- 0.17	86.5 +/- 2.3
Is (N=4)	0.152	1.15 +/- 0.30	84.0 +/- 3.8
Ap (N=5)	0.148	1.53 +/- 0.23	89.3 +/- 1.8

Graph 5.21: Percentage translocation (T) calculated by the 'growth rate method' in 'natural' and reestablished symbioses

(N = 5, except N = 4 for reinfections involving symbionts of A. ballii and I. sulcatus) (+/- S.E.).

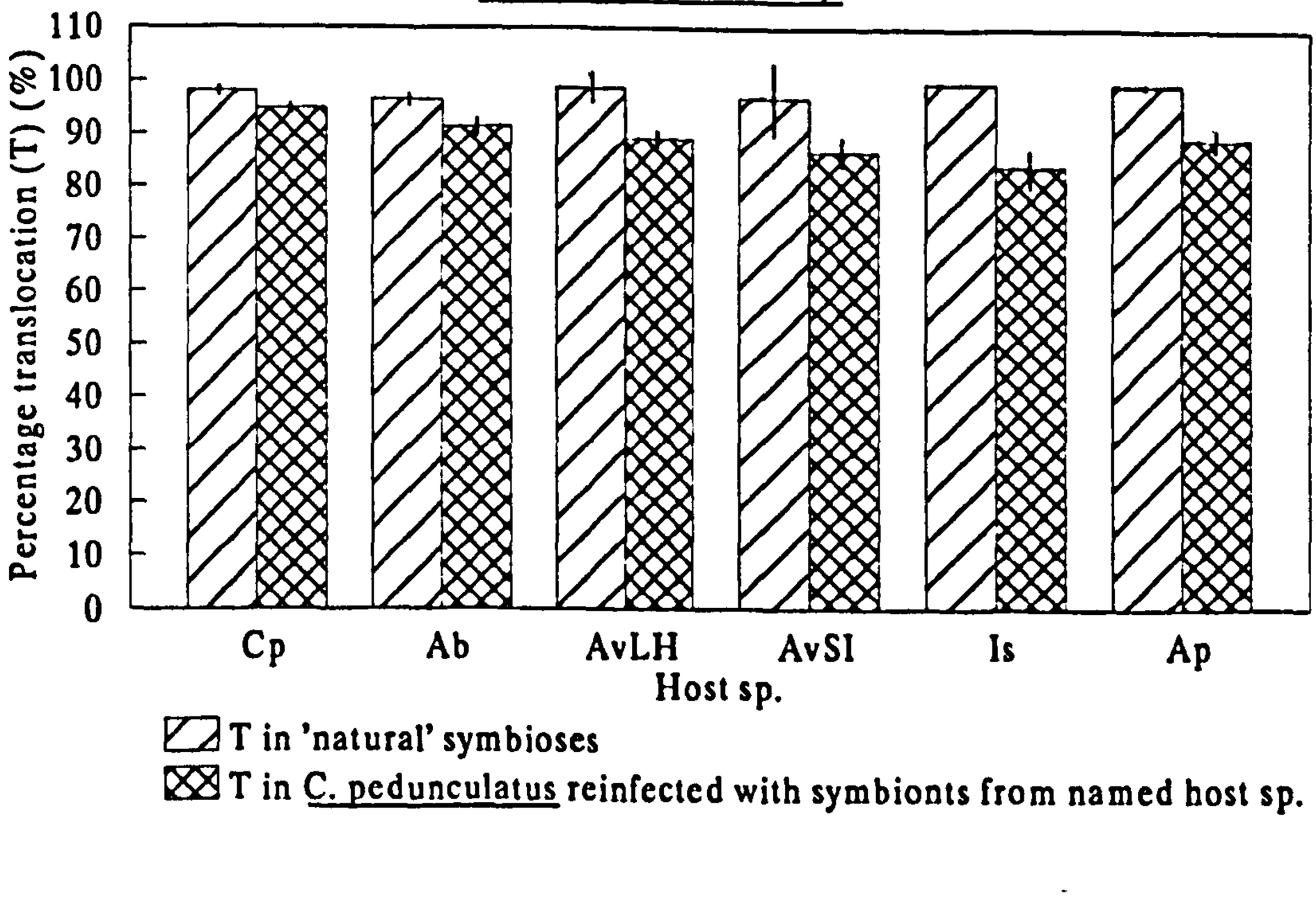


TABLE 5.18 RESULTS OF STUDENT'S T-TEST COMPARING THE PERCENTAGE TRANSLOCATION, AS DETERMINED USING THE 'GROWTH RATE METHOD', BETWEEN SYMBIONTS WHEN IN THEIR ORIGINAL HOST SPECIES AND WHEN IN REINFECTED C. PEDUNCULATUS (SIGNIFICANT DIFFERENCES ($P<0.05$) IN BOLD WITH AN ASTERISK).

Host sp. from which symbionts were originally from.	T statistic and probability
Cp (N=5)	**T=5.69, P=0.0047 ***
Ab (N=5 for 'natural' symbiosis, N=4 for reestablished symbiosis)	**T=3.05, P=0.0220 ***
AvLH (N=5)	**T=6.87, P=0.0024**
AvSI (N=5)	**T=4.32, P=0.0120 ***
Is (N=3 for 'natural' symbiosis, N=4 for reestablished symbiosis)	**T=4.13, P=0.0260 ***
Ap (N=5)	**T=5.66, P=0.0048 ***

(f)**Calculation of the CZAR.**

(i)**The CZAR in the 'natural' symbioses.**

(1)The CZAR calculated using the radioisotopic measurement of the percentage translocation.

The calculation of the CZAR in the 'natural' symbioses from the daily net algal photosynthetic production, the daily animal respiration and the percentage translocation (as estimated from *in vivo* ^{14}C incubations and the 'growth rate method') is shown in table 5.19 (p.307). The CZAR was not calculated using the percentage translocation measured by *in vitro* ^{14}C incubations because of the possible effects of the isolation procedure on the capacity of the symbionts to release photosynthate or the host material to stimulate release.

The CZAR estimated using the percentage translocation derived by ^{14}C labelling was significantly different between the 'natural' symbioses ($H=19.58$, $P=0.002$). Only the CZAR in *A. ballii* differed significantly, being significantly lower than in *A. viridis* (Lough Hyne) and *I. sulcatus*. The CZAR in *A. ballii* was also approaching being significantly less than in *A. viridis* (Shell Is.). The low CZAR in *A. ballii* reflected the low percentage translocation estimated using ^{14}C labelling (33.8 %) and the comparatively high animal respiration : net algal photosynthesis ratio. Despite the CZAR in the other 'natural' symbioses not varying significantly when the percentage translocation was measured using ^{14}C (appendix 13, table 1 (p.544)), it was noted that,

uniquely, the CZAR in I. sulcatus was greater than 100 % and was at least 2 fold greater than in the other anthozoans with the exception of A. viridis (Lough Hyne). This was unsurprising considering that the percentage translocation was highest in I. sulcatus and that net algal photosynthetic production and host respiration were 2.0-3.6 and 1.3-2.5 times greater respectively in I. sulcatus than in the other host species, with the exception of A. pallida.

(2)The CZAR calculated using the percentage translocation as determined by the 'growth rate method'.

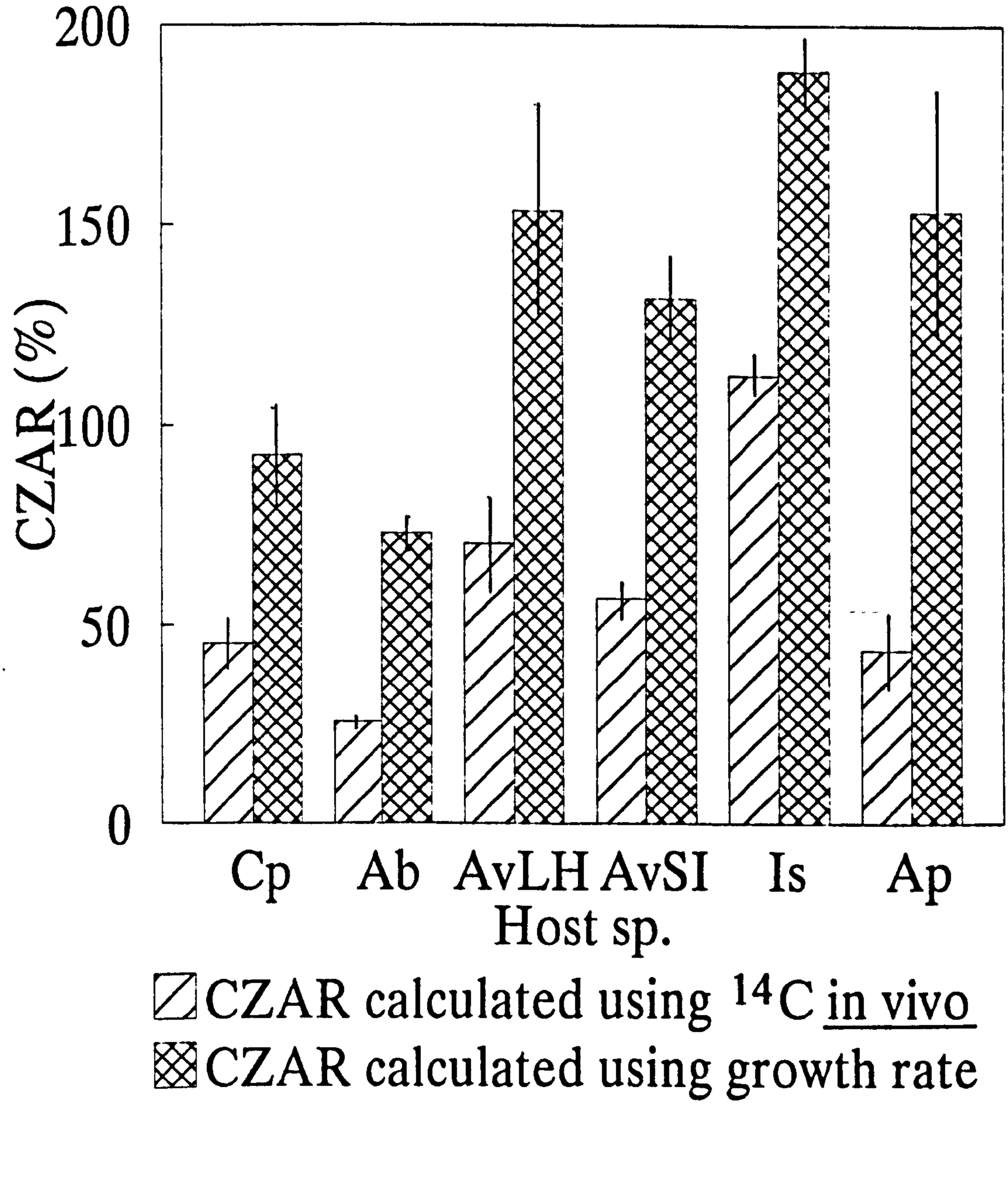
When the CZAR was calculated using the percentage translocation derived by the 'growth rate method' (table 5.19 (p.307)), the CZAR in the different host species was again significantly different (H=18.08, P=0.003). The only significant difference between the CZAR in the different host species was the significantly higher CZAR in I. sulcatus than in A. ballii, although the CZAR in A. viridis (Lough Hyne) was approaching being significantly higher than in A. ballii (appendix 13, table 2 (p.544)). The high percentage translocation estimated by the 'growth rate method' resulted in an average CZAR of more than 100 % in all the 'natural' symbioses except in C. pedunculatus (92.7 %) and A. ballii (73.2 %). The CZAR values in the 'natural' symbioses, as calculated using both the ^{14}C and growth rate methods, are compared in graph 5.22 (p.308).

TABLE 5.19 CALCULATION OF THE CZAR IN THE 'NATURAL' SYMBIOSES (Pz (net)=net algal photosynthesis; Ra=animal respiration; T=percentage translocation).

Host sp.	Cp (N=5)	Ab (N=5)	AvLH (N=5)	AvSI (N=5)	Is (N=3)	Ap (N=5)
Pznet (mgC/ 12h/mg assoc. prot.) +/- S.E.	0.039 +/- 0.011	0.035 +/- 0.013	0.061 +/- 0.011	0.034 +/- 0.007	0.124 +/- 0.020	0.161 +/- 0.004
Ra (mgC/ 24h/mg assoc. prot.) +/- S.E.	0.040 +/- 0.006	0.047 +/- 0.018	0.039 +/- 0.004	0.026 +/- 0.006	0.065 +/- 0.010	0.103 +/- 0.006
T (%) (using ^{14}C *in vivo*) +/- S.E.	48.6 +/- 5.4	33.8 +/- 2.7	45.4 +/- 2.8	41.9 +/- 2.6	59.3 +/- 3.9	28.4 +/- 3.0
T (%) (using growth rate) +/- S.E.	98.0 +/- 0.6	96.5 +/- 1.1	98.7 +/- 0.3	96.9 +/- 0.6	99.5 +/- 0.06	99.6 +/- 0.2
CZAR (%) (using ^{14}C *in vivo*) +/- S.E.	**45.4 +/- 5.8**	**25.7 +/- 1.6**	**70.5 +/- 12.0**	**56.8 +/- 4.4**	**112.2 +/- 5.0**	**43.7 +/- 8.7**
CZAR (%) (using growth rate) +/- S.E.	**92.7 +/- 11.4**	**73.2 +/- 3.8**	**153.5 +/- 26.4**	**131.5 +/- 10.3**	**188.4 +/- 8.5**	**153.3 +/- 30.7**

Graph 5.22: The CZAR in the 'natural' symbioses calculated using ^{14}C and the 'growth rate method'

(N=5, except N=3 for I. sulcatus) (+/- S.E.).



(ii)**The CZAR in the reestablished symbioses**

(1)The CZAR calculated using the radioisotopic determination of the percentage translocation.

The calculation of the CZAR in the reestablished symbioses from the daily net algal photosynthetic production, the daily host respiration and the percentage translocation (as estimated using both ^{14}C labelling and the 'growth rate method') is shown in table 5.20 (p.313).

The CZAR calculated using the percentage translocation measured with ^{14}C in the reestablished symbioses could not be compared statistically because of the method by which the replicate values were calculated. However, the CZAR was highest in the 'homologous' symbioses (76.7 %) and symbioses with algae originally from A. ballii (70.4 %). The CZAR in the other 'heterologous' symbioses was similar, the lowest being in the symbiosis with symbionts from A. viridis (Shell Is.) (58.0 %). The translocation of ^{14}C labelled compounds was not significantly different in these symbioses, therefore the differences in the CZAR reflected differences between the ratios of daily net algal photosynthesis : daily animal respiration in the reestablished symbioses. C. pedunculatus reinfected with 'homologous' symbionts and Symbiodinium sp. from A. ballii had net algal photosynthesis/day (12 hours) : host respiration/day (24 hours) ratios of 1.30 and 1.60 respectively, compared to ratios between the same 2 values ranging from 1.0-1.20 in the other 'heterologous' symbioses.

(2)The CZAR calculated using the 'growth rate method'.

The CZAR in the reestablished symbioses calculated using the percentage translocation derived by the 'growth rate method' (table 5.20 (p.313)) varied significantly between different reinfections (H=24.12, P<0.0001). The CZAR in the 'homologous' reinfections was significantly greater than in polyps reinfected with symbionts from A. viridis (Shell Is.). The CZAR in C. pedunculatus reinfected with algae originally from A. ballii was also significantly greater than in C. pedunculatus reinfected with symbionts isolated from A. viridis (Shell Is.) and I. sulcatus (appendix 13, table 3 (p.545)). The CZAR values in the reestablished symbioses, as estimated using both ^{14}C labelling and the 'growth rate method', are compared in graph 5.23 (p.314).

(3)The CZAR standardized to symbiont density and biomass.

The standardized values of the CZAR in the reestablished symbioses are given in table 5.21 (p.315).

The CZAR/cell/mg association protein in the 'homologous' and 'heterologous' reestablished symbioses was not significantly different when the percentage translocation was estimated using $NaH^{14}CO_3$ (H=8.85, P=0.116) or the 'growth rate method' (H=9.52, P=0.091). These values are compared in graph 5.24 (p.316).

However, significant differences were present between the CZAR/mg algal C/mg association protein in the different reestablished symbioses when the percentage translocation was measured using ^{14}C labelling (F=5.67, P=0.002) or the

'growth rate method' ($F=5.39$, $P=0.002$).

When the CZAR was calculated using the percentage translocation measured by ^{14}C labelling, a significantly greater percentage contribution to animal respiration was made by each mg symbiont carbon in the 'homologous' reinfected *C. pedunculatus* than in symbioses with symbionts originally from *A. ballii* and *A. viridis* (Shell Is.). The CZAR/mg algal C/mg association protein was also approaching being significantly greater in the 'homologous' reestablished symbioses than in the reestablished symbioses with algae from *A. viridis* (Lough Hyne), *I. sulcatus* and *A. pallida* (appendix 13, table 4 (p.545)).

A similar pattern was also observed when the CZAR estimated using the 'growth rate method' was standardized to the algal carbon standing stock/mg association protein. Each mg algal carbon again contributed to a significantly greater percentage of the *C. pedunculatus* respiratory budget in the 'homologous' reestablished symbiosis than in the symbiosis between *C. pedunculatus* and *Symbiodinium* sp. isolated from *A. viridis* (Shell Is.). The CZAR/mg algal C/mg association protein calculated with the 'growth rate method' also approached a significantly greater percentage in the 'homologous' reinfected hosts than in symbioses between *C. pedunculatus* and symbionts isolated from *A. viridis* (Lough Hyne), *I. sulcatus* and *A. pallida* (appendix 13, table 5 (p.546)).

Using either method of estimating the percentage

translocation, the percentage contribution of each unit biomass of symbiont carbon to the daily respiratory budget of C. pedunculatus was not significantly different between any of the 'heterologous' symbiont 'strains' (appendix 13, tables 4 (p.545) and 5 (p.546)). The CZAR calculated using the percentage translocation determined by the ^{14}C labelling method or by the 'growth rate method' and standardized to the algal carbon standing stock/mg association protein is compared between the different reestablished symbioses in graph 5.25 (p.316).

TABLE 5.20 CALCULATION OF THE CZAR IN THE REESTABLISHED SYMBIOSES (Pz(net)=net algal photosynthesis; Ra=animal respiration; T=percentage translocation).

Cp + symbionts from named sp.	Cp (N=5)	Ab (N=4)	AvLH (N=5)	AvSI (N=5)	Is (N=4)	Ap (N=5)
Pznet (mgC/ 12h/mg assoc. prot.) +/- S.E.	0.035 +/- 0.0000	0.041 +/- 0.0001	0.023 +/- 0.0000	0.024 +/- 0.0001	0.021 +/- 0.0001	0.027 +/- 0.0000
Ra (mgC/ 24h/mg assoc. prot.) +/- S.E.	0.027 +/- 0.000	0.026 +/- 0.0001	0.020 +/- 0.000	0.023 +/- 0.0001	0.019 +/- 0.0001	0.025 +/- 0.0001
T(%) (using ^{14}C *in vivo*) +/- S.E.	59.1 +/- 3.7	44.0 +/- 3.7	52.8 +/- 4.3	56.9 +/- 6.3	55.8 +/- 4.7	59.1 +/- 3.9
T(%) (using growth rate) +/- S.E.	94.6 +/- 0.2	91.3 +/- 1.3	89.1 +/- 1.3	86.5 +/- 2.3	84.0 +/- 3.8	89.3 +/- 1.8
CZAR (%) (using ^{14}C *in vivo*) +/- S.E.	**76.7 +/- 0.02**	**70.6 +/- 0.2**	**62.0 +/- 0.1**	**58.1 +/- 0.3**	**61.0 +/- 0.1**	**64.2 +/- 0.1**
CZAR (%) (using growth rate) +/- S.E.	**122.8 +/- 0.21**	**146.5 +/- 1.8**	**104.4 +/- 1.5**	**88.3 +/- 5.0**	**91.8 +/- 3.9**	**96.9 +/- 1.9**

Graph 5.23: The CZAR in C. pedunculatus reinfected with symbionts from the named host sp. calculated using ^{14}C and the 'growth rate method'

(N=5, except N=4 for reinfections involving symbionts of A. ballii and I. sulcatus) (+/- S.E.).

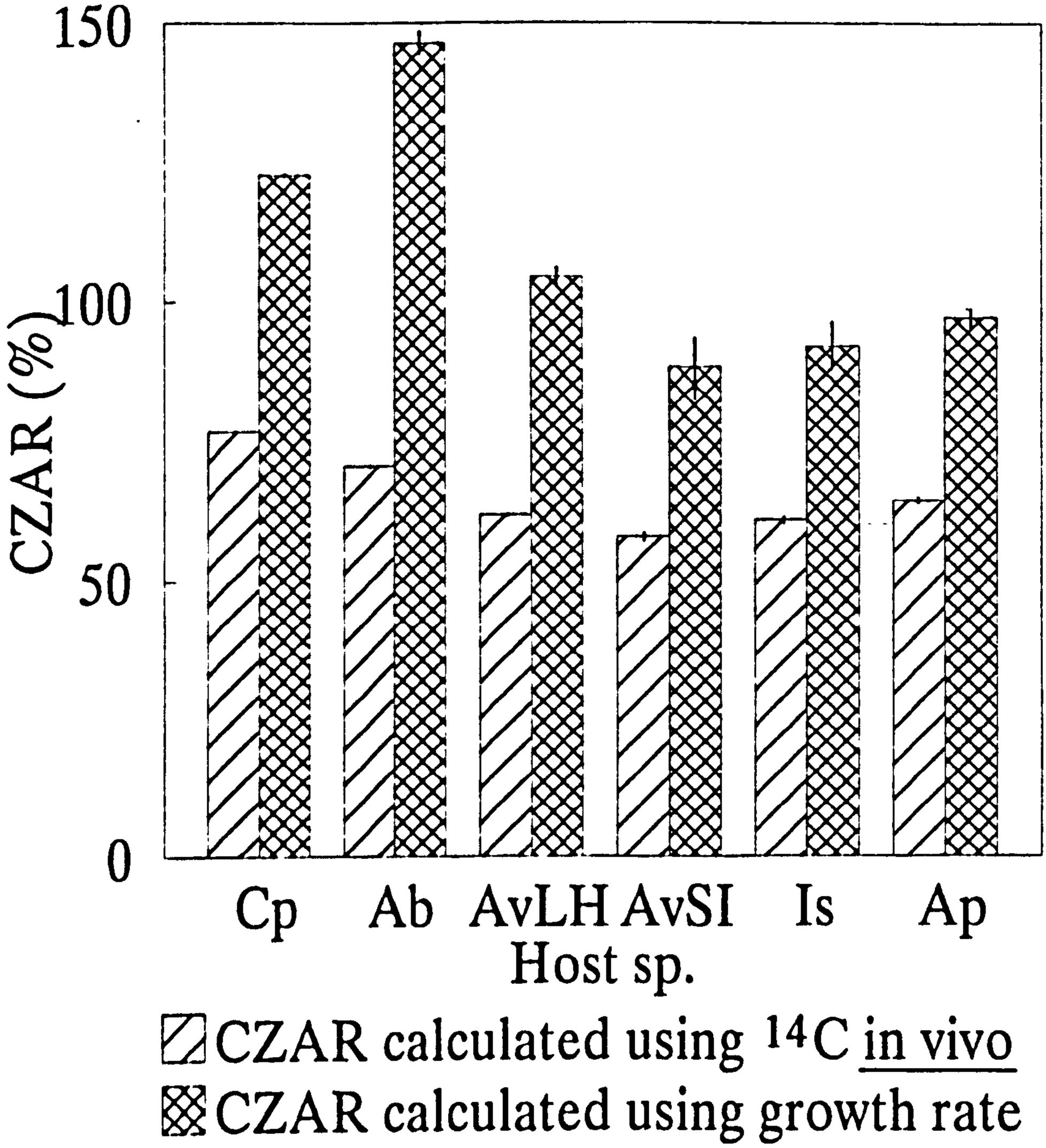
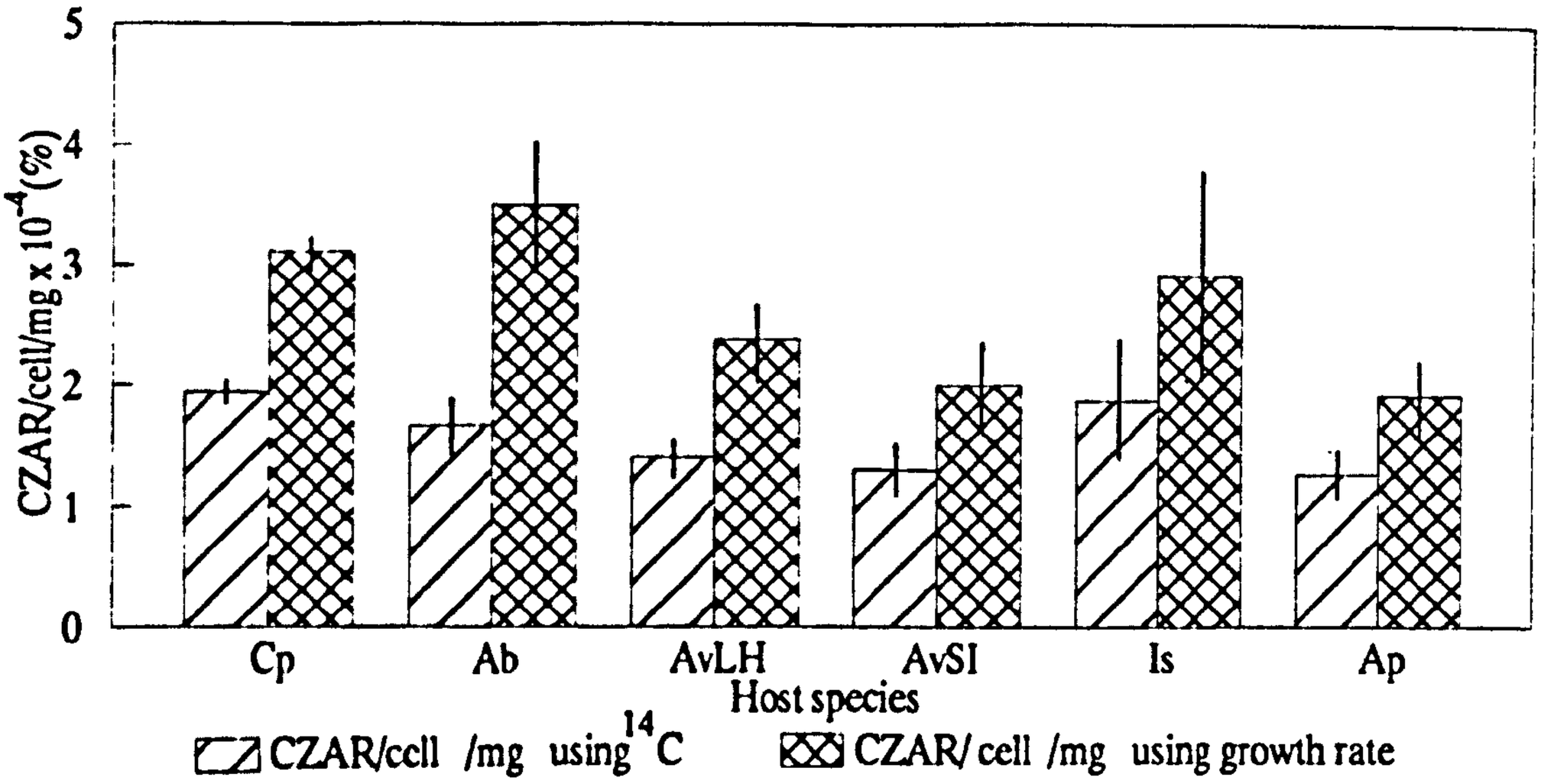


TABLE 5.21 THE CZAR IN THE REESTABLISHED SYMBIOSES STANDARDIZED TO SYMBIONT CELL DENSITY AND CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN.

Cp + symbionts from named host sp.	CZAR/cell/ mg assoc. protein $x10^{-4}$ (%) (using ^{14}C in vivo) +/- S.E.	CZAR/cell/ mg assoc. protein $x10^{-4}$ (%) (using 'growth rate method') +/- S.E.	CZAR/mgC/ mg assoc. protein (%) $x10^{3}$ (using ^{14}C in vivo) +/- S.E.	CZAR/mgC/ mg assoc. protein (%) $x10^{3}$ (using 'growth rate method') +/- S.E.
Cp (N=5)	1.94 +/- 0.06	3.11 +/- 0.10	5.85 +/- 0.17	9.36 +/- 0.29
Ab (N=4)	1.67 +/- 0.23	3.50 +/- 0.53	3.18 +/- 0.44	6.63 +/- 1.00
AvLH (N=5)	1.40 +/- 0.15	2.38 +/- 0.28	3.50 +/- 0.37	5.95 +/- 0.71
AvSI (N=5)	1.30 +/- 0.19	2.00 +/- 0.34	2.71 +/- 0.40	3.73 +/- 0.42
Is (N=4)	1.87 +/- 0.47	2.90 +/- 0.85	3.35 +/- 0.84	5.20 +/- 1.52
Ap (N=5)	1.26 +/- 0.19	1.92 +/- 0.32	3.62 +/- 0.54	5.53 +/- 0.91

Graph 5.24: The CZAR, as calculated using ^{14}C and the 'growth rate method', standardized to symbiont density in C. pedunculatus reinfected with symbionts from the named host sp..

(N=5, except N=4 for reinfections involving symbionts from A. ballii and I. sulcatus) (+/− S.E.).



Graph 5.25: The CZAR, as calculated using ^{14}C and the 'growth rate method', standardized to symbiont carbon standing stock in C. pedunculatus reinfected with symbionts from the named host sp..

(N as in graph 5.24) (+/− S.E.).

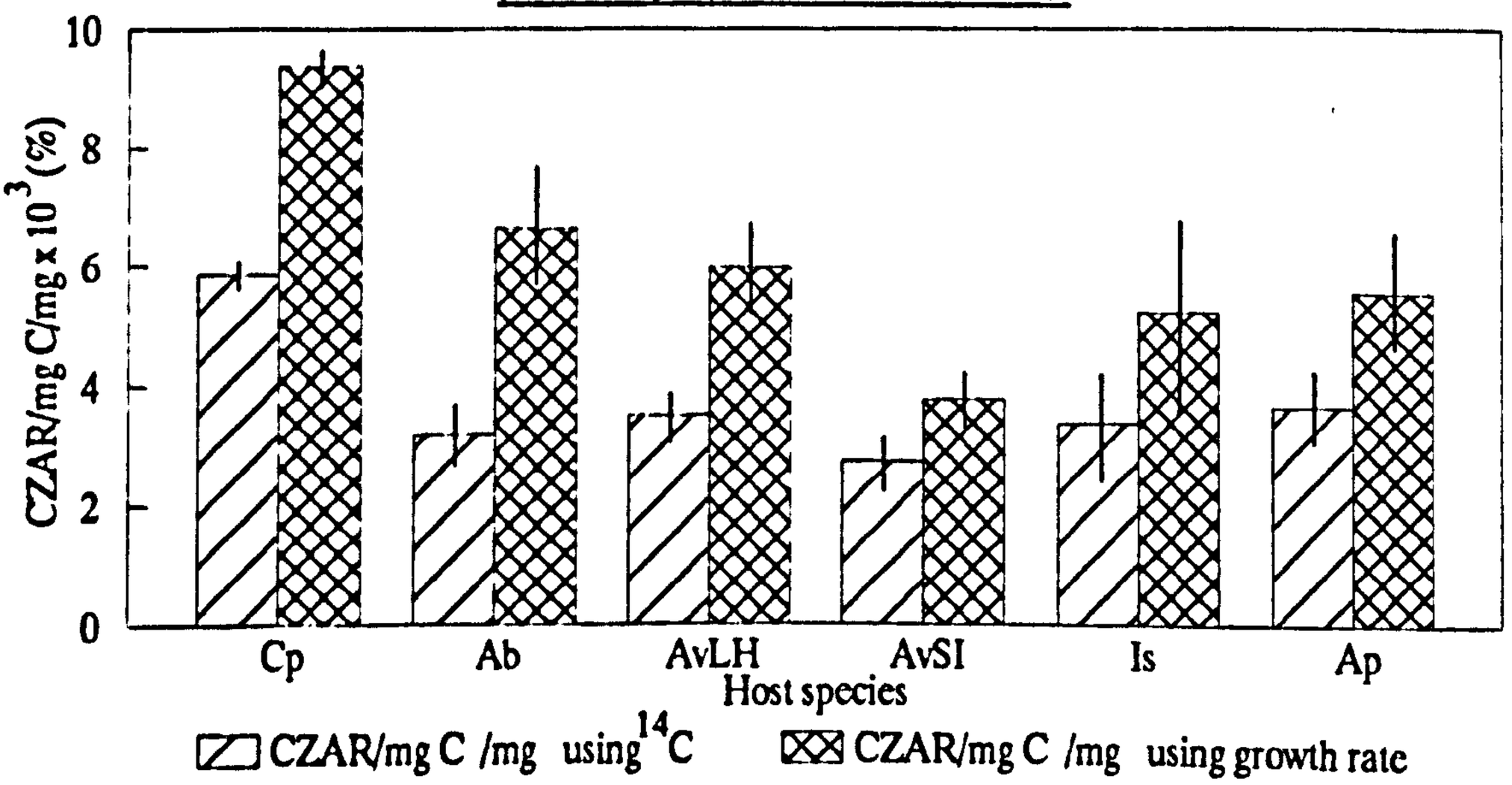


TABLE 5.22 THE INFLUENCE OF A 5 FOLD INCREASE IN THE MITOTIC INDEX (MI) AND AN 8 FOLD INCREASE IN THE ALGAL RESPIRATION RATE (Rz) ON THE CZAR IN THE 'NATURAL' SYMBIOSES (μ=cell specific growth rate (day^{-1}); T=percentage translocation (%); Pz(net)=net algal photosynthesis (mgC/12h/mg association protein); Ra=animal respiration (mgC/24h/mg association protein)).

Host sp.	μ when MIx5	T when MIx5	CZAR (%) when MIx5	Pz net when Rzx8	T when Rzx8	Ra when Rzx8	CZAR (%) when Rzx8	T when MIx5 and Rzx8	CZAR (%) when MIx5 and Rzx8
Cp	0.19	92.6	91	0.04	98.2	0.04	101	91.2	94
Ab	0.15	87.9	65	0.03	96.6	0.04	75	83.4	65
AvLH	0.16	94.8	147	0.06	98.8	0.03	171	94.1	163
AvSI	0.12	86.8	114	0.03	96.4	0.02	155	82.0	132
Is	0.12	97.7	185	0.12	99.5	0.06	215	97.5	211
Ap	0.10	98.6	154	0.15	99.7	0.08	178	98.2	175

(iii)Investigation of the effects of an elevated symbiont mitotic index and respiration rate on the CZAR.

The influence of a 5 fold increase in mitotic index (MI) and an 8 fold increase in symbiont respiration on the CZAR in the 'natural' symbioses is shown in table 5.22 (p.317). The increased MI resulted in a reduced percentage translocation due to net fixed carbon being assumed to be used either in growth or translocated. The CZAR in I. sulcatus and A. pallida was influenced very little as a result of the increased MI. This was because of the comparatively high net algal photosynthetic rates and carbon specific growth rates lessening the impact of the increase in carbon allocated to symbiont growth on the calculation of the percentage translocation. In contrast, the 8 fold increase in algal respiration resulted in an increase in the CZAR of up to approximately 23.5 % (in A. viridis (Shell Is.)). The increased CZAR resulted from a proportionately larger decrease in host respiration over 24 hours than in net algal photosynthesis over 12 hours. A combined 8 fold increase in symbiont respiration and 5 fold increase in MI also gave increased estimates of the CZAR, but due to the opposite effects of the 2 parameters, the differences were generally less than 10 % and were at most 22.1 % (in I. sulcatus).

(g)<u>Carbon budget flow diagrams</u>

Flow diagrams showing the flux of carbon over 24 hours from symbionts to host in the 'natural' and reestablished symbioses are presented in figure 5.2 (pp.320-326).

Figure 5.2: Diagrams illustrating the daily flux of photosynthetically fixed carbon in the named symbioses.

Key for figure 5.2:

P (gross) = gross photosynthetic production

Rz = symbiont respiration

μz = symbiont growth

T = translocation from symbiont to host

Ra = host respiration

μa = host tissue growth

Rep = host reproduction

L = losses from association to sea water

all above values in mg C/day/mg association protein, assuming 12 hours at or above photosynthetic saturating irradiance.

Values in brackets are percentages of P(gross) used by each sink

The percentage translocation was estimated using either the mitotic index (ie. the 'growth rate method'), indicated by 'MI', or *in vivo* ^{14}C labelling, indicated by '^{14}C'.

Figs. 5.2.1 - 5.2.6: 'Natural' symbioses

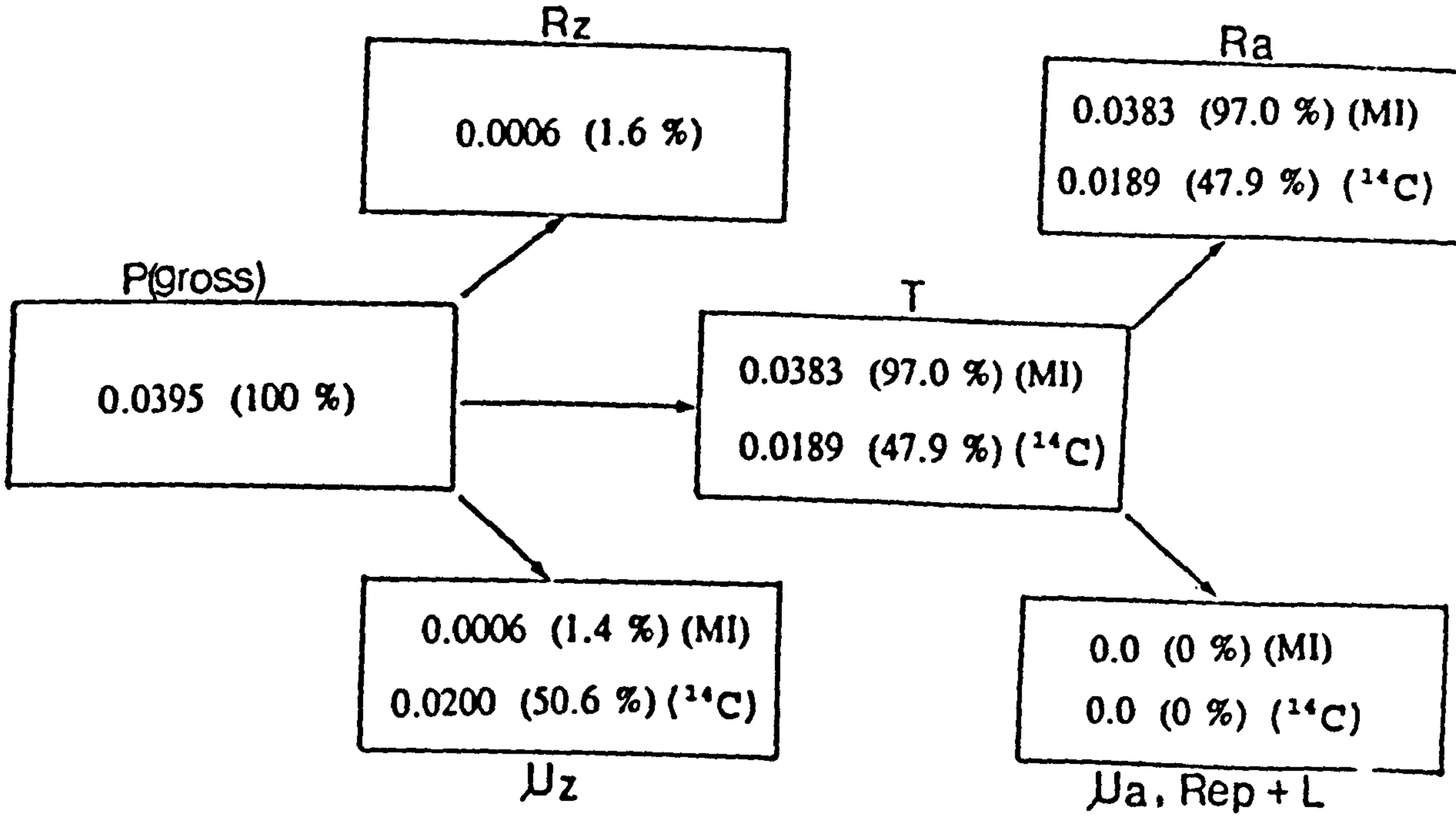


Fig. 5.2.1: Host sp. C. pedunculatus

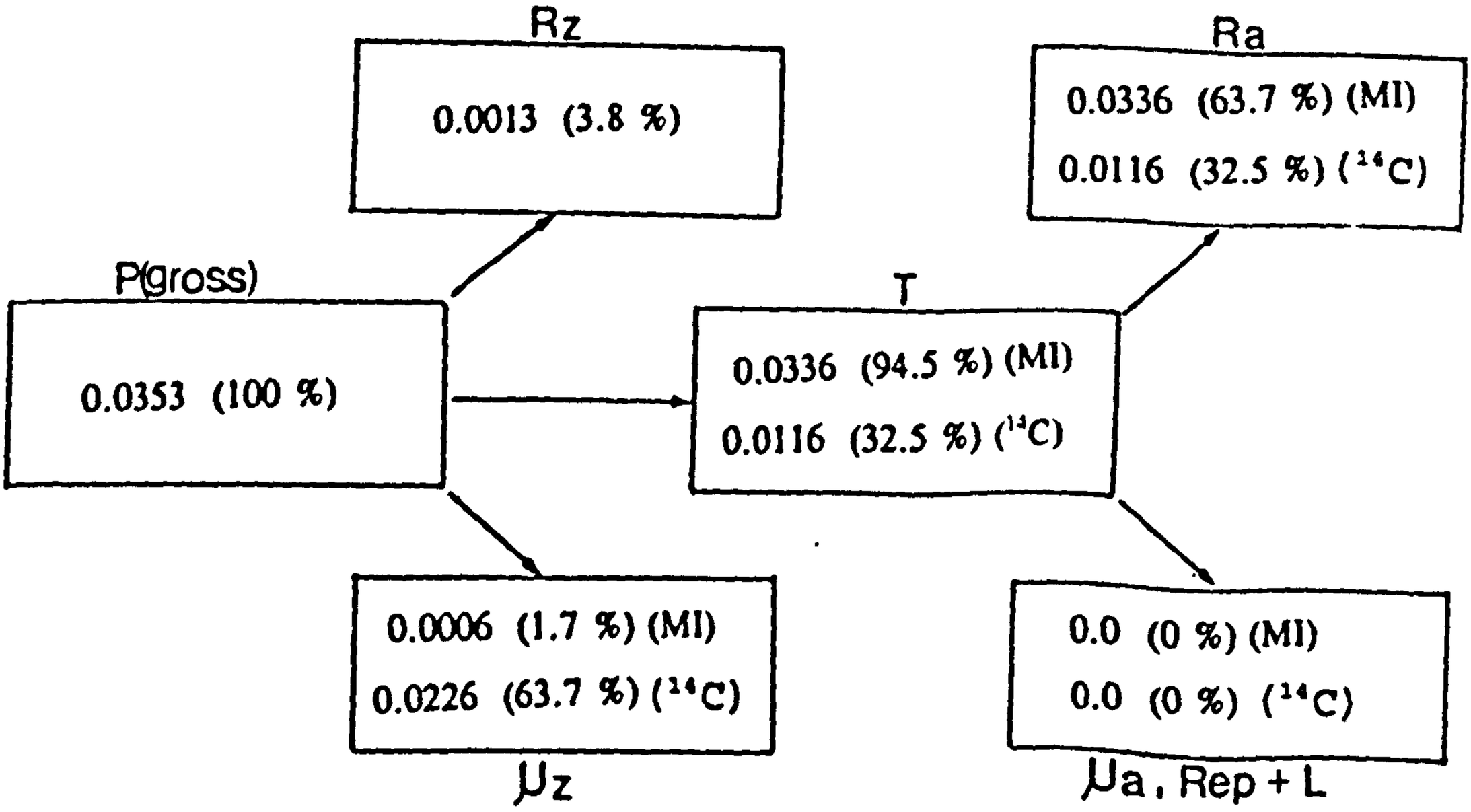


Fig. 5.2.2: Host sp. A. ballii

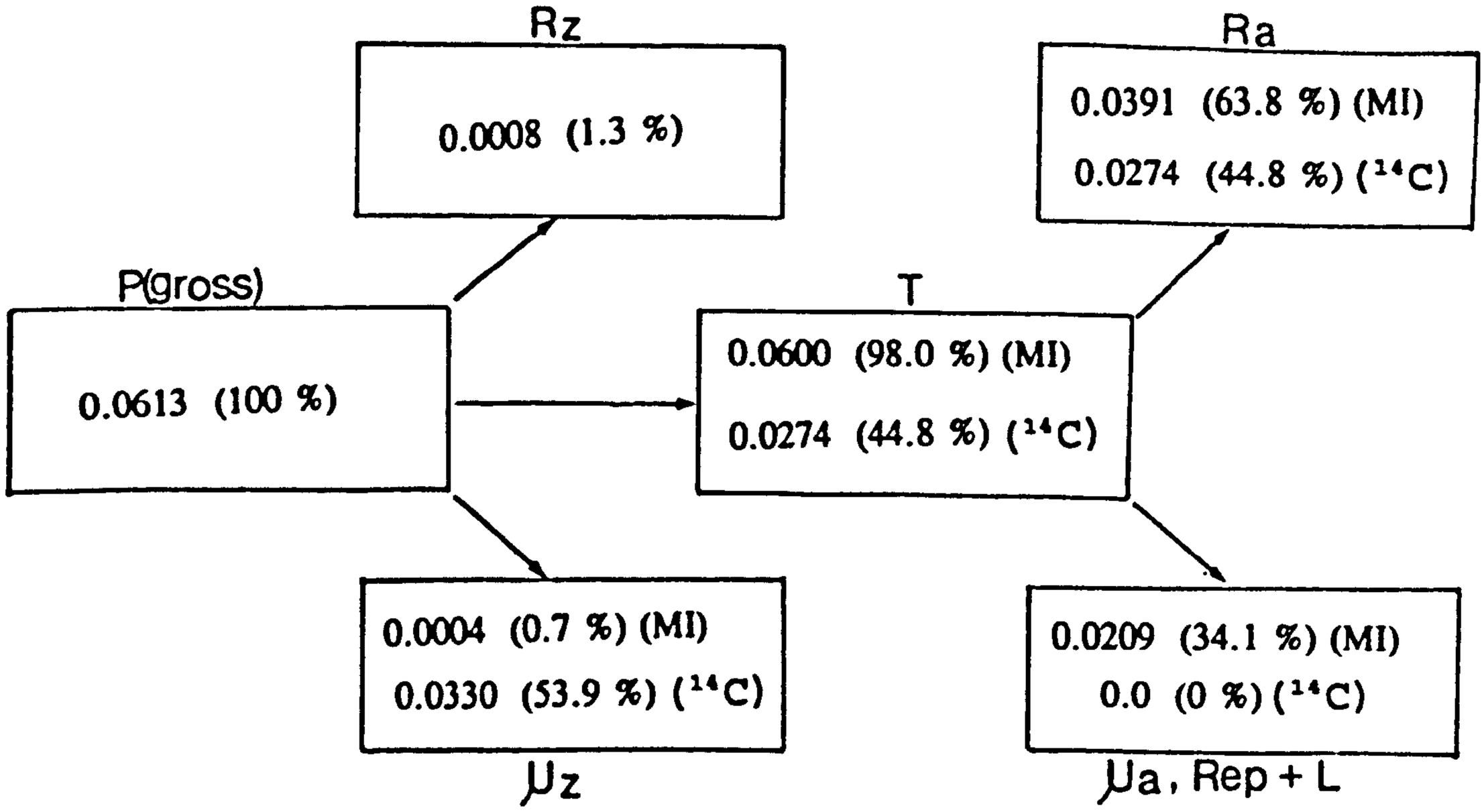


Fig. 5.2.3: Host sp. A. viridis (Lough Hyne)

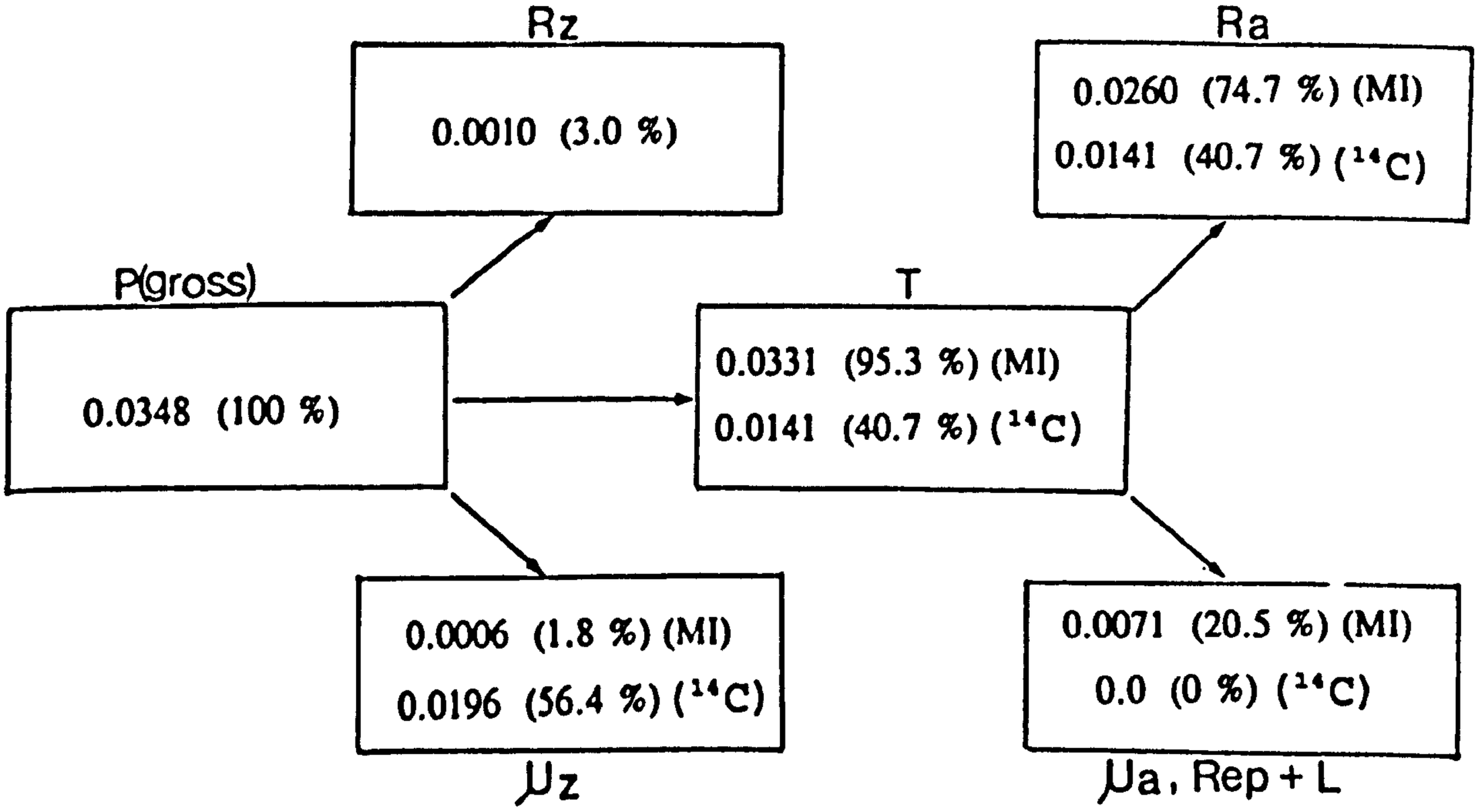


Fig. 5.2.4: Host sp. A. viridis (Shell Is.)

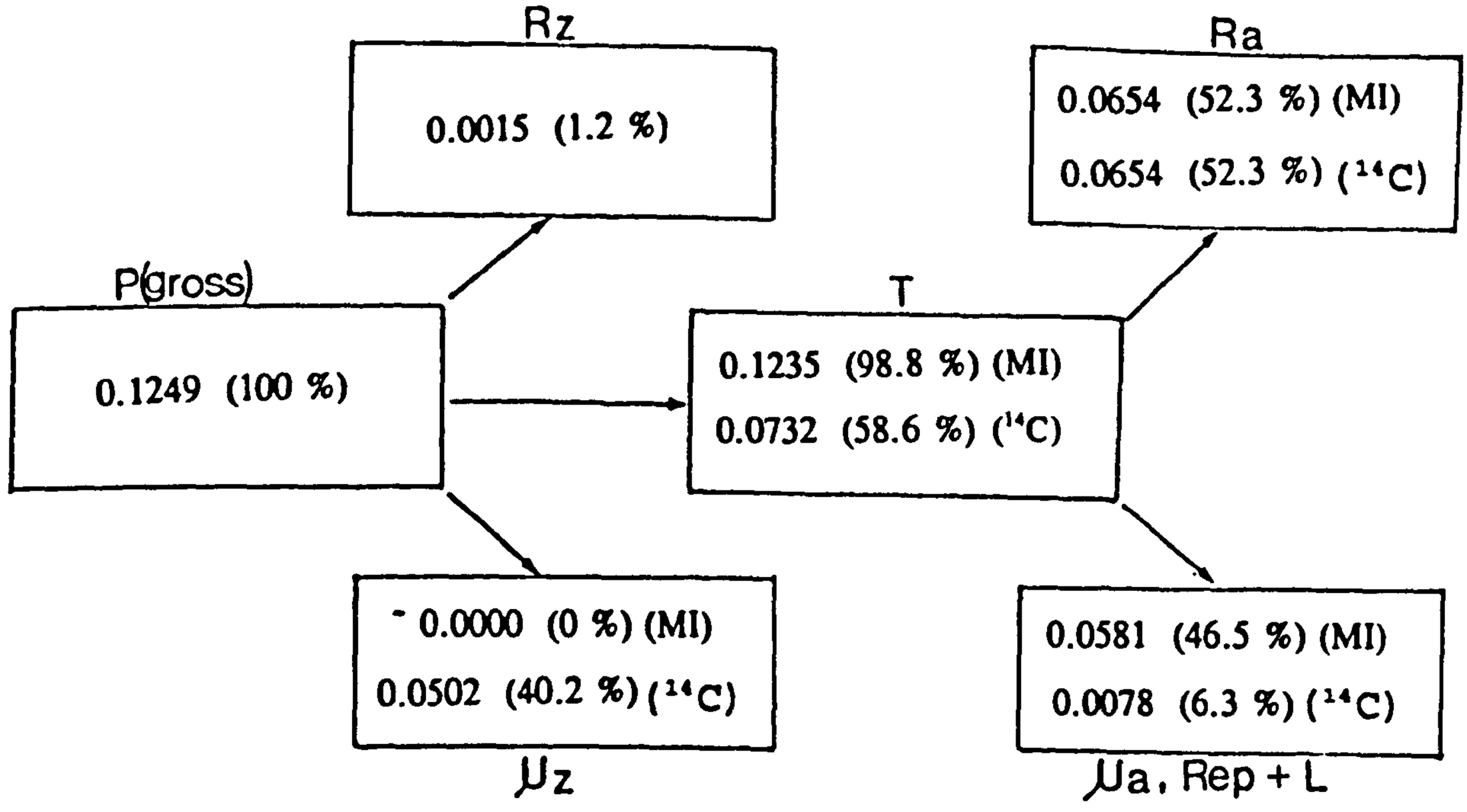


Fig. 5.2.5: Host sp. I. sulcatus

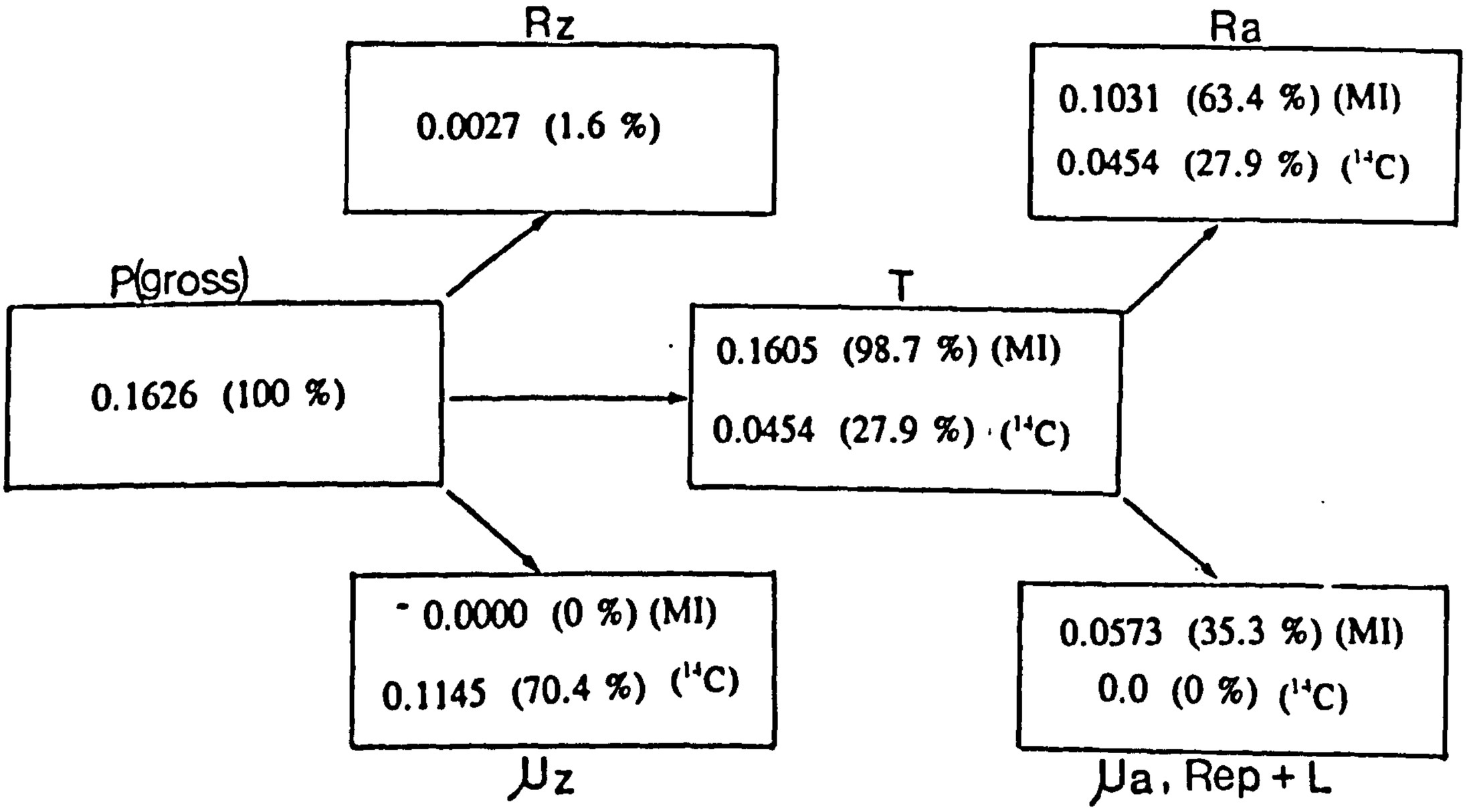


Fig. 5.2.6: Host sp. A. pallida

Figs. 5.2.7 - 5.2.12: C. pedunculatus (Cp) reinfected with symbionts from named host species.

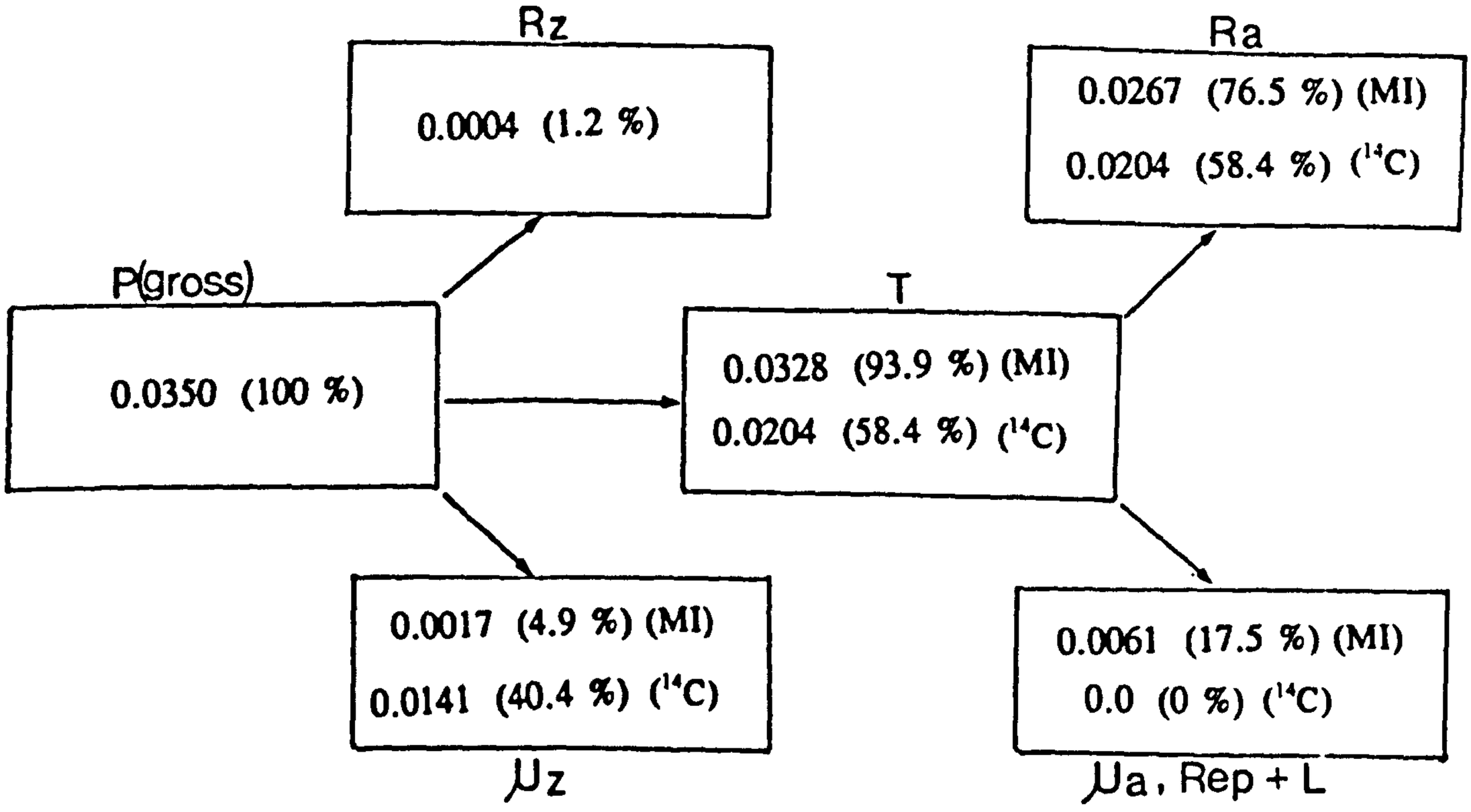


Fig. 5.2.7: Cp + C. pedunculatus

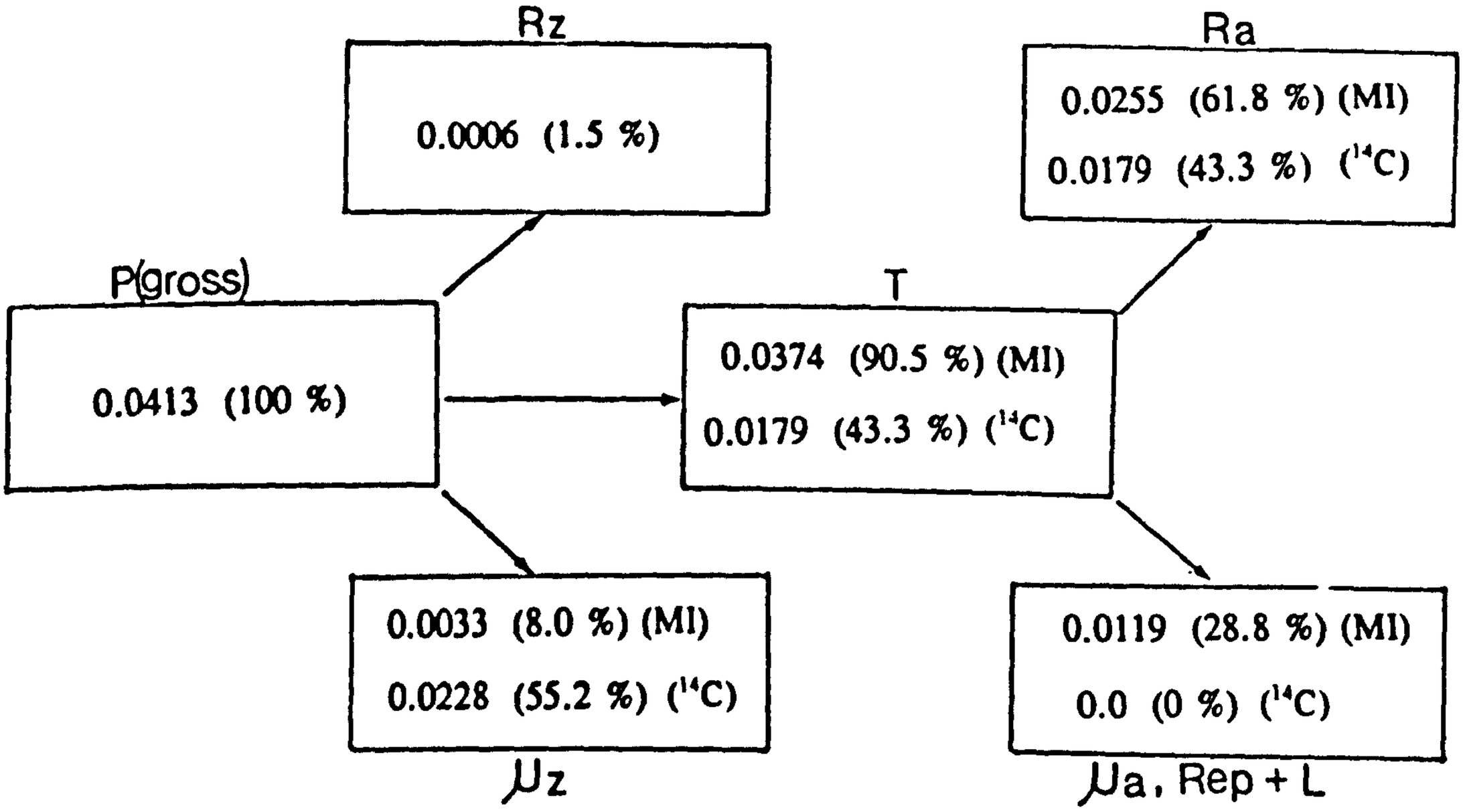


Fig. 5.2.8: Cp + A. ballii

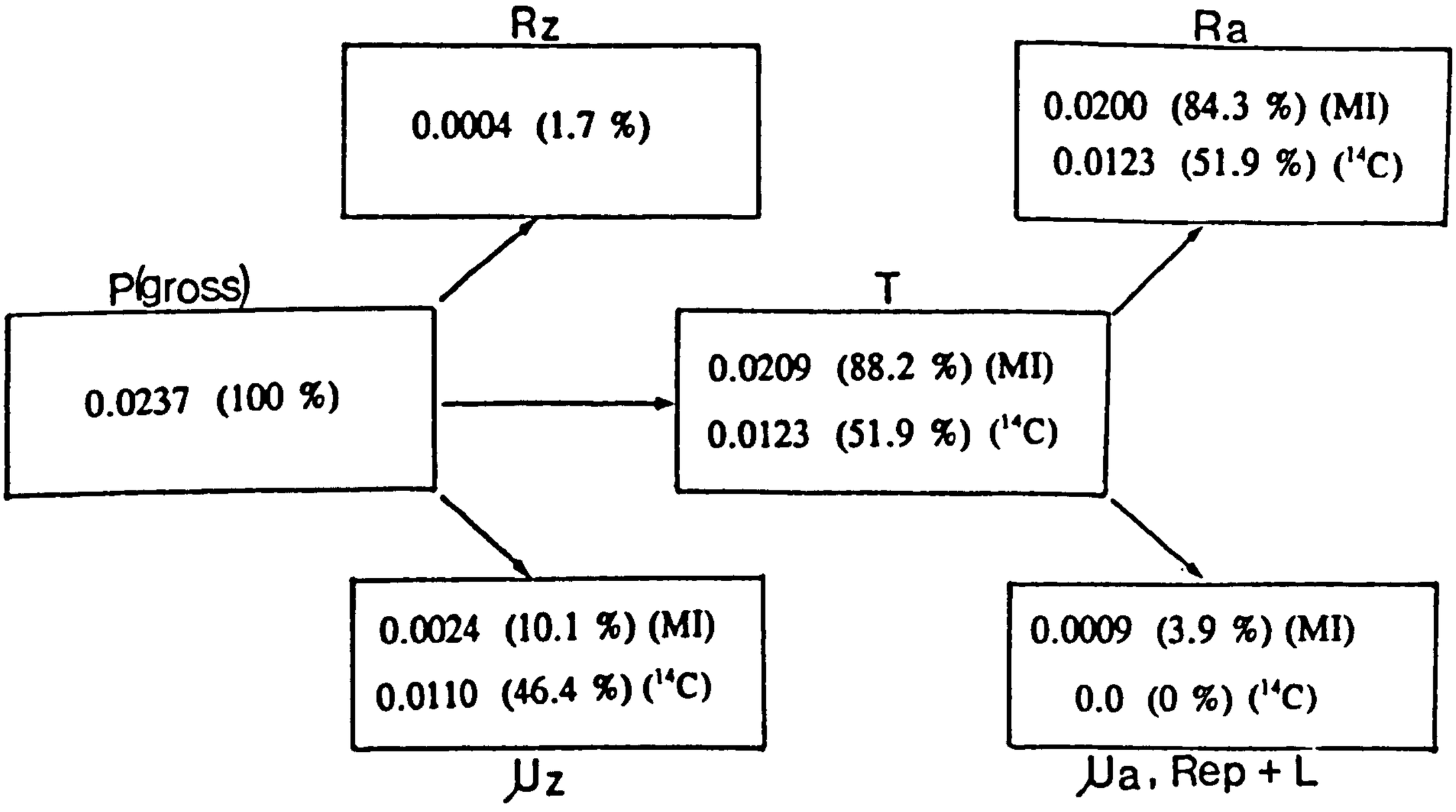


Fig. 5.2.9: Cp + A. viridis (Lough Hyne)

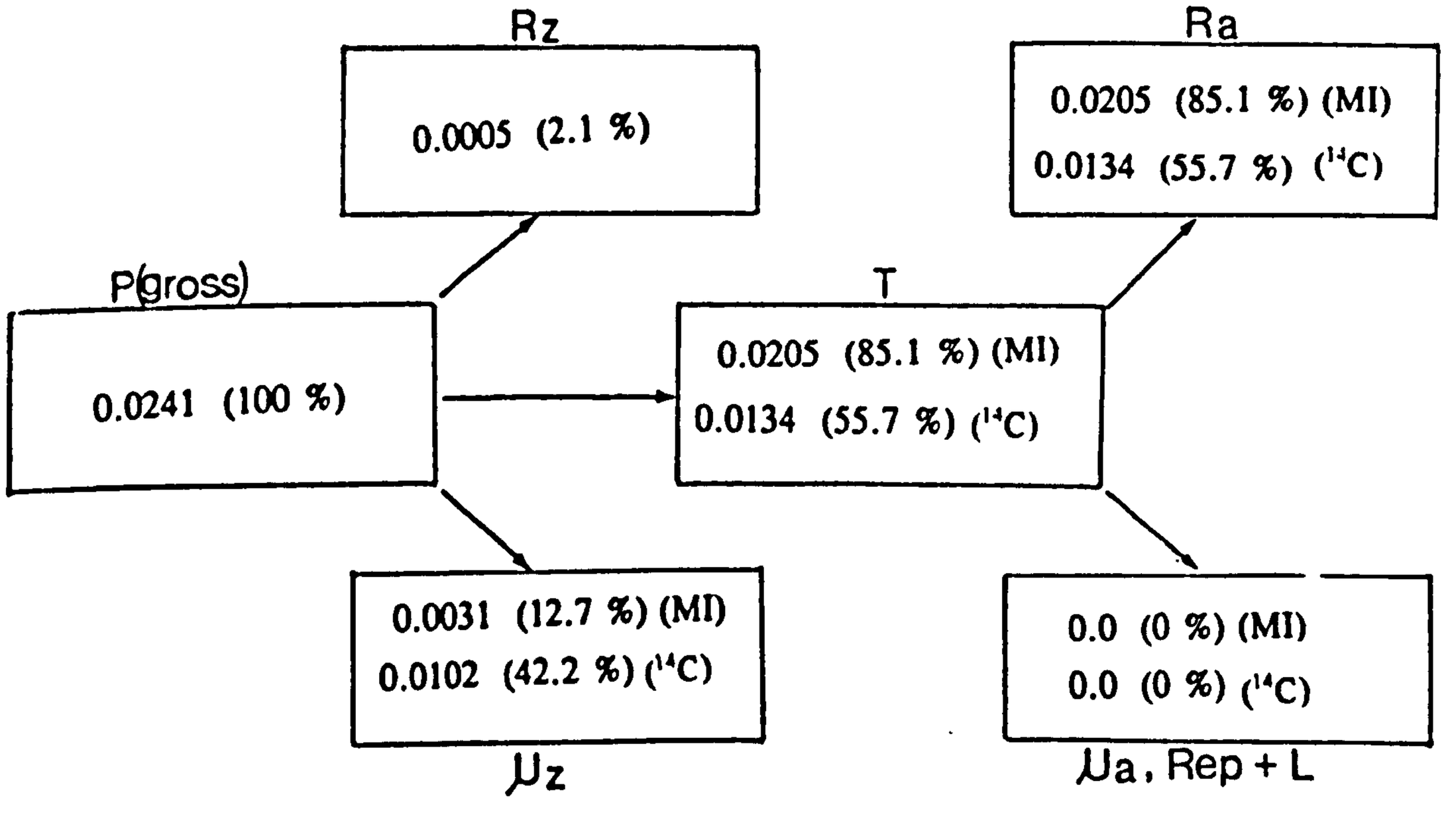


Fig. 5.2.10: Cp + A. viridis (Shell Is.)

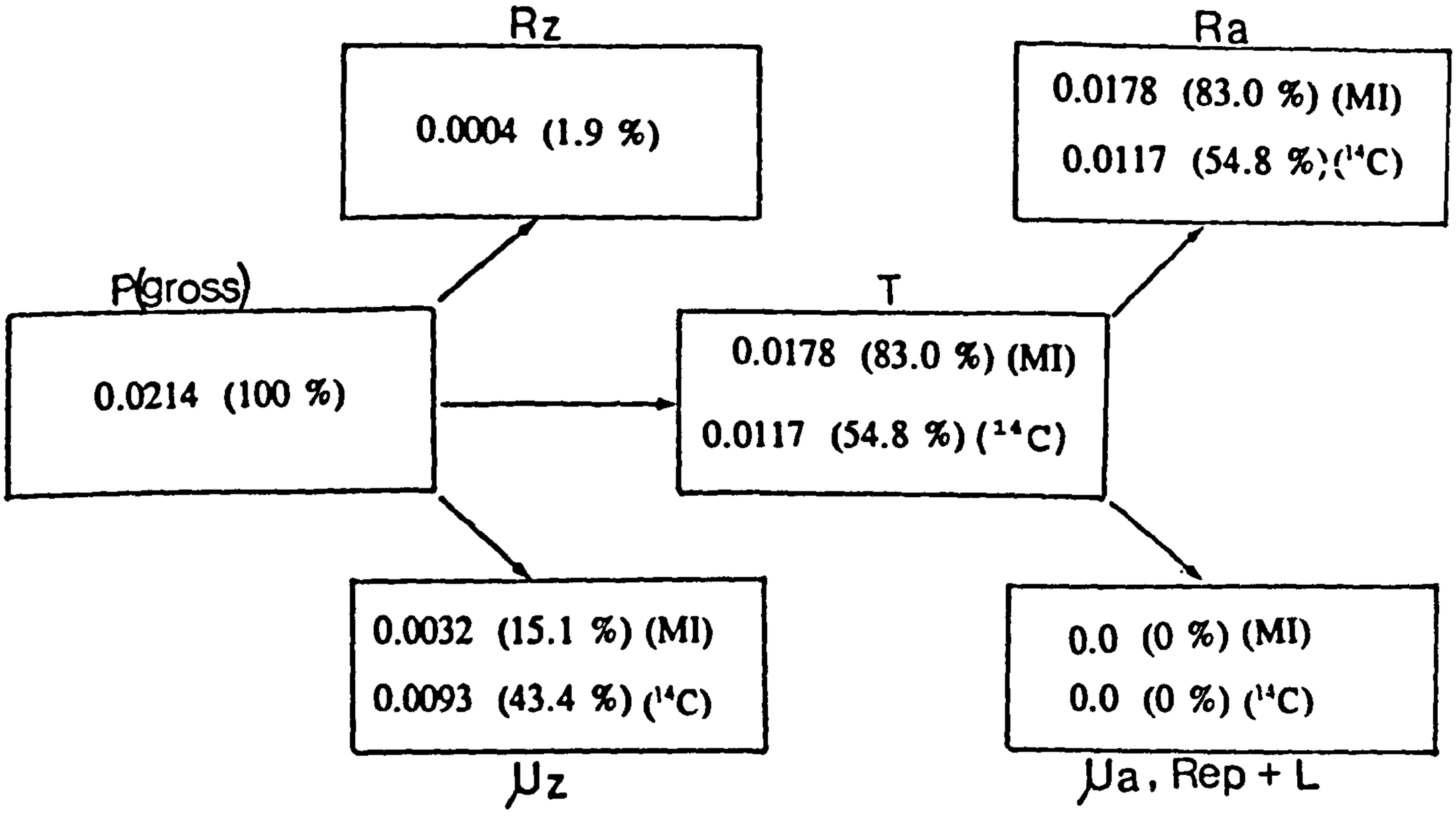


Fig. 5.2.11: Cp + <u>I. sulcatus</u>

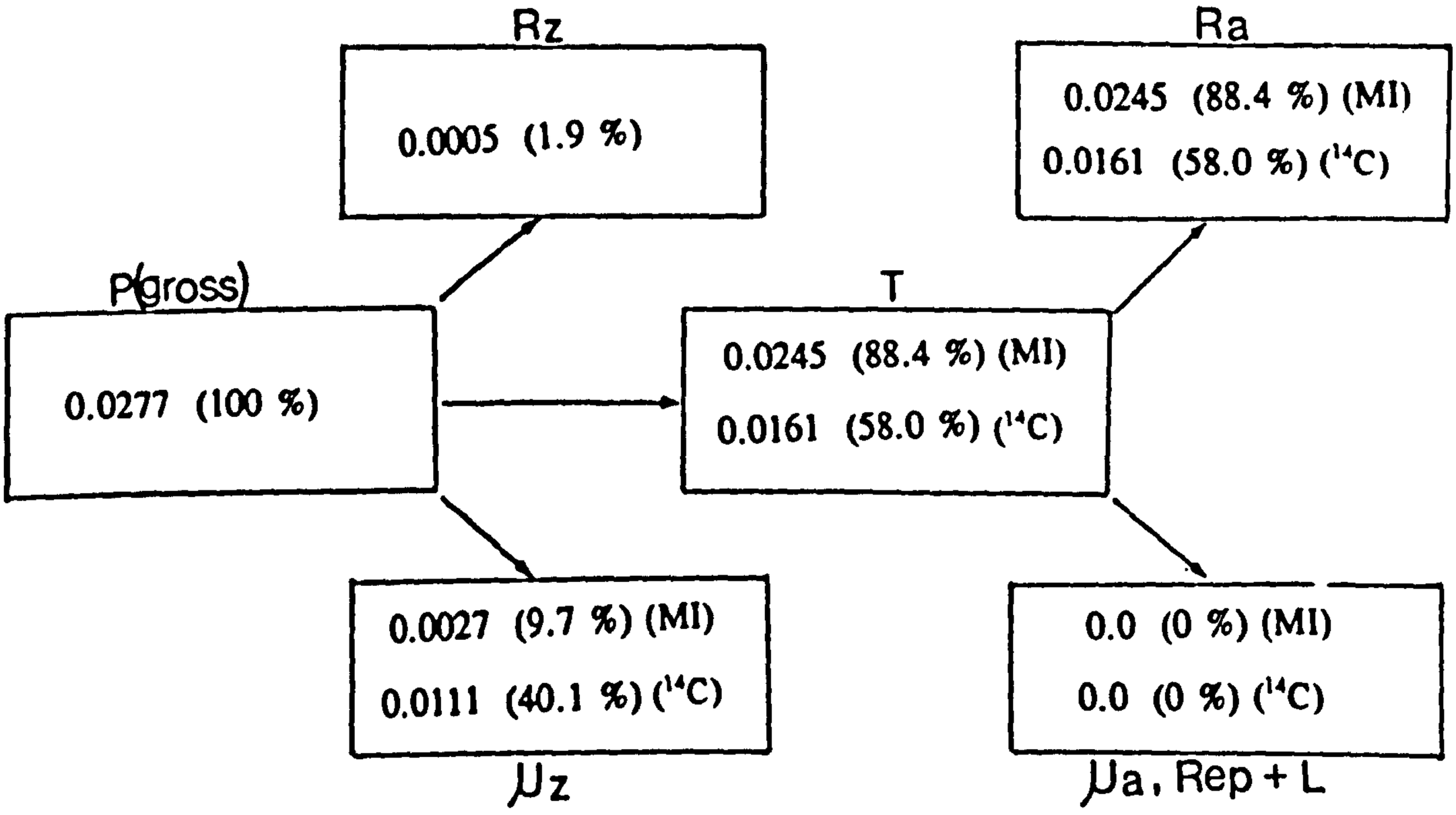


Fig. 5.2.12: Cp + <u>A. pallida</u>

5.4 Discussion

(a) Photosynthetic carbon fixation

(i) Photosynthetic saturation irradiance, the compensation irradiance and the distribution of symbiotic Anthozoa in the field.

The irradiances at which photosynthetic saturation and compensation occurred have implications when considering the distribution of the British Anthozoa and A. pallida in the field and photoadaptations to their light environment.

The steepness of the slope of the light saturation curve prior to saturation ('a') has been shown to increase and the irradiance at which photosynthetic saturation occurs has been shown to decrease in relation to lower light intensities in several species of corals. This indicates that corals are more photosynthetically effective in dim light than their conspecifics in high light environments (Wethey and Porter 1976; Scott and Jitts 1977; Chalker et al. 1983; Porter et al. 1984; Wyman et al. 1987). Photosynthetic efficiency has been expressed as either the ratio of organic matter produced to the available light, or the ratio of photosynthetically assimilated CO_2 to the light quanta absorbed by the photosynthetic apparatus (Dubinsky et al. 1984; Wyman et al. 1987). The anthozoans used in this study were all adapted to white fluorescent lights at 80 $\mu E/m^2/s$. Therefore, any differences in the saturation and compensation irradiances were the result of intra-symbiotic factors. Such factors may include symbiont strain specific

differences (Chang et al. 1983), symbiont self-shading (Crossland and Barnes 1977; Fisher et al. 1985; Wyman et al. 1987) and differences in the pigmentation and light attenuating qualities of the host tissues.

Strain specific differences in saturation irradiances have been demonstrated previously. Cultured symbionts of T. maxima and M. verrucosa have been shown to saturate at lower irradiances than Symbiodinium cells from A. pulchella, when all were adapted to the same light regime (Chang et al. 1983). The higher saturation irradiance of Symbiodinium sp. from C. pedunculatus than the 'heterologous' symbionts in the reinfected animals may suggest a 'strain' specific difference between the symbionts of the British Anthozoa, particularly when it is considered that the symbiont population densities were not significantly different nor were the host animals different. However, once in C. pedunculatus, the symbionts of I. sulcatus and A. pallida no longer saturated at lower irradiances than the other symbiont 'strains'. This suggested that the different irradiances required to saturate the 'natural' symbioses are predominantly determined by either self-shading or light attenuation by the host.

Self shading by symbionts may hinder the passage of light to the algae and result in photoadaptation (Chalker et al. 1983; Wyman et al. 1987). But the average densities of symbionts reported in this thesis were only determined on a per polyp basis. Consequently, the degree of self shading to

which the symbionts were subjected *in situ* cannot be inferred from these values.

There was some evidence that light attenuation by host tissue plays a role in determining the saturation irradiance. The saturation irradiances of the symbionts of *I. sulcatus* and *A. pallida*, which were notably the 2 most translucent host species, were lower than those of the symbionts of the other host species. In contrast, the saturation irradiance of the symbionts of *C. pedunculatus* was very high compared to the symbionts of the other host species. *C. pedunculatus* was unique amongst the studied Anthozoa by possessing accumulations of chromatophores throughout its endoderm. The function of these chromatophores is unknown, but they may attenuate light passing to the symbionts thereby necessitating a greater irradiance to saturate photosynthesis.

The above suggestions regarding the observed differences in the P vs. I curves are speculative. If more detailed analysis of the photosynthetic performance of the symbionts in relation to irradiance is required then measurements would have to be made on isolated symbionts enabling strain specific differences and the degree of light attenuation by host tissue and self-shading to be ascertained.

The saturation irradiances of the anthozoans here, with the exception of *C. pedunculatus*, were low compared to most of the published values for corals. These values range from approximately 300 to 2100 $\mu E/m^2/s$ (Falkowski and Dubinsky

1981; Burris et al. 1983; Porter et al. 1984; Spencer Davies 1984; Edmunds and Spencer Davies 1986; Farrant et al. 1987a). The photosynthetic production of the symbiont population of the clam T. gigas has also been shown not to saturate until irradiances of 600-2000 $\mu E/m^2/s$ (Fisher et al. 1985). Saturation irradiances reported for actiniarians and zoanthids are generally lower than those for corals and have been reported as ranging from 125 to 360 $\mu E/m^2/s$ (Fitt et al. 1982; Tytler 1982; Clayton and Lasker 1984; Steen and Muscatine 1984; Stambler and Dubinsky 1987). Previously, of the anthozoan species used in this investigation, P vs. I curves have only been constructed for A. pallida and A. viridis. The saturation irradiance of A. pallida adapted to 65 $\mu E/m^2/s$ was not specifically given by Clayton and Lasker (1984), but estimates derived from their P vs. I curves indicate photosynthetic saturation occurring between 125 and 200 $\mu E/m^2/s$, similar values to the saturating irradiance of 120 $\mu E/m^2/s$ measured in the present research. The saturating irradiance of A. viridis has been reported as 160 and 200 $\mu E/m^2/s$ by Tytler (1982) and Stambler and Dubinsky (1987) respectively. These values are approaching the saturating irradiances of 240-320 $\mu E/m^2/s$ estimated here for A. viridis. In contrast, the compensation irradiance of A. viridis has been reported as 17 $\mu E/m^2/s$ (Dorsett 1984), a much lower figure than the 64 to 84 $\mu E/m^2/s$ estimated here. This author also measured the linear P vs. I relationship up to 40 $\mu E/m^2/s$, approximately half the irradiance over which

the linear relationship was measured in this work.

Although symbioses in the field are likely to have adapted to the light regime in which they are living, the different compensation and saturation irradiances of the Anthozoa from Lough Hyne measured in the laboratory can be related to the comparative distributions of these animals in the lough, as reported by Turner (1988). C. pedunculatus was predominantly found at -1 to -4 m on an open stretch of sea bed where the photosynthetically active radiation (PAR) was measured as approximately 300-550 $\mu E/m^2/s$ on a sunny day and 50-100 $\mu E/m^2/s$ on a cloudy day. A. viridis were common from -5 to -15 m, A. ballii were most common from -3 to -15 m and I. sulcatus were observed on rocky ledges from -3 to -21 m. The irradiances to which the latter 3 species were subject were measured as between 20 and 300 $\mu E/m^2/s$ on a sunny day and less than 10 to 50 $\mu E/m^2/s$ on a cloudy day. The virtual confinement of C. pedunculatus to open, shallow waters may largely be because of the comparatively high irradiances required for this anthozoan to attain photosynthetic saturation and compensation point. In contrast, the lower compensation and photosynthetic saturation points of the other 3 species would enable these anthozoans to live at greater depths. The habit of I. sulcatus to grow on shaded rocky ledges which provide a suitable substrate, at up to -21 m where there is reduced competition from algal overgrowth (Turner, pers. comm.), could be aided by the comparatively low saturation and compensation points of this

symbiosis.

(ii)Gross photosynthesis at saturation (P(gross)max.).

In addition to total association photosynthesis being influenced by the density of symbionts present (Clayton and Lasker 1984; Kinzie and Hunter 1987), gross photosynthesis at saturation (P(gross)max.) standardized to cell density or chlorophyll-a content may also be influenced by environmental parameters in the field. Cultured symbionts adapted to high light intensities have a higher P(gross)max. than low light adapted algae (Chang et al. 1983), and coral symbionts adapted to blue and red light were found to photosynthesize at faster rates per unit chlorophyll-a than symbionts adapted to low white light at saturation (Kinzie and Hunter 1987). UV light also influences photoadaptation: Symbiodinium cells adapted to PAR + UV light photosynthesized more rapidly when irradiated with UV containing light than symbionts adapted to PAR only (Kinzie 1993). Temperature has been shown to increase primary productivity in proportion to maintenance requirements in tropical corals (Coles and Jokiel 1977), in A. pallida, which photosynthesized most rapidly at 32 ^{o}C (Clark and Jensen 1982), and in the temperate Capnella gaboensis, which had an optimal photosynthetic rate at 25 ^{o}C (Farrant et al. 1987a). Increased water flow has been shown to enhance the photosynthetic rate of reef corals due to a decrease in the length of the diffusional path from the ambient environment into the animal's tissues (Patterson et

al. 1991). Therefore, the adaptation of the symbioses in this research to laboratory conditions may have led to photosynthetic rates different to those that would be measured in the field or in freshly collected specimens. The values of P(gross)max. measured here are difficult to compare with many of the published photosynthetic rates because of the different parameters used to standardize gross photosynthesis. These include chlorophyll-a content (Wethey and Porter 1978; Jokiel and Morrissey 1986; Wyman et al. 1987), buoyant weight of the association (Fitt et al. 1982), unit surface area of the association (Porter et al. 1984), polyp number (Steen and Muscatine 1984), dry weight (Svoboda 1978; Shick and Dykens 1984; Tytler and Spencer Davies 1986; Klumpp et al. 1992), wet weight (Stambler and Dubinsky 1987), symbiont density (Clayton and Lasker 1984), total nitrogen content (McCloskey et al. 1978) and protein content (Chalker et al. 1983; Clayton and Lasker 1984). Clayton and Lasker (1984) demonstrated a gross photosynthetic rate at saturation of approximately 0.0105-0.021 ml O_2/h/mg association protein for A. pallida, depending on the feeding regime of the host. This range is slightly less than the P(gross).max of 0.02708 +/- 0.0066 ml O_2/h/mg association protein estimated here for the same host species.

(iii)Net symbiont photosynthesis per algal cell (ie. the cell specific rate) and per unit symbiont biomass (ie. the biomass specific rate).

The significantly higher cell and biomass specific rates of net algal photosynthesis in I. sulcatus than in A. viridis (Shell Is.), and the significantly higher biomass specific rate of net algal photosynthesis in A. pallida than in A. ballii and A. viridis (Shell Is.) suggested that 'strain' specific differences in photosynthetic performance may exist amongst the symbionts of the studied Anthozoa. Cultured symbionts of T. maxima and M. verrucosa adapted to the same conditions exhibited lower photosynthetic rates at saturation when standardized to chlorophyll-a content than symbionts from A. pulchella (Chang et al. 1983).

But it is also possible that the different net algal photosynthetic rates per symbiont cell or unit biomass measured in the 'natural' symbioses in this work are due to host regulation. The significantly lower biomass specific net algal photosynthetic rate of the symbionts isolated from I. sulcatus, A. pallida and A. viridis (Lough Hyne) when residing in C. pedunculatus than when in their original host species suggests that photosynthesis may be inhibited by the host cell environment. One possible hypothesis to explain this reduced photosynthetic rate in the reestablished symbioses may be host limitation of the photosynthetic inorganic carbon requirement. The symbionts can obtain inorganic carbon either from respiration or the sea water

bicarbonate pool (Burris et al. 1983; Muscatine 1990). The conversion of bicarbonate obtained from sea water to CO_2 is catalyzed by the enzyme carbonic anhydrase, which consequently may control the availability of CO_2 to the symbionts (Weis et al. 1989; Weis 1993; Yellowlees et al. 1993). If the concentration of carbonic anhydrase is lower in the reinfected C. pedunculatus than in I. sulcatus, A. viridis (Lough Hyne) and A. pallida, then the available CO_2 may be insufficient to satisfy the potential symbiont demands in the reestablished symbioses. Aposymbiotic anemones and tridacnid clams, which have been found to contain much lower concentrations of carbonic anhydrase than symbiotic individuals (Weis 1991; Yellowlees et al. 1993), produce carbonic anhydrase upon reinfection with 'homologous' algae (Weis 1991). Weis (1991) measured the concentration of the enzyme to be proportional to the density of the symbiont population. If carbonic anhydrase production is specific to the 'strain' of infecting symbiont, then this hypothesis could explain the significantly higher maximum rates of photosynthesis observed by the 'homologous' symbionts.

A second hypothesis to explain the greater cell and biomass specific photosynthetic rates of the 'homologous' than the 'heterologous' symbionts when in C. pedunculatus is the differential stimulation of photosynthesis as a result of "host factor"-symbiont specificity. Evidence for a "host factor" mechanism by which the host can control symbiont

photosynthesis has been provided by *in vivo* and *in vitro* ^{14}C incubations. Chloroplasts of the alga *Halimeda discoidea* are stimulated to photosynthesize more rapidly when residing in the saccoglossan *Elysia tuca* than when in the alga itself (Stirts and Clark 1980). Similarly, the photosynthetic rate of symbionts from *A. elegantissima* was found to increase by an order of magnitude when the symbionts were in host homogenate rather than sea water (Trench 1971c). In contrast, host limitation of symbiont photosynthesis in the hydrocoral *Millepora alcicornis* was suggested by Muscatine *et al*. (1972), who observed that photosynthetic carbon fixation increased linearly in response to homogenate dilutions. But Sutton and Hoegh-Guldberg (1990) found no evidence of host control of photosynthesis in 4 species of temperate Anthozoa and suggested that all these contradictory results may indicate variability in the host's ability to influence photosynthetic rates. That the lowest rates of photosynthesis in reinfected *C. pedunculatus* were by 'heterologous' symbionts could indicate the suppression of photosynthesis due to different symbiont 'strains' responding variably to the concentration of a stimulatory factor in the tissues of *C. pedunculatus*. Alternatively, a particular photosynthetic stimulus for some symbiont 'strains' may not be present in *C. pedunculatus*.

A third hypothesis that could explain the reduced photosynthetic rate per cell or unit biomass of some symbiont 'strains' when in a 'heterologous' host is a

reduction in the number of thylakoid lamellae per symbiont. A reduction in chloroplast number may occur due to a degree of starvation (Steele 1975), leading to a reduction in the number of photosynthetic units (PSUs) in which light energy is converted to chemical energy (Prezelin 1987).

Contrary to the situation with the symbionts of I. sulcatus, A. pallida and A. viridis (Lough Hyne), the photosynthetic rate of the symbionts of C. pedunculatus and A. ballii per cell was not significantly different when the symbionts were in the 'natural' and reestablished symbioses. This was despite the significantly smaller size of these symbionts after inoculation into C. pedunculatus (table 4.2 (p.200)). It therefore appeared that the higher biomass specific photosynthetic rate of the symbionts in the reinfected animals negated the effect of this size difference. G.J. Smith (1986) suggested that the 2 fold higher cell specific photosynthetic rate in juvenile Aulactinia stelloides than in the adult polyps was a result of the higher metabolic rate in the juveniles. However, the respiration rate of the reinfected C. pedunculatus was lower than that of the hosts in the 'natural' symbioses, suggesting that the metabolic rate of the reinfected animals was lower than that of the symbiotic anthozoans. Two unsupported explanations for the increased biomass specific photosynthetic rates of the symbionts from C. pedunculatus and A. ballii in the reestablished symbioses are an increase in the CO_2 available for photosynthesis, or a more efficient conversion of light

into chemical energy.

The higher cell and biomass specific photosynthetic rates of the symbionts of C. pedunculatus and A. ballii when in the reinfected hosts, rather than the total symbiont population density or biomass, resulted in the higher photosynthetic production of the reestablished symbioses involving these algae. Thus the importance of host-symbiont specificity with regard to the stimulation of photosynthesis and the availability of photosynthetically fixed carbon to sinks in the symbiosis is evident.

(b)**Photosynthetically fixed carbon utilized by the symbionts.**

(i)Symbiont : association protein ratios and the calculation of symbiont respiration.

Published values of the symbiont : symbiosis protein ratio, which is assumed to be equivalent to the symbiont:symbiosis respiratory ratio, are similar to those calculated here. The symbiont:symbiosis biomass ratio in scleractinian corals has been measured as 1:12 to 1:43.0 (Muscatine et al. 1981; McCloskey and Muscatine 1984; Falkowski et al. 1984; Muscatine et al. 1984). The zoanthids Z. sociatus and P. variabilis have algal:total protein ratios of 1:5.8 and 1:28.5 respectively (Steen and Muscatine 1984). A. elegantissima has been shown to possess an average ratio ranging from 1:11 to 1:38.5 (Fitt et al. 1982; Shick and Dykens 1984) and the temperate soft coral C. gaboensis has

an algal:symbiosis ratio of only 1:2.3 (Farrant et al. 1987b). Published protein ratios are not available for the British Anthozoa, but the symbionts of A. viridis were estimated by Harland et al. (1991) to constitute 12.5 % of the dry weight of the symbiosis. In comparison, only 2 to 3.6 % of the biomass was calculated as being attributable to the symbionts in A. viridis using the protein ratio method (this work). It is evident from the majority of published ratios and those presented in this work that if the protein ratio determination of symbiont respiration is applied then symbiont respiration is calculated to be a very small sink for the fixed carbon. Symbiont respiration in the 'natural' and reestablished symbioses required only 1.2-3.8 % of gross photosynthetic production over 24 hours (fig. 5.2 (pp.320-326)).

Respiration rates of symbionts from different host species, calculated using published protein ratio and respirometry data, are similar to the respiration rates of the symbionts in the temperate Anthozoa and A. pallida, which ranged from 6.0×10^{-5} to 2.6×10^{-4} ml O_2/h/mg association protein. Symbiodinium sp. in S. pistillata, which has a similar density of symbionts to the British Anthozoa, consumes 1.91×10^{-5} to 4.02×10^{-5} ml O_2/h/mg association protein during respiration (McCloskey and Muscatine 1984). Symbionts of the zoanthids Z. sociatus and P. variabilis respire at 1.25×10^{-4} and 3.47×10^{-5} ml O_2/h/mg association protein respectively (Steen and Muscatine 1984).

The accuracy of the protein ratio method of calculating symbiont and animal respiration has been questioned, even though this method is undoubtedly the most frequently used. Algal respiration has been measured *in vitro* using isolated *Symbiodinium* cells from the corals *Porites porites* (Edmunds and Spencer Davies 1986), *Pocillopora eydouxi* (Spencer Davies 1984) and *S. pistillata* (McCloskey and Muscatine 1984), and the anemone *A. pulchella* (Muller-Parker 1984). McCloskey and Muscatine (1984), comparing algal respiration rates calculated using isolated symbionts and the protein ratio assumption, found symbiont respiration to be 8 times greater when measured *in vitro*. If the *in situ* rate was the same as that measured *in vitro*, then symbiont respiration would account for 50 % of total symbiosis respiration in *S. pistillata* (McCloskey and Muscatine 1984). Using this technique, Spencer Davies (1984) calculated algal respiration to comprise 24 % of symbiosis respiration in *P. eydouxi*. The percentage contribution of algal respiration to symbiosis respiration was less pronounced in the symbioses under investigation here when the rate of algal respiration was increased by a factor of 8, ranging from 12.2 % in *C. pedunculatus* to 27.0 % in *A. viridis* (Shell Is.). In an attempt to resolve whether the *in situ* rate of symbiont respiration was actually being underestimated by the protein ratio method or whether the elevated *in vitro* rates were a result of the symbiont's isolation from the host, G.J. Smith and Muscatine (1986)

used multiple correlation and analysis of the regression of coral respiration with respect to symbiont density. The regression coefficients generated by the linear model used to investigate the dependence of colony respiration on host and symbiont biomass were equivalent to the biomass specific respiration rates of the host and symbionts. These coefficients indicated that the biomass specific respiration rates of the algal symbionts of S. pistillata and Montastrea annularis may be an order of magnitude greater than those of the hosts and that the protein ratio assumption underestimates symbiont respiration. In contrast, the regression method may overestimate symbiont respiration as it predicts that symbiont respiration accounts for more than 100 % of colony respiration in S. pistillata, which is obviously not possible.

In recognition of the problems associated with measuring symbiont respiration, Stambler and Dubinsky (1987), using A. viridis, estimated the amount of carbon fixed, the quantity required for symbiont growth and the percentage translocated to the host. The remainder of the fixed carbon was assumed to be directed into symbiont respiration. This procedure resulted in an estimated 39.9 % of fixed carbon being used in symbiont respiration and symbiont respiration contributing 45.7 % of the overall symbiosis respiration. But these authors only estimated the percentage translocation using in vivo ^{14}C labelling, which is believed to underestimate translocation (Smith, R.E.H. 1982;

Muscatine et al. 1983; Muscatine et al. 1984). If Stambler and Dubinsky (1987) had measured the percentage translocation by the more accurate 'growth rate method' (Muscatine et al. 1984), then considerably less carbon would have been estimated to be available for algal respiration and growth.

It therefore appears that, unless symbiont respiration can be estimated directly in situ, all estimates of this value and its influence on the overall flow of carbon within the symbiosis will be speculative. The results of the spreadsheet analysis, where the symbiont respiration rate was increased 8 fold, suggest that errors in the calculation of symbiont respiration only have a small influence on the calculation of the CZAR.

(ii)Carbon used in symbiont growth.

From the in situ growth rates of Symbiodinium spp. published, which range from 0.013-0.133/day (Muscatine et al. 1984; Spencer Davies 1984; Hoegh-Guldberg et al. 1987; Wilkerson et al. 1988), and those calculated here, which ranged from 0.019-0.040/day in the 'natural' symbioses, it is apparent that symbiont growth represents a relatively small sink for photosynthetically derived carbon (Muscatine 1990) and energy (Spencer Davies 1984; Stambler and Dubinsky 1987). Spencer Davies (1984) estimated that the cell specific growth rate was only 0.0017/day and that algal growth utilizes 0.09 % of the photosynthetic energy input in P. eydouxi. Similarly, Edmunds and Spencer Davies (1986)

calculated the cell specific growth rate to be 0.0028/day and algal growth to use 0.8 % of the photosynthetic energy input in P. porites. These very low growth rates (cf. Muscatine et al. 1984; Hoegh-Guldberg et al. 1987; Wilkerson et al. 1988) may be a result of the more direct method employed in the determination of the cell specific growth rate when compiling the energy budgets of these 2 corals. Spencer Davies (1984) and Edmunds and Spencer Davies (1986) estimated the cell specific growth rate by measuring host tissue growth and assuming a constant symbiont density in similar coral "nubbins" before and after the experimental period. In contrast, the estimation of the cell specific growth rate by the equations of McDuff and Chisholm (1982) and Wilkerson et al. (1983) relies on assumed periods of cytokinesis which may introduce error into the calculation of the size of the symbiont growth carbon sink.

The size of the symbiont growth carbon sink in the reestablished symbioses was greater than that in the 'natural' symbioses, accounting for 4.89 to 15.1 % of the gross photosynthetic production when calculated via the 'growth rate method' (cf. to approximately 0 % to 1.79 % in the 'natural' symbioses) (figure 5.2 (p.320-326)). The greater carbon sink in algal growth in the reestablished symbioses rendered less carbon available for translocation. Symbiont growth has also been found to substantially effect symbiosis carbon flux in the temperate nudibranch P. ianthina. This nudibranch may contain high or low densities

of Symbiodinium sp., with the cells in the low density populations growing at 0.399/day compared to 0.100/day in the high density populations. The faster growth rates of the low density symbiont populations and the smaller quantities of carbon they fix result in animals with low symbiont densities not obtaining translocated carbon from the symbionts. This is contrary to the situation in hosts with high symbiont densities (Hoegh-Guldberg et al. 1986). The possibility also exists that carbon and energy could be lost from the symbiosis as a result of expulsion of symbionts by the host. This was investigated by Hoegh-Guldberg et al. (1987) using 2 species of stony and 2 species of soft coral. These authors revealed that only 0.1 % of the carbon standing stock is lost daily through expulsion, representing 0.01 % of the daily carbon fixation. The rate of symbiont loss was just 4 % of the rate at which cells were added to the population. The extent of symbiont loss by expulsion from the British Anthozoa is unknown. But if algal expulsion from the British Anthozoa is on a similar scale to that in tropical corals then it represents a very small carbon and energy sink in these associations.

(c)**Translocation of fixed carbon from symbionts to host.**

(i)The nature of the translocated compounds.

The nature of the translocated carbon in Symbiodinium - invertebrate symbioses has been the subject of much work and debate over the past 30 years, and has been reviewed by D.C.

Smith et al. (1969), Trench (1979) and Cook (1983). In comparison to the Hydra-Chlorella symbiosis, where maltose is believed to be the major translocate (Muscatine 1965), in vitro and in vivo radioisotopic labelling has identified glycerol as the major constituent (24.8-95.0 %) of the material translocated from the endosymbionts of corals, sea anemones, zoanthids and tridacnid clams (Muscatine 1967; Muscatine and Cernichiari 1969; Lewis and Smith, D.C. 1971; Trench 1971b; Trench 1974; Schmitz and Kremer 1977; Sutton and Hoegh-Guldberg 1990). In addition, glucose, alanine, and the organic acids fumarate, succinate and glycolate have been identified in the translocated material (Muscatine 1967; von Holt and von Holt 1968b; Muscatine and Cernichiari 1969; Lewis and Smith 1971; Trench 1971b; Trench 1974; Schmitz and Kremer 1977; Schlichter et al. 1983). Other organic acids were also listed amongst the translocated compounds by von Holt and von Holt (1968b), but of these only the translocation of malate and citrate has subsequently been supported (Trench 1971b). In addition to the compounds listed above, Schmitz and Kremer (1977) also identified mannose, several sugar phosphates, aspartate and glutamate. But these authors did not distinguish between intracellular and extracellular algal products or metabolites of algal translocates produced by the host tissue.

Glycerol and glucose are generated in a phosphorylated form, but are dephosphorylated to permit their passage across the

cell membranes (Macon McDermott and Blanquet 1991). Once translocated, glycerol was originally believed to be used primarily for lipid synthesis by the host because of the observation that lipid is the predominant labelled fraction in the host tissue after incubation with $NaH^{14}CO_3$ (Muscatine and Cernichiari 1969; Smith, D.C. et al. 1969; Trench 1971b; Patton et al. 1983). Following labelling with ^{14}C-bicarbonate, deacylated lipids from host tissue have also been shown to be labelled only in the glycerol moiety (Muscatine and Cernichiari 1969; Trench 1971b). However, Patton et al. (1983), after incubating the corals S. pistillata and Stylophora hystrix with ^{14}C-bicarbonate, recovered the majority of the lipid label from the fatty-acyl moieties, leading to speculation concerning the role of translocated glycerol (Battey and Patton 1984).

Fatty acid synthesis within symbionts was previously indicated in isolated symbionts of Pocillopora capitata, which incorporated acetate-1-^{14}C in the light into fatty acids. Identical labelled fatty acids were found in the host fraction of the intact association, where they appeared in triglycerides and wax esters (Patton et al. 1977). These authors concluded that the symbionts converted non-photosynthetically fixed carbon (possibly acetate) from the host into fatty acids which were translocated to the host and used there in lipogenesis, thereby explaining the absence of radiolabelled carbon from the fatty-acyl moiety in the previous ^{14}C-bicarbonate incubations. The

translocation of fatty acids, largely as saturated or monoenoic fatty acids, was later supported by isotope incorporation pattern data (Blanquet et al. 1979). The observation that the majority of label after both acetate-1-^{14}C and $NaH^{14}CO_3$ incubations was located in the fatty-acyl moiety led to the conclusion that earlier investigators had made a methodological error (Patton et al. 1983). In conjunction with this result, the observation of triglyceride containing lipid droplets "blebbing" from symbionts of the anemone Condylactis gigantea resulted in speculation that triglycerides synthesized in the algae were translocated to the host (Kellogg and Patton 1983). This conclusion has more recently been supported by the observation that in aposymbiotic A. viridis, less than 1 % of the host lipid is composed of triglycerides compared to 4 % in symbiotic hosts (Harland et al. 1991). Kellogg and Patton (1983) speculated that the symbionts obtained acetate from their own mitochondria rather than from the host. Patton and Burris (1983) suggested that the increase in lipid synthesis when oxygen concentrations were increased was the result of increased cell catabolism and an acceleration of acetate transfer from the mitochondria to the chloroplasts where lipid synthesis occurs. Acetate may also be derived from dissolved organic matter (DOM) in the sea water or by photosynthetic assimilation of inorganic carbon (Schlichter et al. 1984).

The evidence for both the translocation of low molecular

weight, water soluble compounds, including glycerol, and lipids from the symbionts to the host resulted in considering separate roles for translocated glycerol and lipids. Both modes of carbon translocation are believed to occur simultaneously, the glycerol being used as a short term respiratory substrate and the lipids being directed into long term energy stores to be used during periods of starvation or reproduction. Respiratory quotient measurements of A. elegantissima have indicated that fed anemones switched from carbohydrate to lipid catabolism when starved (Fitt and Pardy 1981) and, in contrast to the temperate A. viridis (Harland et al. 1992), tropical corals may rely extensively on their stored lipids on over-cast days (Spencer Davies 1991). This greater dependence on triglyceride and wax ester stores in tropical species may be because of their faster metabolic rates due to the higher temperature (Harland et al. 1992). Neither the symbiont nor the host can rapidly convert glycerol into fatty acids, but incubations with glycerol-^{14}C result in some labelling of lipids. Therefore, the CO_2 produced by glycerol respiration may be refixed by the algae and translocated back to the host as lipid (Battey and Patton 1984).

The relative proportion of glycerol and lipid translocated has recently been demonstrated to change with depth and hence light regime. As a result of calculations of the respiratory and photosynthetic quotients in corals, Gattuso and Jaubert (1990) suggested that glycerol is the major

TABLE 5.23 EXAMPLES OF PUBLISHED VALUES OF THE PERCENTAGE TRANSLOCATION (T) MEASURED USING IN VIVO ^{14}C LABELLING.

Host sp.	Percentage trans. (T) (%)	Author(s)
Pocillopora damicornis (tropical coral)	32.5 - 41.4	Muscatine and Cernichiari 1969; Muscatine et al. 1981.
Scolymia lacera (tropical coral)	40.0	von Holt and von Holt 1968a.
Stylophora pistillata (tropical coral)	36.7 - 64.0	Muscatine et al. 1984; Gattuso et al. 1993.
Capnella gaboensis (temperate soft coral)	10.0 - 19.1	Farrant et al. 1987b.
Heteroxenia fuscescens (tropical soft coral)	20.0	Schlichter et al. 1983.
Aiptasia pallida (subtropical / tropical anemone)	39.9 - 82.0	Clark and Jensen 1982; Clayton and Lasker 1984.
Anemonia viridis (temperate anemone)	40.2 - 50.0	Taylor,D.L. 1969b; Stambler and Dubinsky 1987.
Anthopleura elegantissima (temperate anemone)	45.0 - 50.0	Trench 1971b.
Anthopleura xanthogrammica (temperate anemone)	0.0 - 3.9	Muscatine 1971; O'Brien 1980.
Condylactis gigantea (tropical anemone)	27.0	von Holt and von Holt 1968a.
Palythoa townsleyi (tropical zoanthid)	20.0 - 25.0	Sutton and Hoegh-Guldberg 1990.
Zoanthus robustus (temperate zoanthid)	11.8 - 35.2	Sutton and Hoegh-Guldberg 1990.
Cassiopeia andromeda (tropical scyphozoan)	5.0 - 10.0	Hofmann and Kremer 1981.
Pteraeolidia ianthina (temperate nudibranch)	20.0 - 50.0	Hoegh-Guldberg et al. 1986; Sutton and Hoegh-Guldberg 1990.

translocate in shallow waters but that lipids are the dominant translocates and catabolic substrates in deeper waters. This conclusion was supported by in vivo $NaH^{14}CO_3$ incubations of S. pistillata from 5-30 m (Gattuso et al. 1993).

Circumstantial evidence also exists for the translocation of sterols from symbiont to host which then act as structural components of membranes. Cnidarians are thought not to be capable of synthesizing sterols, obtaining some from heterotrophic nutrition and others, including cholesterol, gorgosterol and 23-demethylgorgosterol, from the symbionts (Withers 1987).

(ii)The percentage of photosynthetically fixed carbon translocated to the host measured using in vivo radioisotopic labelling.

(1)The percentage translocation in the 'natural' symbioses.

The percentage of carbon translocation estimated previously using in vivo ^{14}C labelling has shown that the percentage translocation differs with respect to the host species (table 5.23 (p.349)). The majority of the published values are similar to those estimated in this work using the same labelling technique, although the percentage translocation in A. pallida measured here was low compared to published figures for this species. The reasons for the lower percentage translocation in A. pallida here are not clear. Clark and Jensen (1982) identified 82 % of photosynthate to be translocated to A. pallida at 12 ^{o}C compared to 63 % at

27 ^{o}C. The A. pallida used in this investigation were maintained at 21 ^{o}C and therefore the temperature regime cannot explain the observed difference. Host feeding regime is not believed to be responsible: A. pallida received similar percentages of ^{14}C-labelled photosynthate when either well fed or starved (Clayton and Lasker 1984).

(2)The percentage translocation in the reestablished symbioses.

Contrary to the situation in the 'natural' symbioses, when the symbionts of the different host species were in C. pedunculatus all the symbiont 'strains' released similar percentages of their photosynthetically fixed carbon to the host. All the symbiont 'strains' released the same or greater percentages of their photosynthate to the reinfected hosts than to their original host species. The percentage of photosynthate translocated by symbionts originally from A. ballii had risen by approximately 10 % , although this value was still nearly 9 % lower than that of the other symbiont 'strains', and the percentage of photosynthate translocated by symbionts from A. pallida had increased by 30.7 %. This observation raises the possibility that photosynthate translocation under constant environmental conditions is predominantly controlled by the host environment rather than being the result of intrinsic symbiont 'strain' specific behaviour. This point will be discussed in detail in the proceeding sections.

(iii)The percentage of photosynthetically fixed carbon translocated to the host estimated by the 'growth rate method'.

(1)Arguments against the use of ^{14}C labelling to determine the percentage translocation.

The results obtained using in vivo ^{14}C labelling conflicted with the values of the percentage translocation calculated with the 'growth rate method'. Using the latter method, the percentage translocation in all the 'natural' symbioses was >95 %, although the percentage translocation was again lowest in A. ballii and highest, but not significantly higher, in I. sulcatus than in the other British Anthozoa. The values of the percentage translocation estimated with the 'growth rate method' were also >80 % in all the reestablished symbioses.

^{14}C is now widely believed to underestimate the percentage translocation. R.E.H. Smith (1982) used models to estimate that excretion by phytoplankton was underestimated by 2-10 times when ^{14}C was used, except when algal growth rates were high and incubation periods were long. This author concluded that the underestimation was due to ^{14}C : ^{12}C disequilibrium. Muscatine et al. (1984) compared the use of ^{14}C labelling and the 'growth rate method' to measure the percentage translocation in S. pistillata. These authors concluded that ^{14}C labelling underestimates the percentage translocation by 30-35 % and that, in addition to ^{14}C :^{12}C disequilibrium, underestimation of the percentage

translocation is caused by the failure of the radioisotope to label all of the translocated compounds. Muscatine et al. (1984), citing the hypothesis of Kellogg (1982, in Muscatine et al. 1984), suggested that ^{14}C label incorporated into phosphoglyceric acid may be lost during fatty acid synthesis. This could occur as a result of decarboxylation in the conversion of phosphoglyceric acid to acetyl CoA. However, this hypothesis was developed to explain the absence of radiolabelled carbon at the fatty-acyl moiety of lipids, which was subsequently found to be present (Patton et al. 1983). Therefore, the suggested mechanism by which translocated compounds lose their radioisotopic label must be questioned. A final explanation for the underestimation of the percentage translocation by ^{14}C labelling is that ^{14}C is refixed in longer term incubations (Muscatine 1990). Further doubt was cast on the validity of using ^{14}C to quantify translocation. This was due to the estimates of the percentage translocation made using the 'growth rate method' which, in contrast to the measurements of the percentage translocation recorded using in vivo ^{14}C labelling, decreased in the case of the symbionts of all the host species following inoculation into C. pedunculatus. The reasons for these contradicting results are unclear. But if all translocated material is not labelled (Muscatine et al. 1984; Gattuso et al. 1993), then the increased percentage translocation measured with ^{14}C in the reestablished symbioses could reflect an increase in the relative

proportion of labelled to unlabelled translocated compounds. This increase could occur irrespective of any changes in the total percentage of fixed carbon being translocated. Translocation is a selective process (Trench 1971b), and the relative proportions of translocated compounds may change as a result of the ambient environment (Gattuso and Jaubert 1990; Gattuso et al. 1993). It is therefore possible that the relative proportions of the translocated compounds may alter following residence in a new cell environment.

This work agreed with Muscatine et al. (1984) and Muscatine (1990), who considered that the 'growth rate method' was the most accurate way to determine the percentage translocation.

(2)The percentage translocation estimated with the 'growth rate method' in the 'natural' symbioses.

The values of the percentage translocation estimated with the 'growth rate method' here were similar to published values. The percentage translocation has been calculated as 95.9-98.0 % in S. pistillata (Muscatine et al. 1984), 88.8 and 95.1 % in the zoanthids P. variabilis and Z. sociatus respectively (Steen and Muscatine 1984) and 59.0-77.0 % in the nudibranch P. ianthina (Hoegh-Guldberg et al. 1986). A similar method to the 'growth rate method' for calculating the percentage translocation, where the portion of the photosynthetically produced energy supply not used in symbiont respiration or growth is assumed to be translocated, has also been employed. This method indicated that the percentage translocation was 36-77 % in A. viridis

(Tytler 1982), 90 % in Pocillopora eydouxi (Spencer Davies 1984) and 78.0 % in Porites porites (Edmunds and Spencer Davies 1986).

(3)The percentage translocation estimated with the 'growth rate method' in the reestablished symbioses.

Due to the questionable accuracy of the ^{14}C labelling method in quantifying the percentage translocation, the most realistic conclusion to be drawn from this investigation is that the percentage of photosynthate translocated to the reinfected hosts was significantly less than occurred in the 'natural' symbioses. This lower percentage translocation calculated with the 'growth rate method' resulted from the low net algal photosynthetic rate of all the reestablished symbioses with the exception of that involving symbionts from A. ballii and, as discussed earlier, the significantly higher mitotic index (MI) in all the reestablished symbioses after 9 months. If, as suggested by Douglas and D.C. Smith (1984), symbiont growth can be suppressed by host stimulation of photosynthate translocation, then partial loss of this stimulatory control, perhaps because of specific "host factor" requirements, could explain the higher symbiont growth rates in the reestablished symbioses. Unlike when the percentage translocation was measured via ^{14}C labelling, the percentage translocation by the 'homologous' symbionts was significantly greater than that by symbionts originally isolated from I. sulcatus and A. viridis (Shell Is.) when residing in C. pedunculatus. This

result suggests host-symbiont specificity reflected in the capacity to release photosynthate. This may arise as a result of differential responses to a "host factor", which will be considered in detail in section (c)(iv) of this discussion (pp.356-368). The lower percentage of photosynthate translocated by the symbionts originally isolated from I. sulcatus and A. viridis (Shell Is.) in the reinfected C. pedunculatus apparently permitted these symbionts to grow at a comparable rate to the 'homologous' symbionts, despite their lower rates of photosynthetic production.

(iv)The measurement of the percentage translocation using in vitro ^{14}C labelling.

(1)Evidence for and the nature of a "host factor".

In vitro incubations of the different 'strains' of symbionts in ^{14}C labelled sea water revealed photosynthate loss to be comparatively small in the absence of a host stimulus, supporting the existence of a "host factor". Pure "host factor" has only recently been isolated (Prof. L. Muscatine, UCLA, pers. comm.) and its precise chemical nature has yet to be elucidated. "Host factor" is believed to be proteinaceous, due to its heat labile nature (Muscatine et al. 1972; Sutton and Hoegh-Guldberg 1990), and greater than 10,000 D in size (Sutton and Hoegh-Guldberg 1990). Other properties of "host factor" were determined by Muscatine et al. (1972), who found that translocation in vitro was not influenced by light intensity or symbiont concentration but

was affected by homogenate concentration and the period of time the homogenate had been in isolation. For example, the stimulatory ability of homogenate from A. agaricites was retained for 3 hours at 26 ^{o}C. Thus, there is considerable evidence for a chemical which stimulates the release of photosynthate from symbiont cells. But it should be noted that it is unknown whether "host factor" activity is an artifact of the preparation procedure or whether it reflects the situation in the intact association. A "host factor" did not appear to stimulate release from all the symbiont 'strains'. The only symbiont 'strains' to release similar or greater percentages of their photosynthetically fixed carbon in response to 'homologous' host homogenates as in vivo were those of C. pedunculatus and A. viridis (Lough Hyne). Symbionts of A. ballii also translocated a similar percentage of photosynthate in vitro and in vivo, but 20.1 % of this was attributed to contamination or natural leakage. In comparison, the percentage of photosynthate translocated in vitro by symbionts of A. viridis (Shell Island), I. sulcatus and A. pallida was very low compared to when in vivo. Thus it appears, in contrast to the conclusion of Muscatine et al. (1984), that in vitro ^{14}C labelling is an even more unreliable method of quantifying carbon translocation than in vivo radioisotopic labelling. The results obtained here were similar to those reported by Hinde (1988) and Sutton and Hoegh-Guldberg (1990), who revealed that the percentage translocation may be influenced

by the *in vitro* preparation procedures. These authors described translocation in *Plesiastrea versipora* as "typical", where 25-55 % of the photosynthetically fixed carbon was released to the host homogenate. "Typical" responses to 'homologous' homogenates by the symbionts of *P. damicornis* and *Tridacna crocea* (Muscatine 1967), *A. elegantissima* (Trench 1971c) and *S. pistillata* (Muscatine *et al*. 1984) have also been reported. These results indicated that the activity of the host homogenate was similar to that which controls translocation *in vivo* (Hinde 1988). In contrast, symbionts of *Zoanthus robustus* released 2.6 % in sea water and only 8.52 % in 'homologous' host homogenate compared to up to 35.2 % *in vivo*. Similarly, symbionts of *P. ianthina* translocated 2.57 % in sea water and 7.28 % in homogenate compared to up to 47.5 % *in vivo* (Hinde 1988; Sutton and Hoegh-Guldberg 1990). Hinde (1988) suggested 3 possible reasons for these results. Firstly, deactivation of the "host factor" during separation of the symbionts and host would prevent the stimulation of translocation. This was thought to be unlikely, because when homogenate from *P. versipora* was diluted with that from *Z. robustus* the release rate of symbionts of *P. versipora* was not affected. Secondly, if *P.ianthina* and *Z.robustus* control translocation by physical interactions between the algal cell membrane(s) and the membrane of the perialgal vacuole, or maintenance of electrical, pH or concentration gradients, then these mechanisms may be destroyed by the homogenization procedure.

The mechanism of creating a concentration gradient to enable glycerol translocation has been supported (Hinde 1988; Sutton and Hoegh-Guldberg 1990). These authors found that homogenate of P. versipora increased the metabolism of 'homologous' symbionts, resulting in the channelling of the early products of the Calvin-Benson (C_3) fixation cycle into the synthesis of glycerol and other neutral compounds, and away from lipid synthesis. These newly synthesized compounds were released to the host. If host homogenate is not present at the time of fixation then the early C_3 cycle products are immediately used to synthesize compounds other than glycerol (Hinde 1988; Sutton and Hoegh-Guldberg 1990). The release of glycerol is believed to occur by diffusion as a result of the concentration gradient created, since biological membranes are known to be freely permeable to glycerol (Macon McDermott and Blanquet 1991). The third hypothesis to explain disruption of the translocation mechanism was that if translocation is increased by a restriction of symbiont cell growth, then removal of the symbionts from the intracellular host environment could release this growth restriction and decrease translocation from the algae. Symbiont growth could be limited by the availability of inorganic nutrients whose supply to the symbionts might be controlled by the host (Hinde 1988). The use of organic molecules obtained from the host by "reverse translocation" is less well documented, although Steen (1987) revealed that cultured symbionts from A. pallida can survive

heterotrophically when supplemented with glycerol and various organic acids, and McAuley (1987) indicated that host derived amino acids were an important source of nitrogen to Chlorella cells in Hydra viridissima. Deane and O'Brien (1981) gave evidence for the active uptake of sulphate, taurine, cysteine and methionine by symbionts isolated from T. maxima, and Na^+-dependent support systems driven by an electrochemical gradient generated by membrane associated Na^+-K^+ ATP'ase have been implicated in the transport of alanine and glucose into Symbiodinium cells (Carroll and Blanquet 1984; Macon McDermott and Blanquet 1991). Disruption of these transport mechanisms, which may be under host control, could remove the ability of the host to limit nutrient supply to the symbionts, enhancing growth and reducing translocation from the algae.

It therefore appears that the translocation control mechanisms in different associations including those of the British Anthozoa and A. pallida differ, and consequently that disruption of the symbiotic associations affects photosynthate translocation in vitro differently. If the mechanisms suggested by Hinde (1988) and Sutton and Hoegh-Guldberg (1990) are applied to the British Anthozoa and A. pallida, then translocation by the symbionts of C. pedunculatus, A. ballii and A. viridis (Lough Hyne) in the 'homologous' incubations can be considered as a "typical" response to a "host factor" whose activity was not affected by separation of the symbiosis. In comparison, the

translocation control mechanism in the A. viridis (Shell Is.), I. sulcatus and A. pallida symbioses was disrupted.

(2)The release of photosynthate by 'homologous' and 'heterologous' symbionts in response to a homogenate of C. pedunculatus.

When the different 'heterologous' symbionts were incubated in C. pedunculatus homogenate they all released significantly less photosynthate than the 'homologous' symbionts. The response of symbionts from I. sulcatus and A. pallida to the C. pedunculatus homogenate was similar to that measured in homogenates of their original hosts. These symbionts released negligible percentages of photosynthate, even though homogenate from C. pedunculatus has a proven ability to stimulate release. This result is similar to that described for symbionts of P. ianthina, which were not stimulated to translocate by homogenate from P. ianthina or P. versipora. In contrast, symbionts of Z. robustus released just 8.52 % of their fixed carbon when in 'homologous' homogenate but released 30.8 % when in homogenate from P. versipora, whose "host factor" did not appear to be affected by the separation procedure (Sutton and Hoegh-Guldberg 1990). Hinde (1988) concluded that these results indicated that both the properties of the alga and the host are involved in the translocation mechanism, a conclusion that is also apparent from this work. That the properties of the 'homologous' and 'heterologous' symbionts differ is also illustrated by the significantly reduced percentage

translocation by the symbionts of A. viridis (Lough Hyne) when in C. pedunculatus homogenate. This indicated that, whilst A. viridis (Lough Hyne) may have a similar translocation stimulation mechanism to C. pedunculatus, the "host factor" in the C. pedunculatus homogenate does not stimulate release by the symbionts of A. viridis (Lough Hyne) to the same extent as the "host factor" in the A. viridis (Lough Hyne) homogenate. This finding suggests that the ability to respond to a particular "host factor" is 'strain' specific. Similarly, Muscatine (1967) measured the percentage of photosynthate translocated by symbionts of P. damicornis in a homogenate of Tridacna crocea to be only 22.2 %, compared to 38.0 % in a 'homologous' P. damicornis incubation.

The apparent specificity between the symbiont and the host homogenate in vitro, if similar events occurred when C. pedunculatus were reinfected, could partly explain the slower depletion rate of endocytosed 'homologous' symbionts rather than 'heterologous' symbionts over the initial 4 day period (graphs 4.2-4.15 (pp.190-191)). However, Trench (1971c) reported that homogenates of aposymbiotic A. elegantissima could only enhance symbiont translocation after the animals had been reinfected with Symbiodinium sp.. Consequently, the extent to which the translocation of photosynthates may be involved in the recognition of symbionts immediately following endocytosis by aposymbiotic C. pedunculatus cannot be determined

without the use of homogenate from these aposymbiotic animals.

Once the reinfecting symbionts are present in the host, or if a symbiont cell infects an already symbiotic host, then the stimulation of photosynthate release could enable the host to recognise a symbiont by the quantity of fixed carbon translocated. The comparatively rapid repopulation of C. pedunculatus by 'homologous' symbionts (graphs 4.16-4.29 (pp.192-193) may be related to these symbionts releasing more photosynthate than other 'strains'. This was indicated by their higher rate of cell specific net algal photosynthesis (if it was not only higher after 9 months, but throughout the establishment of the symbiosis) and higher percentage translocation. In contrast, the symbiont that was 'least successful' in establishing a symbiosis with C. pedunculatus was that from A. viridis (Shell Is.). This 'heterologous' symbiont had a lower cell specific net algal photosynthetic rate than the other symbiont 'strains' and one of the lowest levels of translocation.

If the host-symbiont specificity observed in vitro is also expressed when the algae are introduced into an intact host, then the similar values of the percentage translocation in the reestablished symbioses could illustrate host manipulation of the symbionts or adaptation by the symbionts to the new cell environment (or vice versa). This observation is consistent with the view of Schoenberg and Trench (1980c), who suggested that the patterns of

adjustment between the two partners, which are involved in determining the degree of symbiosis specificity (Dubos and Kessler 1963), may become modified over generational and evolutionary time. The period over which this modification may occur was not determined here, but comparative in vivo studies of translocation at different time intervals following reinfection would help clarify this matter.

(3)Percentage translocation by symbionts of C. pedunculatus when incubated in 'homologous' and 'heterologous' homogenates.

"Host factor" specificity was also indicated by the significantly reduced percentage of photosynthate translocated by the symbionts of C. pedunculatus when they were incubated in homogenates of A. viridis (Lough Hyne) and A. ballii. The complete lack of stimulation of Symbiodinium cells from C. pedunculatus to release photosynthate by homogenates of A. viridis (Shell Is.), I. sulcatus and A. pallida could have been the result of either specificity of a "host factor" or the disruption of the host control mechanism of these host species. The ability of A. viridis (Shell Is.) homogenate to stimulate 43.2 % release by symbionts of A. viridis (Lough Hyne) suggested that even though the translocation control mechanism in A. viridis (Shell Is.) is apparently disrupted upon separation, this host still possesses the ability to stimulate release by symbionts capable of responding. The absence of

photosynthate translocation by symbionts of C. pedunculatus when in A. viridis (Shell Island) homogenate may therefore reflect "host factor" specificity.

(4)The percentage translocation by symbionts of C. pedunculatus and A. viridis in response to homogenates of the same host species but from different geographical locations.

The results of the in vitro ^{14}C incubations investigating C. pedunculatus and A. viridis from different geographical locations were unexpected. These results indicate for the first time that the host translocation control mechanism and/or the specificity of the "host factor" may differ within the same host species but from different locations. There was little evidence indicating differences between C. pedunculatus from Lough Hyne and Netley with respect to translocation stimulation. In contrast, clear differences between the translocatory behaviour and control were observed in A. viridis from different locations. The percentage translocation measured in vivo with ^{14}C label in A. viridis from 4 of the locations is not known, but it will be assumed that it would differ little from that in A. viridis from Shell Island or Lough Hyne (41.9 and 45.4 % respectively), or the percentage translocation in A. viridis reported by Stambler and Dubinsky (1987) and D.L. Taylor (1969b). Therefore, the negligible or substantially reduced percentage translocation in 'homologous' incubations of A. viridis from all locations (0.0-17.0 %), except Lough Hyne

(79.9 %), may have resulted from the disruption of the translocation control mechanism. The low *in vitro* percentage translocation of 2.6 % measured by Gallop (1974) using *A. viridis* from Plymouth may have been the result of a similar disruption.

The *A. viridis* symbionts which release only small percentages of photosynthate *in vitro* in their 'homologous' homogenates were also not induced to release photosynthate when incubated in homogenate from *A. viridis* (Lough Hyne). This finding suggested that symbionts of *A. viridis* from different locations may possess different properties. Also, symbionts from *A. viridis* (Trearddur Bay) translocated only half the percentage of photosynthetically fixed carbon in *A. viridis* (Lough Hyne) homogenate as in *A. viridis* (Trearddur Bay) homogenate, indicating "host factor" specificity. "Host factor" specificity was much more evident when translocation by symbionts of *A. viridis* (Lough Hyne) in response to homogenates of *A. viridis* from other locations was measured. Symbionts of *A. viridis* (Lough Hyne) released similar percentages of translocate when in homogenates of *A. viridis* from Lough Hyne, Loch Sween and Trearddur Bay, suggesting that whilst the translocation control mechanisms of *A. viridis* from Loch Sween and Trearddur Bay may be inactivated during homogenization, the host cells still possess a "host factor". "Host factor" also appeared to be present in homogenates of *A. viridis* from Bembridge, Shell Island and Weymouth, although these stimulated a lower

percentage of photosynthate translocation by symbionts of A. viridis (Lough Hyne) than the 'homologous' homogenate, again indicating "host factor" specificity. Evidence for "host factor" in the A. viridis symbioses whose release control mechanisms were apparently destroyed by homogenization may indicate that "host factor" in these symbioses operates in conjunction with an intact symbiosis dependent mechanism. Alternatively, this observation could indicate that "host factor" is present in these host animals but that the symbionts are not able to respond to it, their translocation being controlled by an independent mechanism.

The possibility of different translocation control mechanisms evolving in A. viridis from different locations is not unreasonable, particularly considering the geographical isolation of the Lough Hyne population from the other populations investigated and that A. viridis sexually reproduces infrequently in Lough Hyne (Dr. J.R. Turner, UCNW, pers. comm.).

(d)**Host respiration and the percentage contribution of the translocated photosynthate to the daily respiratory carbon budget of the host (CZAR).**

(i)Host respiration and arguments against the assumption that host respiration in the dark is equivalent to host respiration in the light.

The translocated carbon was available for host respiration. Measurements of host respiration have been made indirectly as described in this work (Muscatine et al. 1981; Steen and Muscatine 1984; McCloskey and Muscatine 1984; Shick and Dykens 1984; Hoegh-Guldberg et al. 1986). Alternatively, host respiration has been estimated by subtracting the symbiont respiration, which is measured directly in vitro or estimated from the remainder of carbon not translocated or used in symbiont growth, from association respiration (Spencer Davies 1984; Edmunds and Spencer Davies 1986; Stambler and Dubinsky 1987; Spencer Davies 1991). Attempts to use aposymbiotic hosts to measure host respiration have been made (Spencer Davies 1984), but such attempts have not been widespread because of the difficulty of obtaining many aposymbiotic hosts and the probability that animal respiration in aposymbiotic hosts differs from that in the symbiotic association anyway (McCloskey et al. 1978). Svoboda (1978) measured a much lower O_2 consumption by non-symbiotic cnidarians than by symbiotic species, concluding that photosynthate translocation from the symbionts resulted in an elevated host metabolism. This enhanced respiratory

rate due to the input of photosynthetically fixed carbon casts doubt on the assumption that host respiration is the same in the light as in the dark (McCloskey et al. 1978; Muscatine et al. 1981; Muscatine et al. 1983; Farrant et al. 1987b; Spencer Davies 1991). Respiration rate changes with depth and irradiance were first shown by Spencer Davies (1977), and this author later suggested that intraspecific differences in the respiration rate of corals at different depths were due to changes in the nutritional input by the symbionts (Spencer Davies 1980). Likewise, the more rapid respiration rate of the temperate sea anemone Aiptasia diaphana following a period of bright illumination rather than following a period of low illumination was concluded as being the result of increased photosynthate availability (Svoboda and Porrmann 1979). Hoegh-Guldberg and Hinde (1986) showed the linear relationship between symbiont density and symbiosis respiration in P. ianthina and estimated, by assuming that host respiration was constant, that algal respiration accounted for 62 % of symbiosis respiration. These authors suggested that this value was an overestimate due to the probability that a greater symbiont density and hence a greater quantity of photosynthate would enhance animal respiration.

The results obtained in this work supported the observations that an increased photosynthetic rate and supply of fixed carbon to the host enhances host respiration. This was particularly illustrated by the higher respiration rate of

I. sulcatus and A. pallida than of the other host species. The symbionts of I. sulcatus and A. pallida exhibited higher rates of net photosynthesis and translocated a greater percentage of the photosynthate than the symbionts in the other host species. Similarly, animal respiration was highest in those reinfected C. pedunculatus whose symbionts had the highest rates of net algal photosynthesis and translocated the most photosynthate.

Recently, Shick (1990) indirectly demonstrated that O_2 consumption in the light is also enhanced by the hyperoxic conditions generated in symbiotic anthozoan tissues as a result of photosynthesis. Shick measured respiration in a series of symbiotic anthozoans at normoxia (21 % O_2) and hyperoxia (50 % O_2); hyperoxia of 50 % O_2 in symbiotic anthozoan tissues had previously been illustrated by Dykens and Shick (1982). The extent of hyperoxic enhancement of O_2 consumption in the different symbioses varied depending on the degree of diffusion limitation experienced by the anthozoan as a result of the diffusion boundary layer. Diffusion limitation was expected to be least in A. pallida because of its morphological simplicity and small size (cf. zoanthid colonies and corals). Consequently, it was calculated that measurements of the respiration rate of A. pallida in the dark at normoxia underestimate the rate in bright light by about 11 %. This was less than the percentage determined for more complex corals and zoanthids. For example, hyperoxic enhancement of respiration in the

zoanthid Palythoa tuberculosa averaged 46 %. Therefore, gross photosynthesis and animal and algal respiration in the light are underestimated by the use of air saturated sea water. More precise respiratory measurements could in future employ 50 % O_2 sea water to simulate the hyperoxic conditions occurring in the animal's tissues during the day. Other factors may influence host respiration. Feeding with squid mantle resulted in a 196 % increase in the pre-prandial respiration rate of A. viridis (Tytler and Spencer Davies 1984), and fed A. elegantissima respired at double the rate of starved anemones (Fitt et al. 1982). Increased temperature resulted in an increased respiration rate in 4 species of coral (Coles and Jokiel 1977) and the non-symbiotic anemone Metridium senile (Walsh and Somero 1981). A reduced salinity was found to induce a decrease in the respiration rate of M. senile (Shumway 1978). Finally, increased water flow enhances animal respiration due to the same reasons given for the enhanced photosynthetic rate (section (a)(ii) of this discussion (p.332)) (Patterson et al. 1991). However, these factors were constant in the laboratory and would only be expected to lead to variability in the CZAR in the field.

(ii)The percentage contribution of the symbionts to the daily respiratory carbon budget of the host (CZAR).

(1)The CZAR in the 'natural' symbioses when in vivo ^{14}C labelling was used to estimate the percentage translocation.

The potential percentage contribution of photosynthetically fixed carbon to the host's daily respiratory carbon budget (CZAR) has been estimated for many symbioses since the model was originally proposed by McCloskey et al. (1978) and Muscatine (1981). The CZAR in tropical hosts has been calculated, using in vivo ^{14}C labelling to estimate the percentage translocation, as 63 % in P. damicornis and 69 % in Fungia scutaria (Muscatine et al. 1981), and 62-84 % and 108-145 % in the clams T. maxima and T. gigas respectively (Trench et al. 1981b; Klumpp et al. 1992). The importance to the host of symbiont derived photosynthetically fixed carbon in eutrophic temperate waters could be expected to be less than that in oligotrophic tropical seas, and the published CZAR values in temperate symbioses have generally been less than those in tropical hosts. Using the in vivo radioisotopic labelling method, the CZAR in temperate hosts has been calculated as 10.5-34.3 % in C. gaboensis (Farrant et al. 1987b) and 13-79 % in A. elegantissima (Fitt et al. 1982; Shick and Dykens 1984). The lower CZAR values calculated in A. elegantissima, using ^{14}C label to estimate the percentage translocation, were notably similar to the value of 25.7 % measured here in the closely related A. ballii using the same method. However, Stambler and Dubinsky

(1987), using *in vivo* ^{14}C labelling to estimate the percentage translocation, calculated the CZAR in *A. viridis* to be 116 % when the association was exposed to 10 hours of saturating irradiance per day. This CZAR was much higher than the values of 70.5 and 56.8 % calculated for *A. viridis* from Lough Hyne and Shell Island respectively, when *in vivo* radioisotopic labelling was used. The difference between these values partly results from the much higher percentage of the symbiosis respiration attributed to algal respiration by Stambler and Dubinsky. This point illustrates how different assumptions in the calculation of the CZAR can lead to results from which different conclusions can be drawn. If the CZAR in *A. viridis* is 116 % then this anthozoan could survive autotrophically with regard to its carbon requirements and not need to rely on its eutrophic environment (Stambler and Dubinsky 1987). But if the CZAR is only 56.8-70.5 %, then even at saturation irradiance, this anthozoan would require a heterotrophic input to satisfy its carbon requirements. Carbon can be obtained heterotrophically via feeding on zooplankton (Yonge 1930; Johannes *et al*. 1970; Muscatine 1990) and particulate organic matter (POM) (Lasker *et al*. 1983), or via the epidermal uptake of dissolved organic matter (DOM) (Schlichter 1975; Schlichter 1982). The CZAR calculated using ^{14}C was also less than 100 % in *C. pedunculatus*, *A. ballii* and *A. pallida*, again suggesting that all these host species require substantial inputs of heterotrophically

derived carbon to survive. But because of the reasons discussed earlier (section (c)(iii)(1) of this discussion (pp.352-354)), the use of ^{14}C to estimate the percentage translocation probably causes the CZAR to be underestimated. Considering this, the CZAR in I. sulcatus is very high (112.2 %), indicating that this zoanthid is autotrophic with respect to its respiratory carbon needs.

(2)The CZAR in the 'natural' symbioses when the 'growth rate method' was used to estimate the percentage translocation.

The likely underestimation of the CZAR when the percentage translocation was estimated with ^{14}C leads to a much greater emphasis being placed here on the CZAR values calculated using the 'growth rate method'.

The CZAR has been previously estimated using the 'growth rate method' as 58.0-157.0 % in the coral S. pistillata (Muscatine et al. 1984; McCloskey and Muscatine 1984), 48.2 % and 13.1 % in the tropical zoanthids Z. sociatus and P. variabilis respectively (Steen and Muscatine 1984), and 79.0-173.0 % in the temperate nudibranch P. ianthina (Hoegh-Guldberg et al. 1986).

In addition to the CZAR calculated using carbon equivalents, the translocated energy available for host respiration has been calculated in the compilation of energy budgets (using energy contents in joules) for several species of Anthozoa. The percentage translocation in these energy budgets has always been estimated using the direct 'growth rate method' of Spencer Davies (1984). The energy contribution of the

symbionts to host respiration, which is interchangeable with the CZAR (Edmunds and Spencer Davies 1986), has been calculated as 218.1 % in P. eydouxi (Spencer Davies 1984) and 296.6 % in P. porites (Edmunds and Spencer Davies 1986), and 134.6 % in P. damicornis, 173.4 % in M. verrucosa and 173.7 % in Porites lobata on "ideal" days (Spencer Davies 1991). The higher percentage contributions of the symbionts to host respiration in these corals compared to the CZAR figures given earlier partly result from the use of in vitro algal respiration measurements and hence lower estimates of host respiration.

The CZAR values in the British Anthozoa and A. pallida calculated using the 'growth rate method' were similar to those reported previously. The CZAR of less than 100 % in C. pedunculatus and A. ballii suggested that when exposed to saturation irradiance for 12 hours these anthozoans could not survive autotrophically with respect to their carbon requirements. This again indicated that a supplement of heterotrophically derived organic carbon was required. In contrast to when the CZAR was calculated with the radioisotopic labelling method, the CZAR in all the other symbioses was greater than 100 %, suggesting that these Anthozoa can obtain enough carbon to survive autotrophically. Estimates of the ratio of photosynthetically fixed carbon : symbiosis respiratory carbon requirement have led to the conclusion that the cold-temperate coral Plesiastrea urvillei can survive

autotrophically with respect to carbon (Kevin and Hudson 1979), the only report of this ability in another species of temperate anthozoan. But these authors did not calculate the carbon requirements of the symbionts nor the percentage translocation. Hence this conclusion must be regarded as speculative.

(3)<u>The CZAR measured in the laboratory vs. the CZAR in the field.</u>

It is unlikely that the laboratory estimates of the CZAR would be replicated in the field as they were all based on constant irradiances and temperatures, fixed light : dark cycles and regular feeding in the laboratory. As described in sections (a)(ii) (pp.332-333), (c)(ii)(1) (pp.350-351) and (d)(i) (pp.368-371) of this discussion, these environmental parameters can influence net algal photosynthesis, the percentage translocation, host respiration and consequently the CZAR.

Obviously, the anthozoans would be frequently subjected to sub-optimal irradiance in the field, with less than 12 hours of saturating irradiance per day and being situated at different depths. Consequently the CZAR in all these symbioses would be lower in the field. The effect of a variable irradiance regime on the CZAR in the field has been investigated previously. Spencer Davies (1991) constructed energy budgets for <u>P. damicornis</u>, <u>M. verrucosa</u> and <u>P. lobata</u> on days with different amounts of cloud cover at 3 m depth. This revealed that, unlike on "normal" and "ideal" days, on

"overcast" days the energy translocated was insufficient to maintain the host respiratory requirements in P. damicornis and P. lobata. Seasonal changes in light availability were found to decrease the CZAR in P. ianthina with similar symbiont densities to 79 % in winter from a peak in summer of 173 % (Hoegh-Guldberg et al. 1986), necessitating the input of heterotrophically derived carbon in winter. The CZAR in C. gaboensis was also found to be greater during summer (34 %) than in winter (11 %), despite similar estimates of the percentage translocation in both seasons (Farrant et al. 1987b). Similarly, the reduced light availability at depth was shown to cause a decrease in the CZAR of S. pistillata, with hosts at 3 m surviving autotrophically with respect to carbon, with a CZAR of 157 %, but hosts at 35 m only having a CZAR of 78 % and therefore needing a heterotrophic source of carbon (McCloskey and Muscatine 1984). The increased respiratory and growth dependency of 9 species of symbiotic coral on heterotrophic sources of carbon with increasing depth has been illustrated using the stable carbon isotope compositions of the algal and animal fractions (Muscatine et al. 1989b). Exposure of high shore A. elegantissima to air for up to 15 hours during daytime spring low tides has also been found to reduce the CZAR from a maximum of 42 % to 17 %, primarily as a result of the reduced photosynthesis when the anemone contracted (Shick and Dykens 1984).

Thus it is evident from the examples described above that

the CZAR measured in the laboratory is not directly comparable to the situation in the field.

(4)The effect of a 5 fold increase in the mitotic index (MI) and an 8 fold increase in the symbiont respiration rate on the CZAR in the 'natural' symbioses.

It was calculated that a 5 fold increase in the mitotic index (MI) would decrease the CZAR and an 8 fold increase in the symbiont respiration rate would increase the CZAR in the 'natural' symbioses. But these changes in the CZAR only affected the conclusion regarding whether the host would require a heterotrophic carbon input to satisfy its respiratory requirements when symbiont respiration was increased in C. pedunculatus. Contrary to the CZAR determined experimentally, C. pedunculatus would be able to obtain all of its respiratory carbon demand autotrophically if the symbiont respiration rate was 8 times greater. However, when symbiont respiration and the MI were increased simultaneously, the CZAR values in the 'natural' symbioses were similar to those originally estimated. Hence the conclusions regarding the heterotrophic carbon input required did not vary.

(5)The CZAR in the reestablished symbioses when both in vivo ^{14}C labelling and the 'growth rate method' were used to measure the percentage translocation.

Using ^{14}C to measure the percentage translocation, the CZAR in the reestablished symbioses revealed that the contribution of carbon from all the 'strains' of symbionts

was insufficient to meet the respiratory carbon budget of C. pedunculatus.

In comparison, when the percentage translocation was calculated by the 'growth rate method' it was concluded that 'strains' of Symbiodinium from C. pedunculatus, A. ballii and A. viridis (Lough Hyne) reinfected into C. pedunculatus were capable of providing photosynthetically fixed carbon in excess of that needed for host respiration. Again using the 'growth rate method', it was determined that C. pedunculatus reinfected with symbionts from A. viridis (Shell Is.), I. sulcatus and A. pallida required a heterotrophic carbon supplement to survive.

The comparatively high CZAR in the 'homologous' reinfected C. pedunculatus was in spite of the 'homologous' reinfections having comparatively low symbiont population densities and biomasses. This finding is explained by the productivity and translocation behaviour of individual symbionts, and consequently the high CZAR/cell/mg association protein (when calculated using either ^{14}C labelling or the 'growth rate method') and the significantly higher (or higher) CZAR/mg C/mg association protein of the 'homologous' symbionts. If the photosynthetic rate and percentage translocation reflect host-symbiont specificity, then the high standardized CZAR values in the 'homologous' reinfections can be considered as expressions of this specificity. Similarly, the low standardized CZAR in the animals reinfected with Symbiodinium sp. from A. viridis

(Shell Is.) and I. sulcatus reflects the comparatively low affinity between these symbionts and C. pedunculatus. But despite the greater effectiveness of the 'homologous' algae, the CZAR (calculated using the percentage translocation determined via the 'growth rate method') in C. pedunculatus reinfected with Symbiodinium sp. isolated from A. ballii was even higher than that in the 'homologous' reinfections. This was because of the insignificantly higher symbiont density and CZAR/cell/mg association protein, and in spite of the lower CZAR/mg C/mg association protein of the symbionts originally from A. ballii when in C. pedunculatus. Thus the importance of the larger size of the symbionts from A. ballii in counteracting their reduced effectiveness is evident.

(e)**Fixed carbon available for host growth and reproduction, and loss from the symbiosis.**

(i)Carbon used in host growth.

The carbon available to the host in the 'natural' or reestablished symbioses that is in excess to that required for respiration can be used for host growth. The importance of symbiont production to host growth is evident from observations of aposymbiotic P. ianthina being smaller than symbiotic individuals (Rudman 1982), symbiotic tridacnid clams growing an order of magnitude faster than other fast growing non-symbiotic bivalves (Klumpp et al. 1992), Cassiopeia sp. growing rapidly in the absence of

large food supplies (Drew 1972) and the slow growth of aposymbiotic C. pedunculatus compared to symbiotic individuals (pers. obs.). The potential contribution of translocated carbon to coral growth has rarely been estimated because of the difficulty of measuring tissue growth independent of skeletal growth (Spencer Davies 1984; Muscatine 1990). But by relating coral skeletal growth or colony area to tissue growth, the percentage of translocated material utilized in host growth has been estimated as only 0.9-8.9 % (Spencer Davies 1984; Muscatine et al. 1985; Edmunds and Spencer Davies 1986).

Despite tissue growth being easier to measure in sea anemones than corals, the measurement of host growth in actiniarians has also seldom been performed. Tytler and Spencer Davies (1986) calculated growth in A. viridis by relating buoyant weight to dry weight, showing that when A. viridis was starved in the light at an irradiance above its daily compensation point, it lost weight. This result contrasted with the indications of the daily photosynthesis : respiration ratio of A. viridis under this light regime, which suggested that the animal could survive autotrophically (Tytler and Spencer Davies 1984). The observations of Tytler and Spencer Davies (1986) revealed that, whilst the carbon potentially available to and used in host growth can be estimated and conclusions made regarding the autotrophic nature of a symbiosis, to survive and grow a host may require a heterotrophic input to obtain other

essential dietary components. The translocated carbon contains amino acids (particularly alanine), but it is believed to be nitrogen deficient and may be considered as "junk food" (Spencer Davies 1984). It is probable that the diet needs to be supplememted with nitrogen-rich organic material, which comes from zooplankton and the uptake of dissolved nitrogen (Muscatine 1990).

The results of this work indicate that, if the CZAR calculated with the percentage translocation derived by the 'growth rate method' is taken as the more accurate determination of this value, A. viridis from Lough Hyne or Shell Island, I. sulcatus and A. pallida all obtain excess translocated carbon which can be directed into tissue growth. The same situation also exists in C. pedunculatus reinfected with 'homologous' symbionts or symbionts originally from A. ballii and A. viridis (Lough Hyne). But the extent to which the translocated carbon satisfies the host's growth demands and the quantity of additional heterotrophically obtained compounds needed to supplement this growth await investigation.

The high CZAR measured in the 'homologous' reinfected C. pedunculatus, compared to that in most of the 'heterologous' reinfected animals, is consistent with previous investigations showing that 'homologous' symbionts promote faster host growth than 'heterologous' symbionts. D.L. Taylor (1971) demonstrated that when the flatworm Amphiscolops sp. was reinfected with 'homologous' symbiont

cells and 2 species of 'heterologous' symbiont, the 'heterologous' symbionts could not support host growth to the same degree as the 'homologous' species. Similarly, Kinzie and Chee (1979) observed that A. pulchella grew more rapidly when in symbiosis with 'homologous' rather than 'heterologous' symbionts. However, the developmental rate of the gorgonian Pseudopterogorgia bipinnata was found to be independent of the infective Symbiodinium 'strain' (Kinzie 1974). Whether the higher CZAR in 'homologous' reinfected C. pedunculatus rather than the hosts reinfected with 'heterologous' symbionts is accompanied by a faster growth rate is an interesting question for future research.

(ii)Translocated carbon used in host reproduction.

The translocated carbon not respired or used in growth may be directed into host reproduction. The use of photosynthetically fixed carbon in host reproduction has only recently been demonstrated directly, using ^{14}C labelling (Rinkevich 1989), but has been indirectly shown in the nudibranch P. ianthina. This nudibranch was only observed to produce egg masses during the summer and autumn, when the CZAR was at its highest, and individuals with low densities of symbionts which did not supply sufficient carbon for host respiration were not seen to reproduce (Hoegh-Guldberg et al. 1986).

Despite these observations, the use of photosynthetically fixed carbon for host reproduction has rarely been incorporated into the carbon or energy budgets of anthozoan

symbioses. For example, otherwise comprehensive carbon or energy budgets of S. pistillata (Muscatine et al. 1984), P. eydouxi (Spencer Davies 1984) and A. viridis (Stambler and Dubinsky 1987) fail to even consider that carbon may be used in reproduction, as did Muscatine's review (1990) of the fate of fixed carbon in symbiotic corals. But the daily energy expenditure on the production of planulae was estimated by Edmunds and Spencer Davies (1986) for P. porites. The percentage of the total energy content of the translocated photosynthate that was used in P. porites reproduction was calculated as only 0.5 %. That host reproduction may be a very small sink for photosynthetically fixed carbon has also been suggested with respect to the asexual reproductive effort of A. pulchella (Hunter 1984). The estimation of the percentage of translocated carbon that is used for host reproduction is further complicated by the fact that the host reproductive effort may differ on a seasonal basis. This was demonstrated by Rinkevich (1989), who reported that ^{14}C labelled photosynthate appeared less in the tissues of S. pistillata during the reproductive season than at other times. The influence of seasonal variations in reproductive effort on carbon budgets in symbioses has not been investigated, although Muscatine et al. (1983) suggested that enhanced symbiont division to supply planulae with symbionts in 'closed' reproductive systems could lead to a reduced translocation of photosynthate.

The quantity of carbon needed for reproduction by the British Anthozoa is unknown, although from figures 5.2.1 and 5.2.2 (p.321) it is evident that C. pedunculatus and A. ballii may both require a heterotrophic input into their diets to reproduce at all. Little is known of the seasonality of reproduction in these host species. There are no records of sexual reproduction in I. sulcatus, C. pedunculatus has been found to brood juveniles throughout the year in the laboratory (pers. obs.) and observations that A. ballii and A. viridis release ova and sperm annually (Dr. J.R. Turner, pers. comm.) are speculative. Reproductive patterns in the British Anthozoa need to be investigated before reproductive effort can be incorporated into the carbon and energy budgets of these species in a meaningful way.

The different quantities of photosynthetically fixed carbon available for host growth, reproduction and loss in the reestablished symbioses (figs. 5.2.7-5.2.12 (pp.324-326)), that arise as a result of host-symbiont specificity, suggest that the different 'strains' of symbionts could support host reproduction to varying degrees. It is possible that C. pedunculatus reinfected with symbionts originally from A. viridis (Shell Is.), I. sulcatus or A. pallida would not reproduce at all without heterotrophic feeding. That the host's ability to reproduce is influenced by the 'strain' of symbiont residing within its tissues has been demonstrated by Trench et al. (1981a) and Colley and Trench (1983). These

authors reported that Cassiopeia sp. only strobilated when in symbiosis with compatible 'strains' of Symbiodinium. Also, D.L. Taylor (1971) noted that aposymbiotic Amphiscolops sp. only reached sexual maturity after 'homologous' symbionts had been inoculated into them. How the estimates of the translocated carbon not used in host respiration are reflected by the reproductive rate of reinfected C. pedunculatus awaits investigation.

(iii)The loss of translocated carbon from the association.

The fact that host growth and possibly reproduction represent comparatively small carbon and energy sinks has led to the conclusion that a large percentage of the translocated carbon is released from the association. Cooksey and Cooksey (1972) were the first to directly demonstrate substantial loss of fixed carbon from corals, revealing that 50 % of photosynthetically fixed ^{14}C was released from Sidastrea siderea and M. annularis during the 20 hours following labelling. Similarly, Crossland et al. (1980a) reported that 50-60 % of fixed carbon was lost over 40 hours from Acropora formosa and Crossland et al. (1980b) reported that 40 % of the daily fixed carbon was lost from Acropora acuminata. Muscatine et al. (1984) also measured that light adapted S. pistillata released 6 % and shade adapted S. pistillata released 50 % of the ^{14}C labelled carbon fixed daily. It is likely that part of the measured loss in these ^{14}C experiments was attributable to respiration but that the majority of the loss was as mucus

and dissolved organic carbon (DOC) (Spencer Davies 1984). This statement is supported by indirect estimates of fixed carbon loss from corals. Spencer Davies (1984) calculated the daily fixed carbon loss from P. eydouxi as 48 % of the total originally fixed by subtracting the energy attributed to symbiont and host respiration and growth from the total produced by photosynthesis, thereby balancing the energy budget. Using the same method, Muscatine et al. (1985) reevaluated the potential daily loss of fixed carbon from S. pistillata as 26-52 % and Edmunds and Spencer Davies (1986) calculated the daily loss from P. porites as 45 %. Similar percentages of fixed carbon released have also been estimated for sea anemones. By balancing the energy budget, it has been estimated that A. pulchella releases 50-80 % (Hunter 1984), A. elegantissima releases 30 % (Zamer and Shick 1987) and A. viridis releases 40 % of the energy assimilated daily (Tytler and Spencer Davies 1986). These high estimates of the percentage of fixed carbon released were in contrast to the results of Stambler and Dubinsky (1987). These authors measured DOC loss from A. viridis using ^{14}C as only 3.7-5.9 % of the total carbon fixed in fed anemones and 1.5-3 % in starved anemones. These percentages are in agreement with those obtained here for A. viridis using an identical method, which averaged 0.2 % for A. viridis (Lough Hyne) and 1.9 % for A. viridis (Shell Is.). The DOC release from the other host species also appeared small and consequently it seems that the majority of carbon

lost from the associations is in the form of mucus. In particular, A. viridis has been observed to produce copious quantities of mucus (pers. obs). This observation is consistent with the carbon budgets shown for the British Anthozoa and A. pallida (figs. 5.2.1-5.2.6 (pp.321-323)). From these figures it is evident that if host growth and reproduction are small sinks of carbon, as suggested by published data, then A. viridis, and also I. sulcatus and A. pallida, must release large percentages of photosynthetically fixed carbon to the sea water.

(f) **General conclusion**

From this discussion, the advantages and shortcomings of estimating the contribution of the symbionts to the host with the CZAR model are apparent. Estimations of the CZAR in different 'natural' and reestablished symbioses proved useful in investigating the reflection of symbiosis specificity in the ability of the symbiont population to satisfy the respiratory carbon requirements of the host, and hence indicate whether carbon would also be available for host growth and reproduction. From the results it was again clear that the symbionts of the British Anthozoa and A. pallida are different and that the relationship between C. pedunculatus and its symbionts exhibits specificity. The host-symbiont specificity reflected by the percentage translocation and photosynthetic rate, and hence the CZAR and carbon available for host growth and reproduction, has

implications when considering the 'fitness' and propagation of novel symbioses. These implications will be discussed in chapter 6. However, to appreciate fully the potential contribution of different symbiont 'strains' to a host's carbon budget, it would be necessary to determine the carbon required for host growth and reproduction, and the carbon excreted. Hence a detailed carbon budget for the symbiosis could be compiled.

5.5 Summary

(i)Net photosynthetic oxygen production by the symbiosis at a series of irradiances and dark symbiosis respiration were measured for C. pedunculatus, A. ballii, A. viridis (Lough Hyne), A. viridis (Shell Is.), I. sulcatus and A. pallida, and for reinfected C. pedunculatus which had been in symbiosis with Symbiodinium sp. from each of these host species for 9 months. Oxygen production and consumption were measured using standard O_2 electrode equipment and rates calculated as ml O_2/h.

(ii)Gross photosynthetic O_2 production at each irradiance was calculated by adding dark respiration to net photosynthesis.

(iii)The protein content of each anthozoan used in the respirometry work was estimated using the method of Lowry et al. (1951). The carbon content of each symbiont was calculated from the average volume of the algal cells from each host species, according to the equation of Strathmann

(1967). The algal carbon standing stock per animal was determined as described by Muscatine et al. (1983) and converted to the protein equivalent as described by Hoegh-Guldberg et al. (1986).

(iv)Gross photosynthesis by the symbionts of each host species at each irradiance was standardized to the total protein content and photosynthesis (P) vs. irradiance (I) curves plotted. The saturation irradiance was determined 'by eye' and the average gross photosynthetic rate at and above saturation (P(gross)max.) was calculated. The P vs. I curves were described with respect to the slope 'a', the irradiance at which 'a' intersected P(gross)max. (Ik) and I(0.95), as described by Chalker et al. (1983). The saturation irradiances were similar in the reestablished symbioses, but in the 'natural' symbioses the comparatively high saturation irradiance of C. pedunculatus could be related to the distribution of this anthozoan in the field.

(v)The symbiont and host respiration rates were calculated from total symbiosis respiration by assuming the rates to be proportional to their biomass ratio, according to Muscatine et al. (1981).

(vi)Net algal photosynthesis was calculated by subtracting symbiont respiration from P(gross)max..

(vii)The net algal photosynthetic rate was considerably higher in I. sulcatus and A. pallida than in the other 'natural' symbioses, as was animal respiration. Animal respiration was highest in the reestablished symbioses

involving symbionts originally from C. pedunculatus and A. ballii, which photosynthesized at a significantly higher rate than the other 'strains' of symbionts.

(viii)Net algal photosynthesis was standardized to symbiont cell density and carbon standing stock/mg association protein. It was evident that the higher total photosynthetic rates observed in those symbioses discussed above were predominantly due to higher biomass specific rates. The higher cell and biomass specific photosynthetic rates of symbionts of C. pedunculatus and A. ballii than the other symbiont 'strains' in reinfected C. pedunculatus, and the reduced rates of symbionts of I. sulcatus and A. pallida in the reestablished symbioses from when in the 'natural' symbioses, indicated host-symbiont specificity reflected in the photosynthetic capacity.

(ix)The percentage of photosynthetically fixed carbon translocated to the host was estimated by 3 methods. These were in vivo and in vitro labelling with $NaH^{14}CO_3$, as adapted from Stambler and Dubinsky (1987) and Muscatine et al. (1972) respectively, and the 'growth rate method' described by Muscatine et al. (1983). The 'growth rate method' involved the calculation of the carbon specific growth rate. This required the conversion of net algal photosynthesis/24 h into carbon units by the standard method (Strickland and Parsons 1972; McCloskey et al. 1978).

(x)The percentage translocation measured in vivo ranged from 28.4-59.3 % in the 'natural' symbioses, comparing well with

published values, and from 44.0-59.1 % in the reestablished symbioses. The percentage translocation in the reestablished symbioses was not significantly different between the different symbioses when estimated using ^{14}C in vivo.

(xi)The percentage translocation estimated using the 'growth rate method' was significantly higher than that measured using ^{14}C in vivo, suggesting that ^{14}C underestimates translocation.

(xii)The percentage translocation estimated by the 'growth rate method' was comparable to the percentage translocation calculated with this method in published work. The percentage translocation estimated with the 'growth rate method' ranged from 96.5-99.6 % in the 'natural' symbioses and 84.0-94.6 % in the reestablished symbioses. The lower percentage translocation in the reestablished symbioses reflected the higher symbiont growth rate in all the reinfected animals and the lower carbon specific growth rate in some of the reinfected animals.

(xiii)The percentage translocation in the reestablished symbioses, calculated with the 'growth rate method', was significantly lower by symbionts originally from I. sulcatus and A. viridis (Shell Is.) than by 'homologous' symbionts, suggesting host-symbiont specificity.

(xiv)The lower percentage translocation in the reestablished symbioses than in the 'natural' symbioses, when estimated by the 'growth rate method', and the higher percentage translocation in the reestablished than the 'natural'

symbioses, when measured using *in vivo* ^{14}C labelling, may indicate a change in the percentage composition of unlabelled compounds in the translocate.

(xv)Release of translocate by symbionts in response to homogenate indicated the presence of a "host factor".

(xvi)The percentage translocation measured in 'homologous' *in vitro* ^{14}C incubations was similar to that measured *in vivo* for *C. pedunculatus*, *A. viridis* (Lough Hyne) and *A. ballii*, but was substantially less *in vitro* than *in vivo* for the other symbioses. This indicated the possible presence of different translocation control mechanisms in the symbioses.

(xvii)'Homologous' and 'heterologous' combinations of symbionts and homogenates of different host species indicated that the symbionts of different host species possess different properties and that the "host factor" is 'strain' specific. It is possible that when different 'strains' of symbionts are first endocytosed by *C. pedunculatus*, the degree of photosynthate release resulting from the specificity could partly enable host-symbiont recognition. That 'heterologous' symbionts released much greater percentages of photosynthate to *C. pedunculatus* after 9 months in the host than *in vitro* also raised the question of adjustment by the partners over time. But the homogenates were prepared from symbiotic hosts, so whether these incubations replicated the situation when the aposymbiotic *C. pedunculatus* were reinfected is unknown.

(xviii)'Homologous' and 'heterologous' *in vitro* ^{14}C incubations of *A. viridis* from 6 different geographical locations were performed. These revealed "host factor" specificity between symbionts and homogenates from different locations, symbionts with different properties depending upon their geographical origin and the possibility of different translocation control mechanisms in *A. viridis* collected from different sites. No evidence of "host factor" specificity or different translocation control mechanisms was observed with respect to *C. pedunculatus* from 2 different sites.

(xix)Net algal photosynthesis/12h and animal respiration/ 24h were converted to carbon units by the standard procedures.

(xx)The percentage contribution of translocated carbon to the daily respiratory carbon budget of the host (CZAR) was calculated according to Muscatine *et al*. (1981). The CZAR was calculated using the percentage translocation estimated by both the *in vivo* ^{14}C labelling method and the 'growth rate method'. The CZAR in the reestablished symbioses was standardized to symbiont cell density and carbon standing stock/mg association protein.

(xxi)The CZAR estimated using the percentage translocation measured with ^{14}C label in the 'natural' and reestablished symbioses was similar to that in published reports, and showed that all the anthozoans, with the exception of *I. sulcatus*, could not survive autotrophically. When ^{14}C was

used, the CZAR in the reinfected symbioses was higher in the animals containing 'homologous' symbionts than in the animals containing 'heterologous' symbionts.

(xxii) When the more accurate 'growth rate method' was used to estimate the percentage translocation, the CZAR in all the 'natural' symbioses was greater than 100 %, except in C. pedunculatus and A. ballii, indicating that these animals could survive autotrophically with respect to carbon. In the reestablished symbioses, the C. pedunculatus reinfected with symbionts from A. viridis (Shell Is.), I. sulcatus and A. pallida could not survive autotrophically, in contrast to the animals reinfected with symbionts from the other 3 host species. The CZAR in animals reinfected with Symbiodinium sp. from C. pedunculatus was significantly higher than that in the 'heterologous' symbioses involving algae from I. sulcatus and A. viridis (Shell Is.).

(xxiii)The different CZAR values in the reestablished symbioses were reflected in the standardized CZAR values. This was expected because of the similar symbiont densities and carbon standing stocks/mg association protein in the reinfected animals. The CZAR/cell/mg association protein calculated using ^{14}C labelling was highest by symbionts originally from C. pedunculatus, as was the CZAR/mg C/mg association protein when either the 'growth rate method' or ^{14}C labelling was used to calculate the percentage translocation. The CZAR/cell/mg association protein, calculated using the percentage translocation

determined by the 'growth rate method', was higher in C. pedunculatus reinfected with symbionts of A. ballii than in C. pedunculatus reinfected with 'homologous' symbionts. This result demonstrated that, whilst Symbiodinium cells originally from C. pedunculatus were more effective at supplementing their reinfected hosts carbon requirements, the larger volume of the algal cells from A. ballii resulted in the overall CZAR being similar. The significantly lower CZAR/mg C/mg association protein and overall CZAR in the C. pedunculatus reinfected with symbionts from A. viridis (Shell Is.) than in the 'homologous' reinfected C. pedunculatus indicated that the specificity observed in the reinfection and symbiont persistence work was also expressed through the CZAR.

(xxiv)The influence of a 5 fold increase in the mitotic index (MI) (Brown and Zamani 1992) and an 8 fold increase in the algal respiration rate (McCloskey and Muscatine 1984) on the CZAR in the 'natural' symbioses was investigated using a spreadsheet. The increases in these values had little effect on the conclusions which could be drawn from the CZAR values determined.

(xxv)Flow diagrams showing the flux of carbon within the symbioses revealed that in those symbioses where the CZAR was greater than 100 %, carbon was available for host growth and reproduction, or release from the symbiosis as dissolved organic carbon (DOC) or mucus. The size of these carbon sinks was not determined here.

CHAPTER 6

BROAD DISCUSSION - SYMBIOSIS SPECIFICITY AND THE EVOLUTION OF ANTHOZOAN - DINOFLAGELLATE SYMBIOSES.

By achieving its specific objectives (chapter 1, section 1.3 (p.13)), this project revealed that the dinoflagellate endosymbionts of different species of British Anthozoa, and conspecific anthozoans from geographically separated populations, are morphologically and physiologically different from one another. It was also evident that Symbiodinium sp. from the British Anthozoa is fundamentally different from the tropical, sub-tropical and other temperate Symbiodinium spp. observed here and in previously reported studies, because of the apparent absence of a motile stage in the cell cycle. This project also demonstrated that the host-symbiont relationship in temperate Anthozoa exhibits specificity, and that this specificity is reflected in the ability of the symbionts to satisfy the host's carbon demands. Consequently, host-symbiont specificity may influence the 'fitness' of the symbiosis. This chapter discusses how the morphological and physiological differences observed amongst the symbionts of the British Anthozoa may have arisen, and how the specificity associated with these differences may determine the extent to which 'novel' symbioses propagate in nature. Little is known of the evolutionary history of gymnodinioid dinoflagellate symbionts. The broad range of dinoflagellate

and invertebrate taxa involved in marine symbioses indicates that there have been many initiations of these symbioses (Trench 1981; Rudman 1982; Trench 1987). It therefore seems quite reasonable to propose that the algae now identified as belonging to the genus Symbiodinium may have originated from either isolated dinoflagellate populations or even different, but related species. Alternatively, if the genus Symbiodinium did itself originate from a single algal species, it is unlikely that the alga would not have shown genetic variation in response to the genetic divergence of the host animals themselves (Trench 1979). Such genetic divergence is evident within free-living marine phytoplankton (Brand 1988). This author stated that small genetic variations occur within local populations, while phytoplankton species that live in more than one habitat or water mass have developed genetic differences which may be considered to be as large as the differences between species. Unfortunately, because of the absence of suitable evidence in the fossil record (Trench 1987), whether the dinoflagellate endosymbionts of the British Anthozoa evolved from a single species and diverged, or originated from different algal taxa remains unresolved. The morphological differences between the cultured symbionts of different hosts suggested that genotypically distinct entities were being observed. However, detailed genetic sequencing is required before it can be stated conclusively that the symbionts of the British Anthozoa are genotypically

different. When the nature of mutualistic symbioses and the reproductive methods of both the symbionts and hosts are considered, it is arguable that genetic divergence of the symbionts has occurred since the establishment of the symbioses with British Anthozoa. This point is particularly well illustrated by the endosymbionts of A. viridis from geographically separated populations. A. viridis predominantly reproduces asexually, by longitudinal fission, and possesses a 'closed' system of symbiont acquisition during sexual reproduction, where symbionts are transferred directly from adult to ova (Turner 1988; Turner 1989). It is therefore probable that, even if an infecting 'heterologous' symbiont could persist in a host, it would be 'out-competed' by the resident symbiont population. This lack of opportunity for symbiosis recombination in nature may lead to prolonged periods of time over which an alga and host are in symbiosis, resulting in the development of more intimate associations. Any morphological or physiological adaptations by the symbionts would be expected to be maintained by the apparent absence of sexual recombination in the life cycle in Symbiodinium spp. (Schoenberg and Trench 1980a). The maintenance of these adaptations in a relatively stable environment (ie. the intracellular environment of the host) has been suggested as a selective force in favour of asexual reproduction and against genetic change in Symbiodinium spp. (Law and Lewis 1983). However, the lack of sexual recombination provides less opportunity for the development

of potentially advantageous adaptations.

Despite the morphological and physiological differences observed between the symbionts investigated in this study, and the associated host-symbiont specificity, it was evident that Symbiodinium cells, even from populations 3000 miles apart and from different climates, remained similar enough to enable the initiation of a symbiosis with the same host species. But due to physiological limitations, it would be highly unlikely that sub-tropical Symbiodinium cells would persist in a temperate host in nature. Once 'heterologous' symbionts have been endocytosed they may be subject to morphological and physiological changes, for example with respect to translocation behaviour and photosynthetic rate, in response to the new host cell environment. The physiological changes following inoculation into another host species that were reported in this thesis indicate that the observed symbiont physiological variability may be the result of physiological plasticity rather than a deeper genetic difference. This suggestion was also made by Rudman (1982) with respect to the symbionts of nudibranchs. This physiological plasticity may enable the persistence of recombined symbioses. As discussed by Roughgarden (1975), the mutualistic response by the symbiont is always adjusted to the current properties of the host and cannot be based on the expectation of reciprocation in future host generations. Adjustment to a new host environment may enable the symbionts to provide a net advantage to the host and

consequently the symbionts would be expected to obtain a favourable response by the host, enabling the symbiosis to persist (Smith, D.C. 1980).

Whilst possibilities for the recombination of Symbiodinium-anthozoan symbioses appear to be few in British waters, in tropical associations occasions arise where hosts become aposymbiotic. As discussed in chapter 4, section 4.1 (pp.167-169), many tropical host species have 'open' symbiont acquisition systems (Trench 1987) or are rendered symbiont-free via "bleaching" (Fankboner and Reid 1980; Brown and Howard 1985; Goreau, T.J. and MacFarlane 1990; Gates et al. 1992). These symbiont-free periods leave the hosts susceptible to reinfection by 'heterologous' symbionts, leading to the possible evolution of 'novel' symbioses (Rowan and Powers 1991a). Rowan and Powers (1991a; 1991b) and Rowan (1991) revealed that genotypically similar populations of Symbiodinium cells were present in taxonomically different tropical host species and suggested that symbioses evolve by the "shuffling" of algal and animal lineages. The contrasting hypothesis, that the evolution of symbioses occurs by the diversification of permanently associated lineages, would be supported if the animal/symbiont relationship were more rigidly maintained. The apparently different symbionts of A. viridis from different locations could indicate the establishment of a 'novel' symbiosis rather than the diversification of a permanently associated symbiont, as was discussed earlier.

But whether there is genetic evidence for "shuffling" in British waters remains to be investigated. Inferences can be made from the results obtained here using British anthozoans regarding the potential for the establishment of 'novel' symbioses in both temperate and tropical seas.

The more rapid attainment of the maximum population density by 'homologous' symbionts following endocytosis observed here indicates that if an aposymbiotic animal was reinfected by 'homologous' and 'heterologous' Symbiodinium cells in the field then the 'heterologous' symbionts would be 'out-competed'. However, if the 'heterologous' symbionts remained 'unchallenged' in the symbiosis then they would become further integrated with the host, eventually dividing at similar rates and attaining similar population densities and biomasses to 'homologous' symbionts. Once such a 'novel' symbiosis was established, the symbiont would become the 'de facto' 'homologous' alga. It is unlikely that this symbiont would be 'out-competed' by infecting symbionts from the same host species, although the co-existence of symbiont strains or species could be possible. Genetically distinct populations of Symbiodinium may occur simultaneously in the same individuals of Pocillopora meandrina and P. damicornis (Rowan and Powers 1991a).

Even though it is possible that the 'heterologous' symbionts could eventually simulate the performance of the 'homologous' symbionts, over the period investigated in this study, host-symbiont specificity appeared to influence

symbiosis 'fitness'. The greater contribution of photosynthetically fixed carbon to host respiration, growth and reproduction in 'homologous' reestablished symbioses, compared to all but one of the 'heterologous' symbioses, suggests that a 'homologous' reinfection would lead to greater survivorship and propagation of the symbiosis. This awaits specific investigation. The propagation of a more successful symbiosis would also be amplified by the predominantly asexual reproductive method of anthozoans (Shick et al. 1979). Therefore, in addition to host-symbiont recognition during the earlier stages of symbiosis establishment, the probable greater 'fitness' of 'homologous' reestablished symbioses may explain the genotypic homogeneity of the symbiont populations in most of the host species investigated by Rowan and Powers (1991a).

In conclusion, this work has demonstrated that the dinoflagellate symbionts of the British Anthozoa and the sub-tropical actiniarian A. pallida are phenotypically, and perhaps genotypically, different. Host-symbiont specificity was associated with these differences, but this did not prevent the establishment of symbioses between a host and 'heterologous' symbionts. The responses of the symbionts to different host species exhibited plasticity. This may have enabled 'heterologous' symbionts, initially exhibiting a low affinity with a particular host species, to persist in a recombined symbiosis and potentially increase the 'fitness' of the two partners. But, whilst the establishment of a

symbiosis between 'heterologous' partners is of benefit to both symbiont and host, the findings of this project suggest that, where 'homologous' symbionts are available, the likelihood of a 'novel' symbiosis persisting is small. The initial recognition of 'homologous' symbionts by the host and the fact that, even after nine months in symbiosis, 'homologous' symbionts confer a greater nutritional advantage upon the host than most 'heterologous' symbionts indicates that host-symbiont specificity limits the potential for the propagation of 'novel' anthozoan-dinoflagellate symbioses in nature.

CHAPTER 7

GENERAL CONCLUSIONS

(i)Morphological and photosynthetic pigment analyses of the endosymbiotic algae found within the endodermis of the British anthozoans C. pedunculatus, A. ballii, A. viridis and I. sulcatus, and the sub-tropical A. pallida showed that these algae are all dinoflagellates belonging to the genus Symbiodinium.

(ii)The symbionts of the British Anthozoa differ from those of the sub-tropical and tropical A. pallida, A. pulchella, Z. sociatus and Cassiopeia sp., and the temperate Pacific anemone A. elegantissima and other previously described symbiotic hosts by a motile stage apparently being absent in culture.

(iii)Coccoid Symbiodinium cells from different species of British Anthozoa and A. pallida differ morphologically in culture, suggesting that they may be genotypically different. These morphological differences enabled the allocation of the symbionts from the different host species to one of 4 groups.

(iv)The coccoid symbionts of A. viridis from different geographical locations differed morphologically in culture, suggesting that they may be genotypically different. The

different sizes of these cells enabled their allocation to one of 2 different groups.

(v)The concentrations of photosynthetic pigments in the symbionts of the British Anthozoa and A. pallida may differ, but conclusions based upon this data were limited by the fact that freshly isolated Symbiodinium cells were used.

(vi)Symbiodinium cells from A. pallida may be genotypically different from those of the British Anthozoa on account of their mitotic index (MI) in culture.

(vii)Surface recognition may play little part in the discrimination between Symbiodinium cells from different host species by C. pedunculatus.

(viii)Recognition of 'homologous' and 'heterologous' symbionts by C. pedunculatus appears to be predominantly post-endocytotic.

(ix)The symbiosis between Symbiodinium sp. from different host species and C. pedunculatus exhibits specificity, reflected in the persistence and proliferation rate of the different symbiont populations in C. pedunculatus.

(x)'Homologous' symbionts are most 'successful' at reestablishing a symbiosis with C. pedunculatus.

(xi)The similarity of Symbiodinium sp. from different locations and climates enables the symbionts of different host species to be recognised by and persist in symbiosis with C. pedunculatus.

(xii)The host cell environment partly determines the photosynthetic rate of the symbiont population.

(xiii)The percentage of photosynthate translocated is reduced in 'homologous' reestablished symbioses from that in 'natural' symbioses on account of the greater utilization of carbon in symbiont growth in reestablished symbioses.

(xiv)Host-symbiont specificity is reflected by the percentage translocation in reestablished symbioses. This specificity is possibly due to "host factor" specificity or differences in the translocation control mechanism between different host species.

(xv)Different "host factors" and translocation control mechanisms, and symbionts with different properties may exist in A. viridis from separate geographical locations.

(xvi)The rate at which the symbiont populations proliferate upon infection of hosts may be partly related to recognition based upon the amount of photosynthate translocated.

(xvii)Different compounds may be translocated by symbionts in reestablished symbioses than by the same symbiont 'strains' in 'natural' symbioses.

(xviii)As a result of symbiont plasticity in response to the host cell environment, symbionts appear to become further integrated into a 'novel' symbiosis with time.

(xix)Only C. pedunculatus and A. ballii of the Anthozoa studied cannot survive autotrophically with respect to organic carbon under optimal irradiance. These 2 host species require a heterotrophic carbon input to survive, grow and reproduce.

(xx)C. pedunculatus can survive autotrophically with respect to organic carbon when in symbiosis with symbionts from C. pedunculatus, A. ballii and A. viridis (Lough Hyne), but not in symbiosis with Symbiodinium sp. from I. sulcatus, A. viridis (Shell Is.) or A. pallida. These latter 3 symbioses therefore require a heterotrophic carbon input to survive, grow and reproduce.

(xxi)The differences in the CZAR in reestablished symbioses reflect the specific host-symbiont relationship.

(xxii)'Novel' symbioses may be initiated in nature. But the more rapid proliferation of 'homologous' symbionts upon reinfection of an aposymbiotic host and the greater 'fitness' of 'homologous' symbioses than most 'heterologous' symbioses suggest that "shuffling" of animal and algal lineages, and the propagation of the resultant symbioses is not likely to occur frequently.

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APPENDIX 1: INVESTIGATION OF THE ELEVATED EXPERIMENTAL TEMPERATURE ON SYMBIOSIS STABILITY.

INVESTIGATION OF THE INFLUENCE OF THE ELEVATED EXPERIMENTAL TEMPERATURE ON SYMBIOSIS STABILITY

Introduction

The aim of this experiment was to establish whether the symbioses were under stress when maintained at 21 ^{o}C, the temperature at which it was proposed to perform all experimental work. As discussed in chapter 2, section 2.2.3.1 (pp.33-37), reductions in the densities of symbiont populations are observed when symbioses are subjected to stressful environmental conditions. This work therefore compared the symbiont density in anthozoans subjected to a temperature the anthozoans were expected to commonly encounter in the field to the symbiont density when the host was at the experimental temperature.

Method

5 A. viridis (Lough Hyne and Shell Is.), A. ballii and C. pedunculatus and 2 colonies of I. sulcatus were placed in an aerated, sea water filled aquarium. The A. viridis, A. ballii and C. pedunculatus had oral disc diameters of approximately 2 cm. The I. sulcatus polyps were approximately 3 mm high. The aquarium was positioned in a water bath at 13 ^{o}C. The C. pedunculatus and I. sulcatus were partitioned off from the A. viridis and A. ballii to prevent the larger anthozoans eating the C. pedunculatus and I. sulcatus, or killing them by stinging. Another tank

containing the above temperate species was set up, but this was maintained at 21 °C. A second tank maintained at 21 °C was also set up, containing 20 A. pallida of oral disc diameter 1 cm. A final tank, maintained at 25 °C, contained the stock population of A. pallida. The anthozoans in these tanks were fed twice weekly with freshly hatched Artemia nauplii and were under a 12 hour light : dark cycle at 80 $\mu E/m^2/s$. Salinity was measured twice weekly with a salinometer and adjusted to 34 °/oo if necessary. After 1 month, 4 similarly sized tentacles were excised from each A. viridis, C. pedunculatus and A. ballii, and 2 similarly sized tentacles were excised from each of 10 A. pallida in each tank. The A. pallida used in the stock tank were the same size as those in the 21 °C tank. 10 similarly sized polyps of I. sulcatus were removed from each colony in each tank (ie. a total of 20 polyps per treatment) using a razor blade. All the polyps and tentacles were relaxed by placing them in a drop of 7.5 % $MgCl_2.6H_2O$ in sea water on a slide for 10 minutes. Using a light microscope and ocular micrometer, the length of the tentacle or polyp and its width at 25, 50 and 75 % along the length was measured. The area of tentacle was approximated by multiplying the length by the average width. The tentacles and polyps were then homogenized individually in a hand held glass tissue homogenizer in 0.5 ml filtered sea water (FSW). The number of symbionts present in each homogenate was estimated using a haemocytometer and the density of cells expressed as

TABLE 1 DENSITY OF SYMBIONTS (CELLS/ MM HOST TISSUE^2) IN TENTACLES AND POLYPS AFTER MAINTENANCE AT 13 OR 25 °C, AND 21 °C (N = 20) (+/- S.E.).

Host sp.	C. pedunc-ulatus	A. ballii	A. viridis (Lough Hyne)	A. viridis (Shell Is.)	I. sulca-tus	A. pall-ida
cells/ mm^2 at 13°C or 25°C	197899 +/- 7211	124878 +/- 6689	99338 +/- 3498	76322 +/- 3441	44924 +/- 13580	48657 +/- 3184
cells/ mm^2 at 21°C	266055 +/- 11279	131134 +/- 5818	116531 +/- 3987	104302 +/- 4179	71426 +/- 3548	69363 +/- 3084

TABLE 2 T-TEST STATISTICS FOR COMPARISONS BETWEEN SYMBIONT DENSITIES AT 13 or 25 °C AND 21 °C (N = 20)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host species	T-test statistic
C. pedunculatus	**T = 3.8 ***
A. ballii	**T = 0.9**
A. viridis (Lough Hyne)	**T = 3.2 ***
A. viridis (Shell Is.)	**T = 5.3 ***
I. sulcatus	**T = 5.6 ***
A. pallida	**T = 4.7 ***

cells/mm^2. The densities of symbionts in the same host species at 21 and 13 ^{o}C or at 21 and 25 ^{o}C were compared statistically using Student's t-test.

Results

The densities of symbionts in the tentacles and polyps at 13, 21 and 25 ^{o}C are shown in table 1 (p.465). The results of t-test comparisons between the symbiont densities in the same host species at different temperatures are presented in table 2 (p.465). The symbiont densities were significantly higher at 21 ^{o}C than at 13 or 25 ^{o}C in all the host species, with the exception of A. ballii. The symbiont density in A. ballii was not significantly different between 13 and 21 ^{o}C.

Discussion

The higher or similar symbiont densities in the anthozoans maintained at 21 ^{o}C than in the animals maintained at 13 or 25 ^{o}C suggested that the symbioses were not under stress at 21 ^{o}C. In fact, the symbioses may have been under reduced stress at 21 ^{o}C than at the other temperatures used. It was concluded that 21 ^{o}C was an acceptable temperature at which to maintain animals and perform experiments in this work.

APPENDIX 2: LIGHT MICROGRAPHS.

Plate 1: Light micrographs of tentacle squashes showing the location of the symbionts within the endoderm.

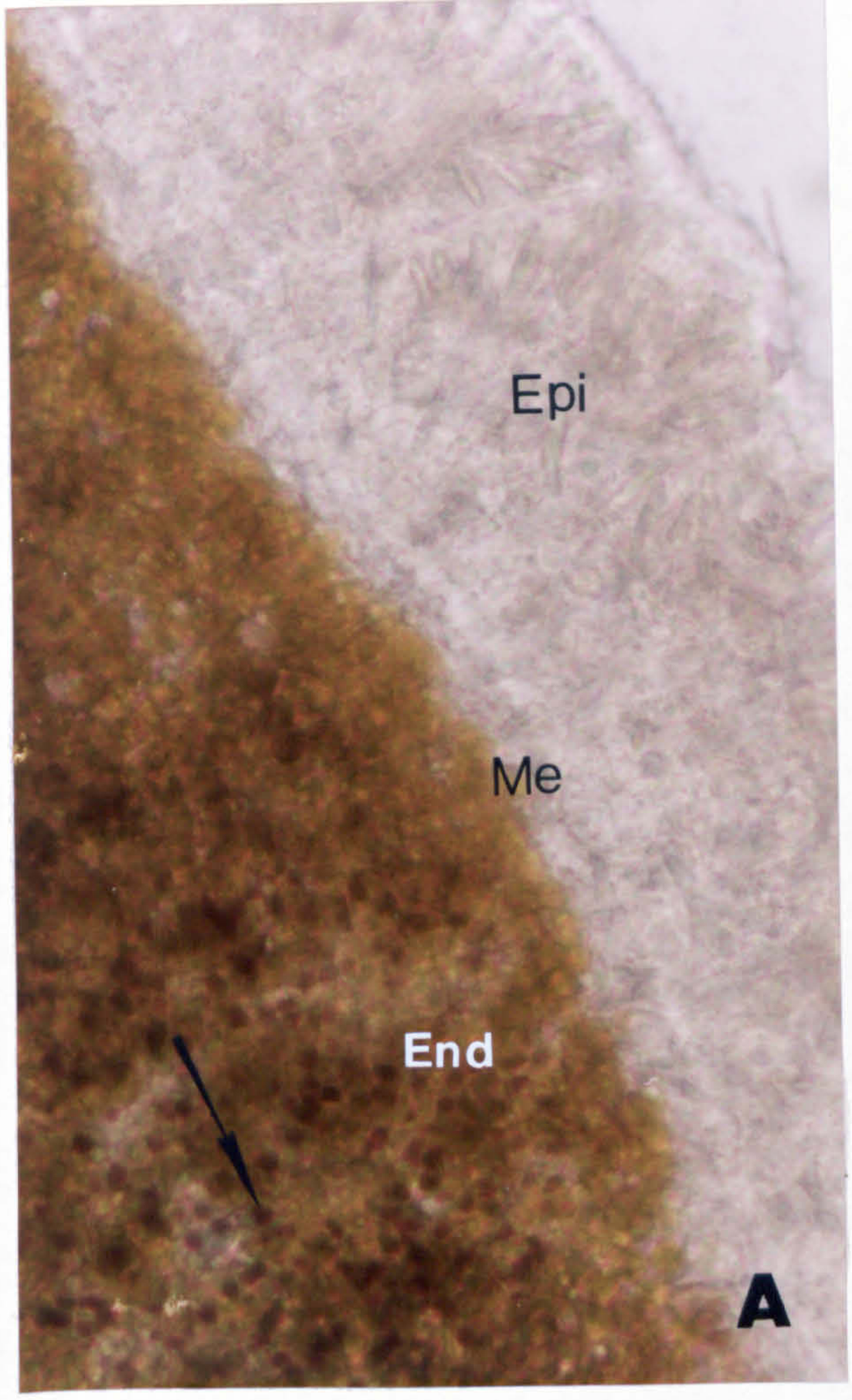
(A) Tentacle squash of C. pedunculatus (magnification approx. x230).

(B) Tentacle squash of A. ballii (magnification approx. x230).

(C) Tentacle squash of I. sulcatus (magnification approx. x360).

(D) Tentacle squash of A. pallida (magnification approx. x360).

End = endoderm; Me = mesogloea; Epi = epidermis; arrow (plate A only) indicates the position of a chromatophore.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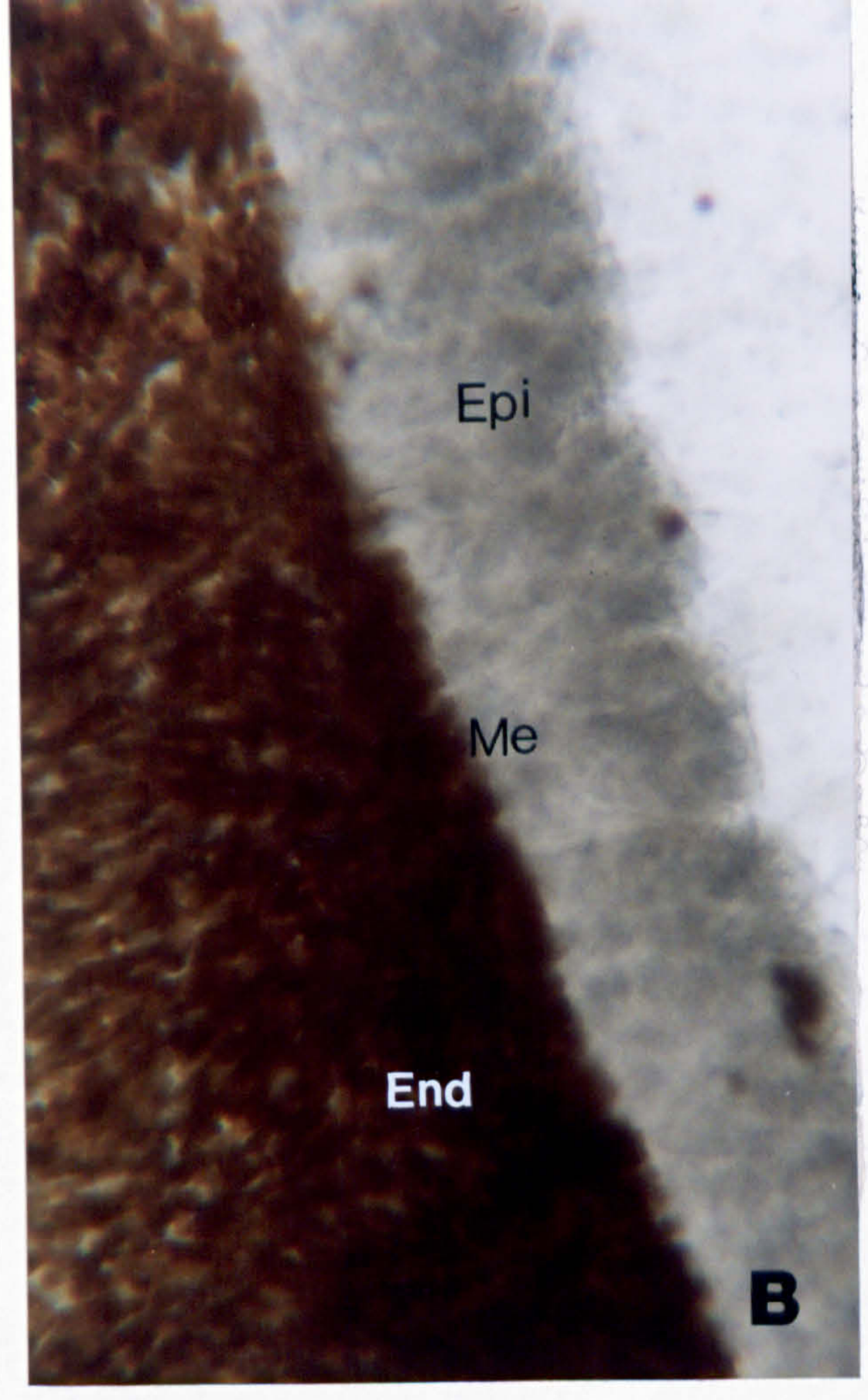
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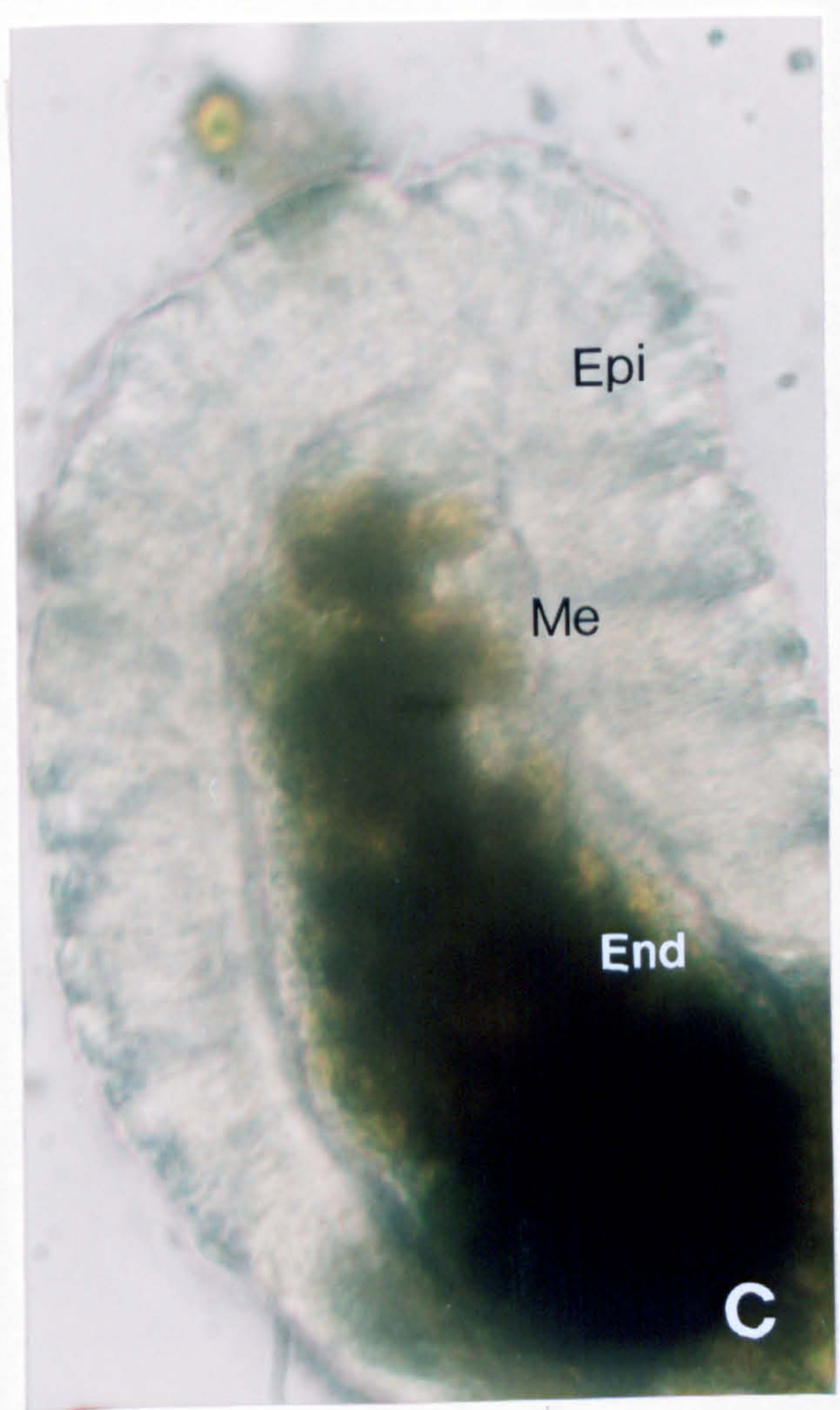
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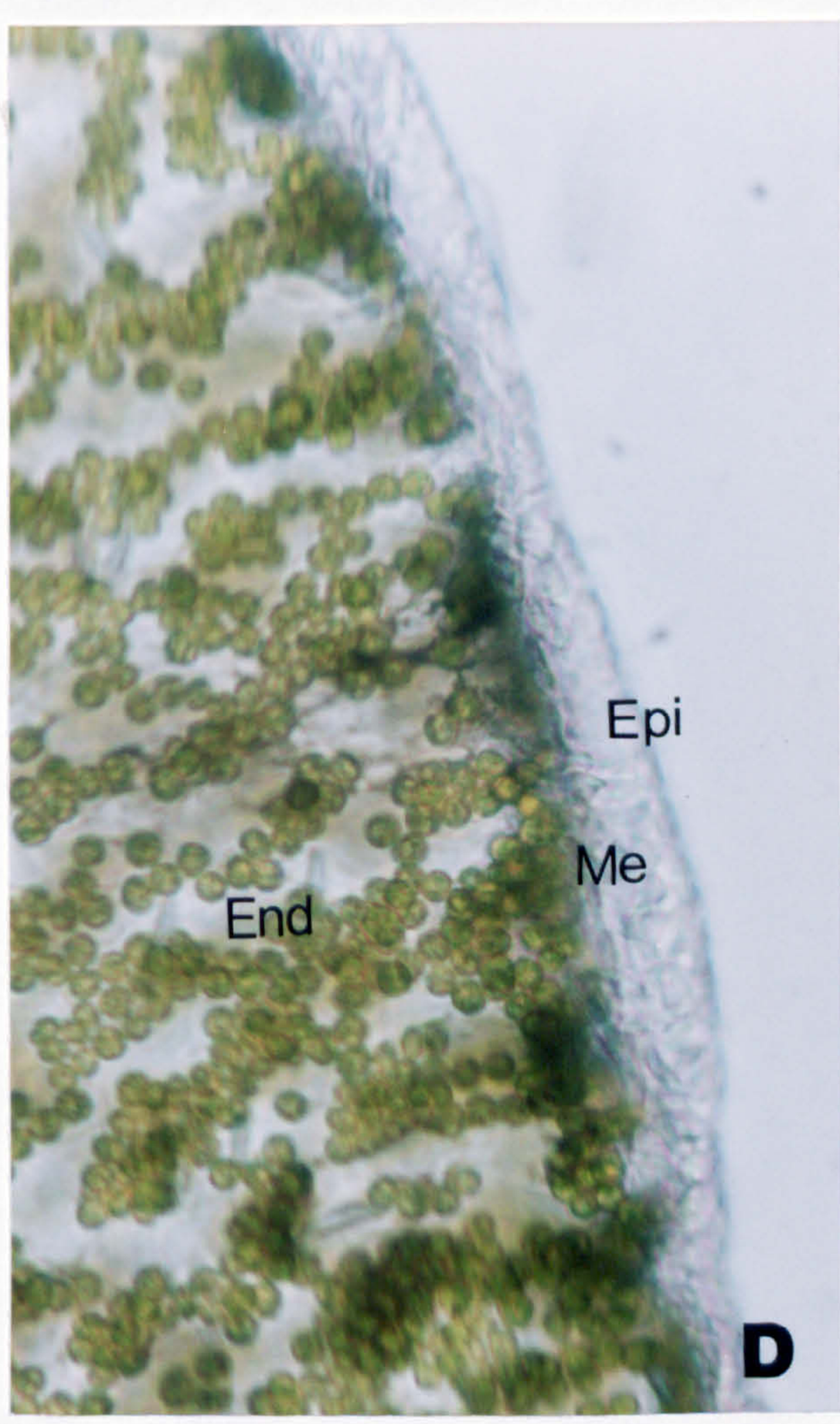
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Plate 2: Light micrographs of Symbiodinium sp. cells in host homogenates.

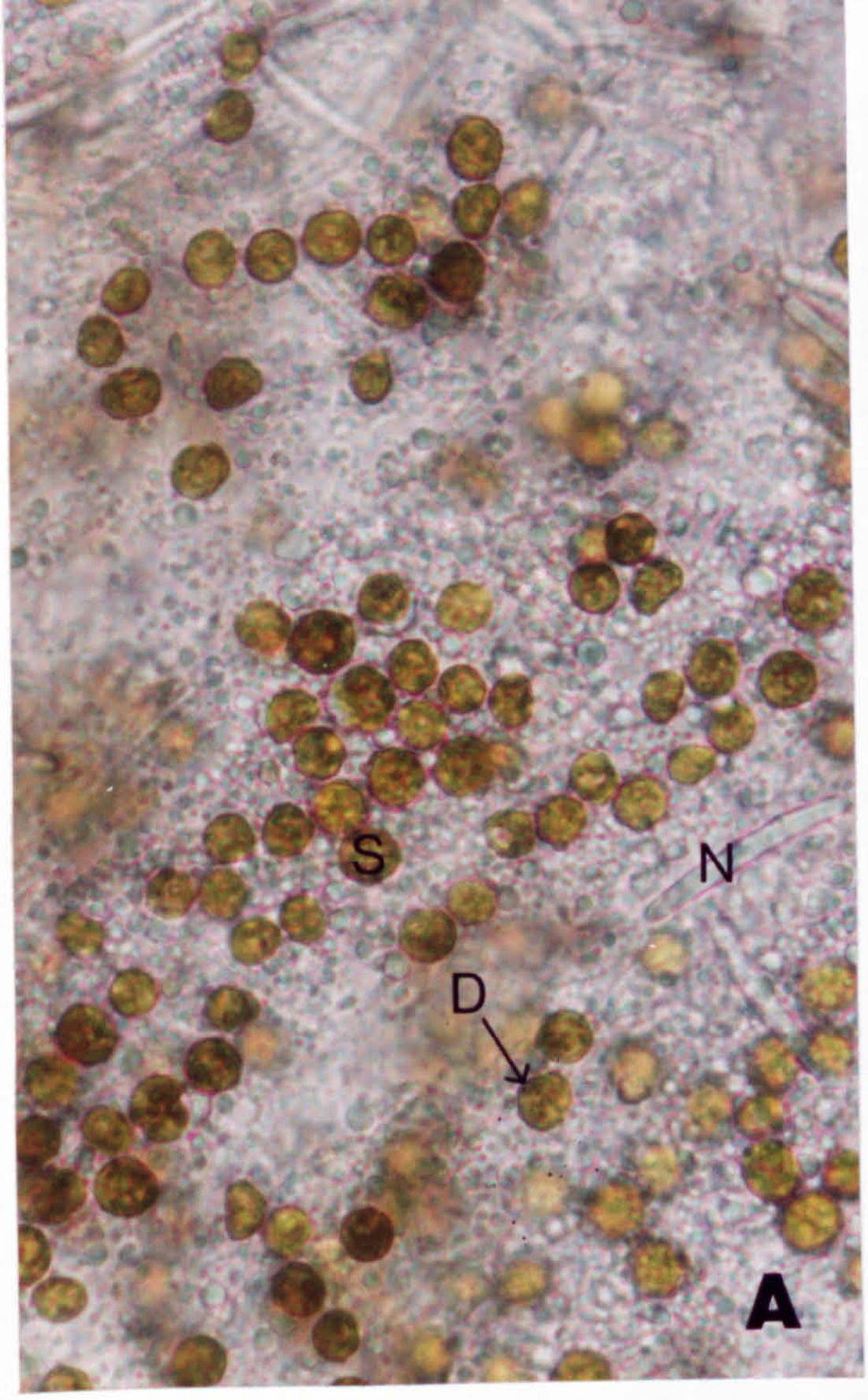
(A) Symbiodinium sp. from A. viridis (Lough Hyne) (magnification approx. x500).

(B) Symbiodinium sp. from A. ballii (magnification approx. x500).

(C) Symbiodinium sp. from I. sulcatus (magnification approx. x650).

(D) Symbiodinium sp. from A. pallida (magnification approx. x500).

S = Symbiodinium sp. cell; N = host nematocyst; D = symbiont in "2-cell dividing stage".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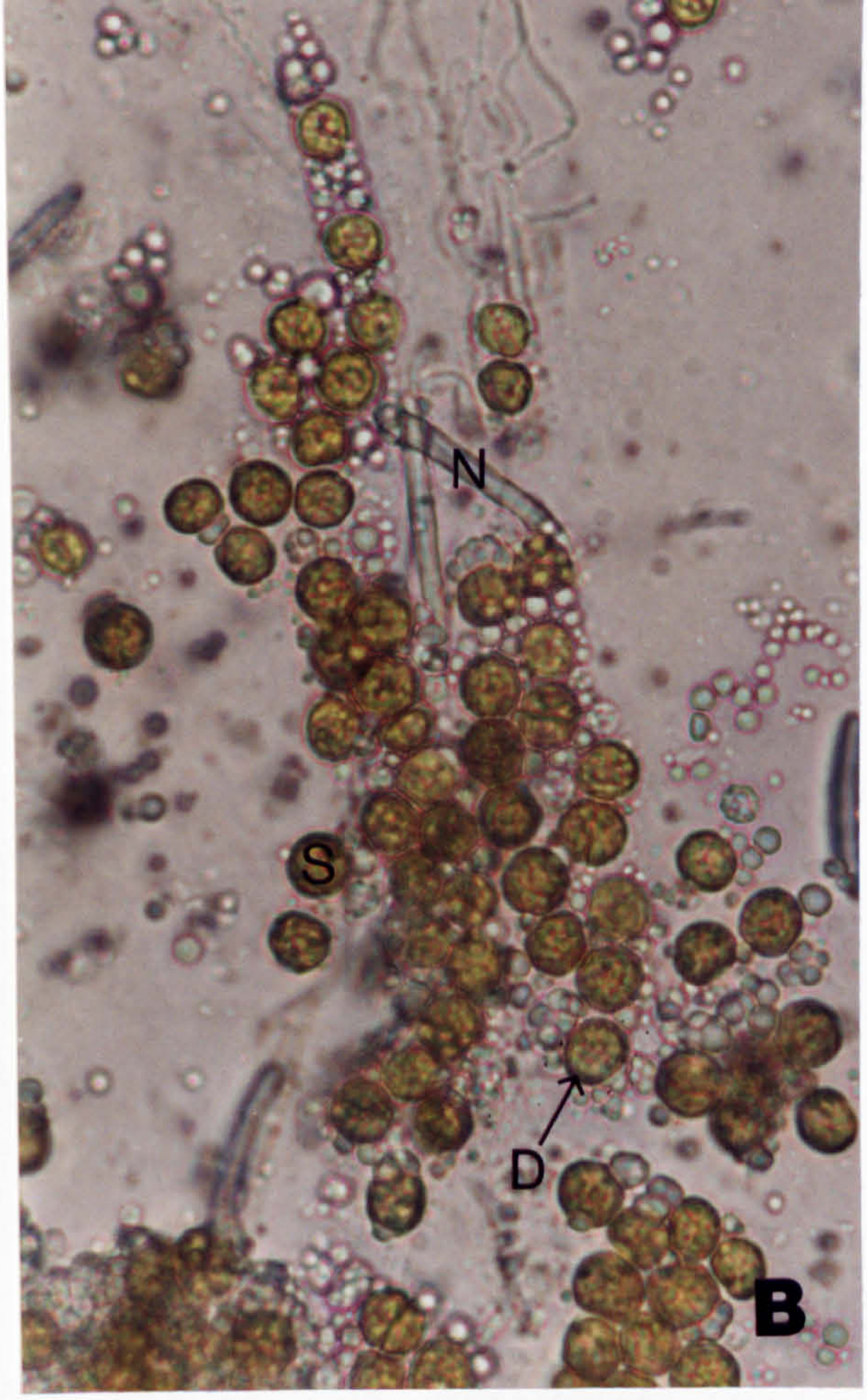
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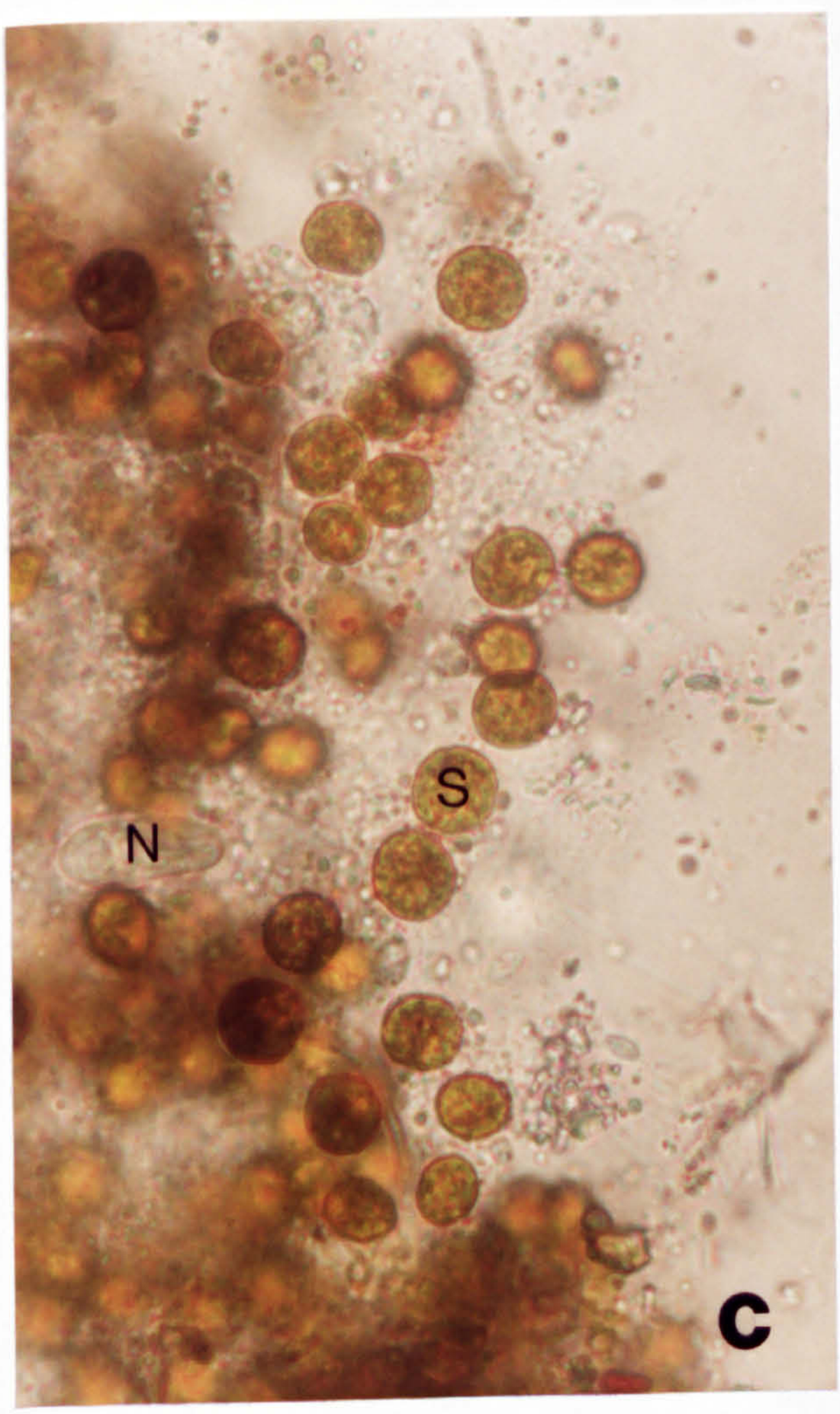
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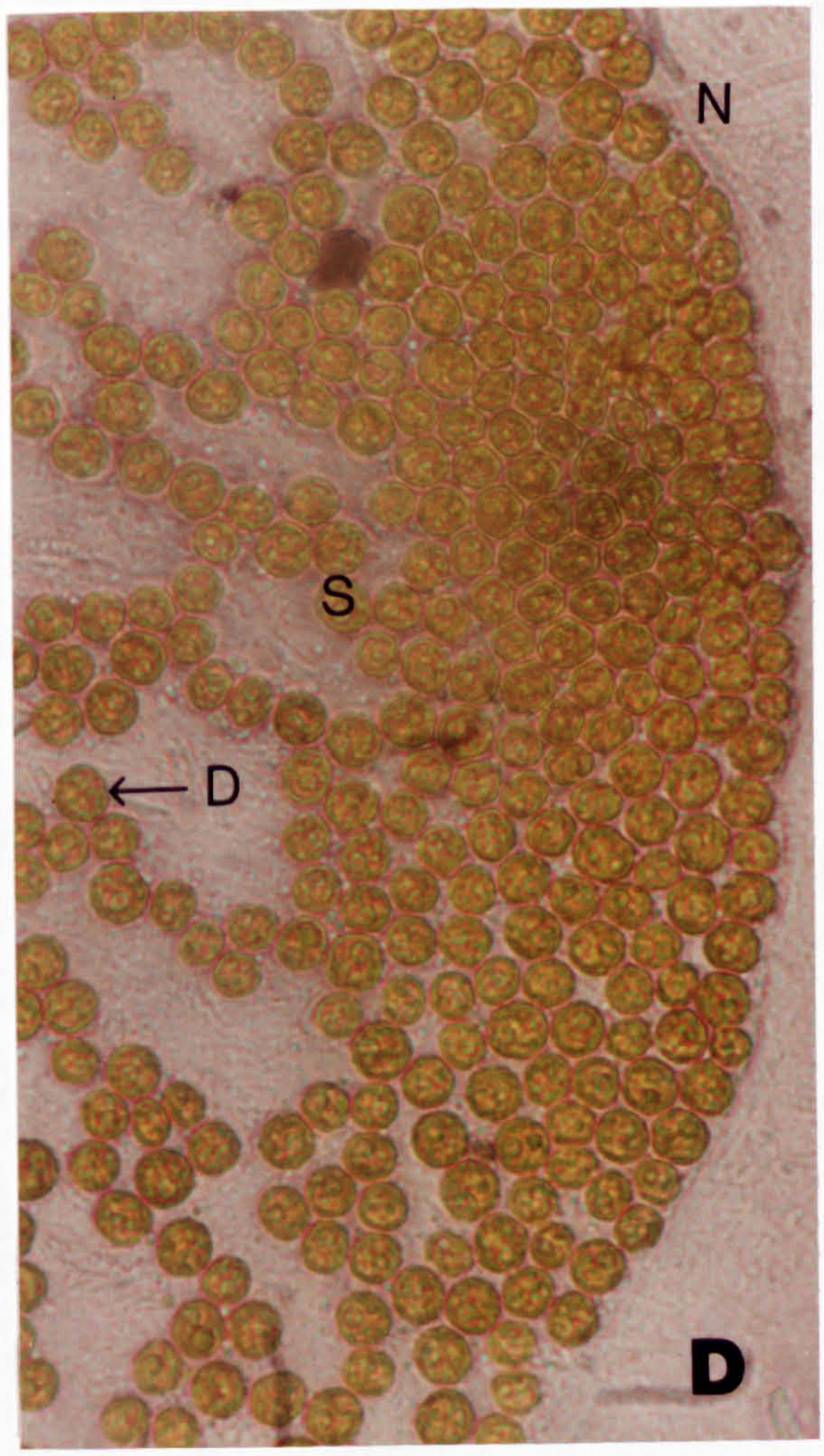
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APPENDIX 3: STATISTICS RELATING TO SYMBIONT DIMENSIONS.

TABLE 1 SIGNIFICANCE TESTS (T-TESTS AND MANN-WHITNEY TESTS) BETWEEN DIMENSIONS OF "2-CELL STAGE" IN CULTURE AND FRESHLY ISOLATED (N=40) (SIGNIFICANT DIFFERENCES IN BOLD WITH ASTERISK)

HOST SPECIES	VALUE OF T FOR COMPARISONS OF CELL LENGTH	VALUE OF T FOR COMPARISONS OF CELL WIDTH
Ap	**T=3.44, P=0.0011 ***	**T=6.30, P<0.0001 ***
Cp	**T=2.54, P=0.013 ***	**T=2.31, P=0.024 ***
AvLs	T=0.71, P=0.48	T=0.05, P=0.96
CpNt	T=0.31, P=0.76	T=0.69, P=0.49
AvLH	T=0.96, P=0.34	T=1.86, P=0.07
AvWH	T=0.30, P=0.76	T=0.66, P=0.51
AvBm	T=0.46, P=0.64	T=0.68, P=0.50
AvTB	T=0.00, P>0.9999	T=1.15, P=0.25
AvSI	T=1.54, P=0.13	T=0.19, P=0.85
Ab	**W=1341.0, P=0.001 ***	**T=2.34, P=0.02 ***
Is	W=1595.5, P=0.82	W=1745.5, P=0.23

TABLE 2 SIGNIFICANCE TEST (MULTIPLE COMPARISON TEST) Z VALUES OF COMPARISONS BETWEEN LENGHTS OF "2-CELL STAGE" OF CULTURED SYMBIONTS FROM DIFFERENT HOST SPECIES (N=40) (CRITICAL VALUE OF Z=3.29) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host	Apul	Ca	Ap	AvLS	Cp	CpNt	AvLH	AvSI	AvWH	AvBm	AvTB	Ab	Is	Zs	Ae
Apul	-	0.6	0.9	2.6	2.6	3.1	**5.3***	**6.8***	**7.1***	**7.5***	**7.8***	**9.2***	**9.7***	**9.8***	**9.8***
Ca	-	-	0.2	2.0	2.0	2.4	**4.6***	**6.1***	**6.4***	**6.9***	**7.1***	**8.5***	**9.0***	**9.1***	**9.2***
Ap	-	-	-	1.7	1.7	2.2	**4.4***	**5.9***	**6.2***	**6.6***	**6.8***	**8.3***	**8.8***	**8.9***	**8.9***
AvLS	-	-	-	-	0.0	0.4	2.7	**4.2***	**4.5***	**4.9***	**5.1***	**6.6***	**7.1***	**7.1***	**7.2***
Cp	-	-	-	-	-	0.4	2.7	**4.2***	**4.5***	**4.9***	**5.1***	**6.6***	**7.1***	**7.1***	**7.2***
CpNt	-	-	-	-	-	-	2.2	**3.7***	**4.0***	**4.5***	**4.7***	**6.2***	**6.6***	**6.7***	**6.8***
AvLH	-	-	-	-	-	-	-	1.5	1.8	2.2	2.5	**3.9***	**4.4***	**4.5***	**4.6***
AvSI	-	-	-	-	-	-	-	-	0.3	0.8	1.0	2.4	2.9	3.0	3.1
AvWH	-	-	-	-	-	-	-	-	-	0.4	0.7	2.1	2.6	2.7	2.8
AvBm	-	-	-	-	-	-	-	-	-	-	0.2	1.7	2.2	2.2	2.3
AvTB	-	-	-	-	-	-	-	-	-	-	-	1.5	2.0	2.0	2.1
Ab	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5	0.6
Is	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2
Zs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
Ae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3 SIGNIFICANCE TEST (MULTIPLE COMPARISON TEST) Z VALUES OF COMPARISONS BETWEEN CELL WIDTHS OF "2-CELL STAGE" OF CULTURED SYMBIONTS FROM DIFFERENT HOST SPECIES (N=40) (CRITICAL VALUE OF Z=3.49) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host	Apul	Ap	Ca	Cp	AvLS	CpNt	AvLH	AvBm	AvSI	AvWH	AvTB	Ab	Zs	Is	Ae
Apul	-	0.9	1.1	1.8	2.3	3.3	5.6 *	6.8 *	7.0 *	7.3 *	8.4 *	8.6 *	9.5 *	10.0 *	10.1 *
Ap	-	-	0.2	0.9	1.4	2.4	4.7 *	5.9 *	6.1 *	6.4 *	7.5 *	7.6 *	8.6 *	9.1 *	9.2 *
Ca	-	-	-	0.7	1.2	2.2	4.5 *	5.7 *	5.9 *	6.2 *	7.3 *	7.4 *	8.4 *	8.9 *	9.0 *
Cp	-	-	-	-	0.5	1.5	3.8 *	5.0 *	5.1 *	5.4 *	6.6 *	6.7 *	7.6 *	8.2 *	8.3 *
AvLS	-	-	-	-	-	1.0	3.3	4.5 *	4.7 *	5.0 *	6.1 *	6.3 *	7.2 *	7.7 *	7.8 *
CpNt	-	-	-	-	-	-	2.3	3.5 *	3.7 *	4.0 *	5.1 *	5.3 *	6.2 *	6.7 *	6.8 *
AvLH	-	-	-	-	-	-	-	1.2	1.4	1.7	2.8	3.0	3.9 *	4.4 *	4.5 *
AvBm	-	-	-	-	-	-	-	-	0.2	0.5	1.6	1.8	2.7	3.2	3.3
AvSI	-	-	-	-	-	-	-	-	-	0.3	1.4	1.6	2.5	3.0	3.1
AvWH	-	-	-	-	-	-	-	-	-	-	1.1	1.3	2.2	2.7	2.8
AvTB	-	-	-	-	-	-	-	-	-	-	-	0.1	1.1	1.6	1.7
Ab	-	-	-	-	-	-	-	-	-	-	-	-	0.9	1.4	1.6
Zs	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.6
Is	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
Ae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 4 SIGNIFICANCE TEST (MULTIPLE COMPARISON TEST) Z VALUES OF COMPARISONS BETWEEN LENGTHS OF "2-CELL STAGE" OF FRESHLY ISOLATED SYMBIONTS OF DIFFERENT HOST SPECIES (N=40) (CRITICAL VALUE OF Z=3.29) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host-t sp.	Ap	Av LS	Cp Nt	Cp	Av LH	Av SI	Av WH	Av Bm	Av TB	Is	Ab
Ap	-	3.2	**3.8 ***	**5.1 ***	**6.0 ***	**7.5 ***	**7.9 ***	**8.4 ***	**8.6 ***	**11.2 ***	**12.6 ***
Av LS	-	-	0.5	1.8	2.8	**4.3 ***	**4.6 ***	**5.1 ***	**5.3 ***	**8.0 ***	**9.4 ***
Cp Nt	-	-	-	1.3	2.2	**3.7 ***	**4.1 ***	**4.6 ***	**4.8 ***	**7.4 ***	**8.8 ***
Cp	-	-	-	-	1.0	2.5	2.8	**3.3 ***	**3.5 ***	**6.2 ***	**7.6 ***
Av LH	-	-	-	-	-	1.5	1.9	2.4	2.6	**5.2 ***	**6.6 ***
Av SI	-	-	-	-	-	-	0.3	0.9	1.1	**3.7 ***	**5.1 ***
Av WH	-	-	-	-	-	-	-	0.5	0.7	**3.3 ***	**4.8 ***
Av Bm	-	-	-	-	-	-	-	-	0.2	2.8	**4.3 ***
Av TB	-	-	-	-	-	-	-	-	-	2.6	**4.1 ***
Is	-	-	-	-	-	-	-	-	-	-	1.4
Ab	-	-	-	-	-	-	-	-	-	-	-

TABLE 5 SIGNIFICANCE TEST (MULTIPLE COMPARISON TEST) Z VALUES OF COMPARISONS BETWEEN WIDTHS OF "2-CELL STAGE" OF FRESHLY ISOLATED SYMBIONTS FROM DIFFERENT HOST SPECIES (N=40) (CRITICAL VALUE OF Z=3.29) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host-t sp.	Ap	Av LS	Cp	Cp Nt	Av LH	Av SI	Av WH	Av Bm	Av TB	Is	Ab
Ap	-	3.2	**4.5 ***	**4.6 ***	**5.6 ***	**8.1 ***	**8.3 ***	**8.5 ***	**9.0 ***	**11.1 ***	**12.3 ***
Av LS	-	-	1.4	1.4	2.4	**4.9 ***	**5.1 ***	**5.4 ***	**5.8 ***	**7.9 ***	**9.5 ***
Cp	-	-	-	0.1	1.0	**3.5 ***	**3.7 ***	**4.0 ***	**4.4 ***	**6.6 ***	**8.1 ***
Cp Nt	-	-	-	-	1.0	**3.5 ***	**3.7 ***	**4.0 ***	**4.4 ***	**6.5 ***	**8.1 ***
Av LH	-	-	-	-	-	2.5	2.7	3.0	**3.4 ***	**5.5 ***	**7.1 ***
Av SI	-	-	-	-	-	-	0.2	0.5	0.9	3.0	**4.6 ***
Av WH	-	-	-	-	-	-	-	0.3	0.7	2.8	**4.4 ***
Av Bm	-	-	-	-	-	-	-	-	0.4	2.6	**4.1 ***
Av TB	-	-	-	-	-	-	-	-	-	2.1	**3.7 ***
Is	-	-	-	-	-	-	-	-	-	-	1.6
Ab	-	-	-	-	-	-	-	-	-	-	-

TABLE 6 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) WHEN INVESTIGATING HOST INTRASPECIFIC GEOGRAPHICAL VARIATION IN CULTURED SYMBIONT LENGTH (N=40) (CRITICAL VALUE OF Z = 2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	AvLS	AvLH	AvWH	AvBm	AvTB	AvSI
AvLS	-	**3.1 ***	**4.7 ***	**5.2 ***	**5.5 ***	**5.8 ***
AvLH	-	-	1.6	2.1	2.4	2.6
AvWH	-	-	-	0.4	0.8	1.0
AvBm	-	-	-	-	0.4	0.6
AvTB	-	-	-	-	-	0.2
AvSI	-	-	-	-	-	-

TABLE 7 RESULTS OF TUKEY'S TEST (CONFIDENCE LIMITS) WHEN INVESTIGATING HOST INTRASPECIFIC GEOGRAPHICAL VARIATION IN CULTURED SYMBIONT WIDTH (N=40) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	AvLS	AvLH	AvBm	AvSI	AvWH	AvTB
AvLS	-	**0.5/1.7 ***	**0.9/2.0 ***	**1.0/2.1 ***	**1.1/2.2 ***	**1.4/2.6 ***
AvLH	-	-	-0.9/0.2	-1.0/0.1	-1.1/0.0	**-1.4/-0.3 ***
AvBm	-	-	-	-0.5/0.6	-0.4/0.7	-0.0/1.1
AvSI	-	-	-	-	-0.7/0.4	-1.0/0.1
AvWH	-	-	-	-	-	-0.2/0.9
AvTB	-	-	-	-	-	-

TABLE 8 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) WHEN INVESTIGATING HOST INTRASPECIFIC GEOGRAPHICAL VARIATION IN FRESHLY ISOLATED SYMBIONT LENGTH (N=40) (CRITICAL VALUE OF Z = 2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	AvLS	AvLH	AvSI	AvWH	AvBm	AvTB
AvLS	-	**3.4 ***	**5.1 ***	**5.5 ***	**6.2 ***	**6.5 ***
AvLH	-	-	1.7	2.2	2.9	**3.1 ***
AvSI	-	-	-	0.5	1.2	1.4
AvWH	-	-	-	-	0.7	0.9
AvBm	-	-	-	-	-	0.2
AvTB	-	-	-	-	-	-

TABLE 9 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) WHEN INVESTIGATING HOST INTRASPECIFIC GEOGRAPHICAL VARIATION IN FRESHLY ISOLATED SYMBIONT WIDTH (N=40) (CRITICAL VALUE OF Z = 2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	AvLS	AvLH	AvSI	AvWH	AvBm	AvTB
AvLS	-	2.6	**5.7 ***	**5.9 ***	**6.3 ***	**6.8 ***
AvLH	-	-	**3.0 ***	**3.3 ***	**3.7 ***	**4.1 ***
AvSI	-	-	-	0.3	0.6	1.1
AvWH	-	-	-	-	0.4	0.8
AvBm	-	-	-	-	-	0.5
AvTB	-	-	-	-	-	-

APPENDIX 4: TRANSMISSION ELECTRON MICROGRAPHS.

Plate 1: Transmission electron micrographs of cultured and freshly isolated Symbiodinium sp. cells.

(A) *Symbiodinium* sp. freshly isolated from *C. pedunculatus* (Lough Hyne) (magnification approx. x13,000).

(B) Cultured *Symbiodinium* sp. from *A. ballii* (magnification approx. x9,800).

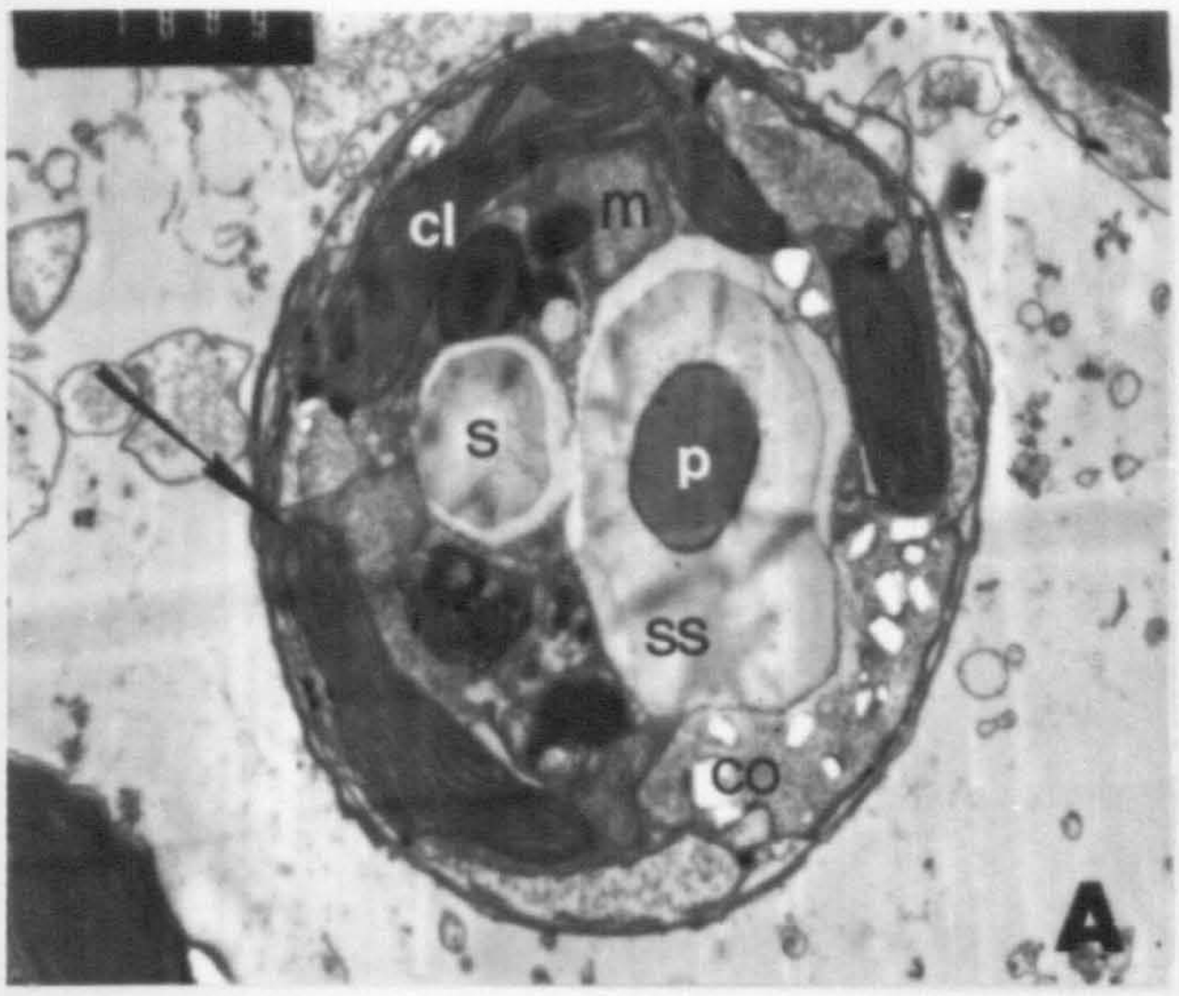
(C) *Symbiodinium* sp. freshly isolated from *A. viridis* (Lough Hyne) (magnification approx. x13,000).

(D) Cultured *Symbiodinium* sp. from *A. pallida* (magnification approx. x9,800).

(E) *Symbiodinium* sp. freshly isolated from *I. sulcatus* (magnification approx. x13,000).

(F) *Symbiodinium* sp. freshly isolated from *A. viridis* (Shell Island) (magnification approx. x13,000).

cl = chloroplast; m = mitochondrion; s = starch; p = stalked pyrenoid; ss = starch sheath of pyrenoid; co = calcium oxalate crystals; ch = chromosome; n = nucleus; nu = nucleolus; fl = possible flagellar-hair producing cavity; arrows indicate peripheral thylakoid lamellae.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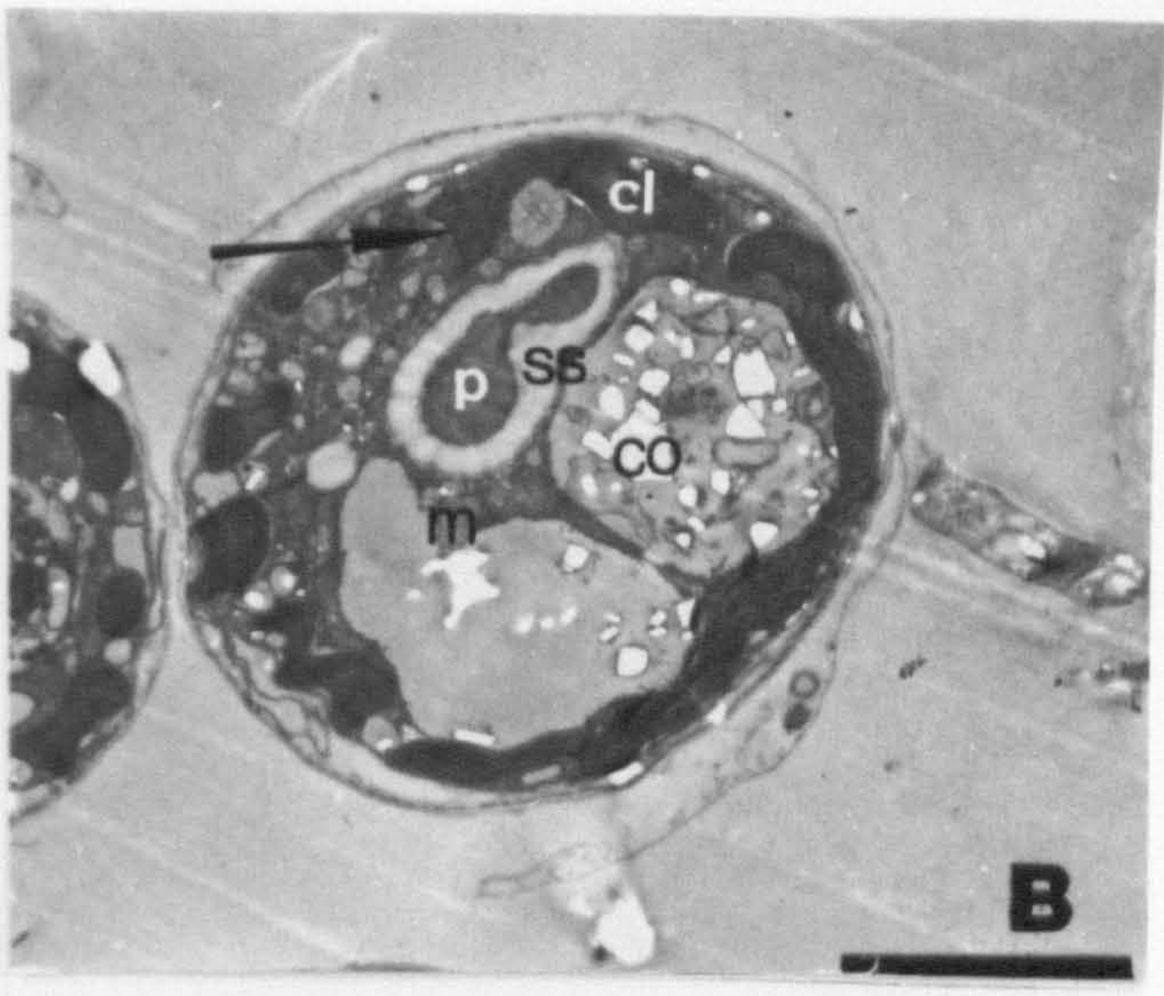
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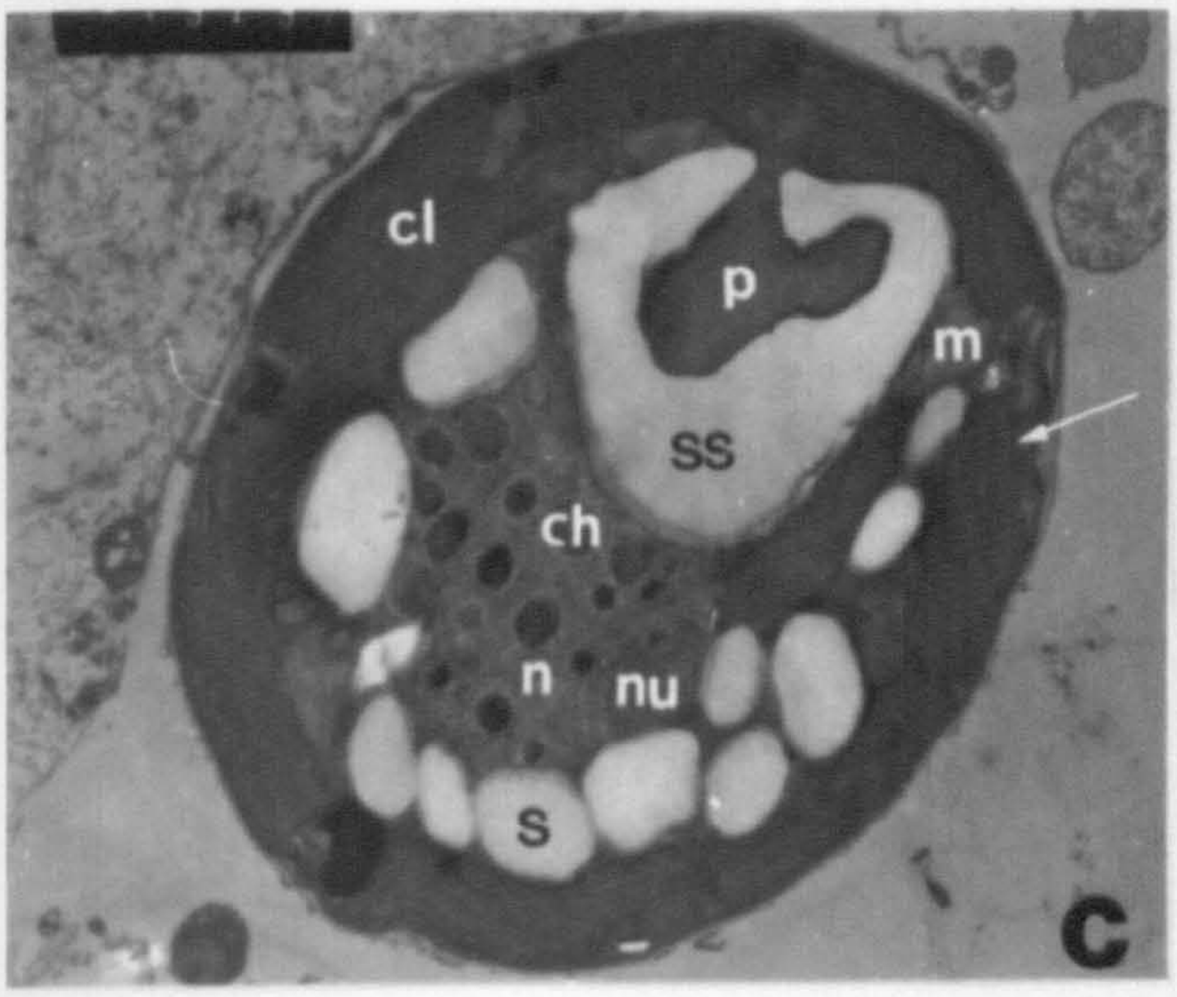
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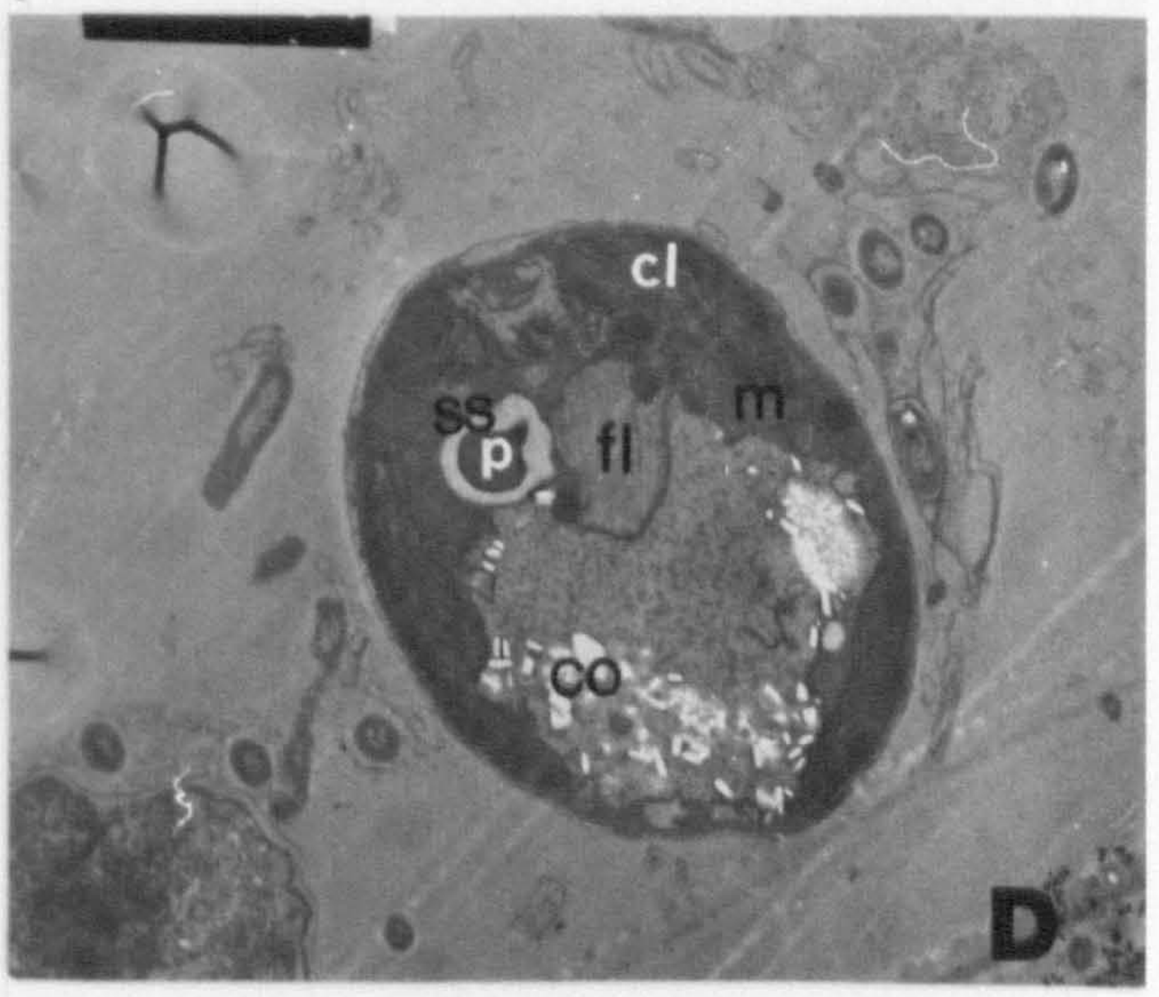
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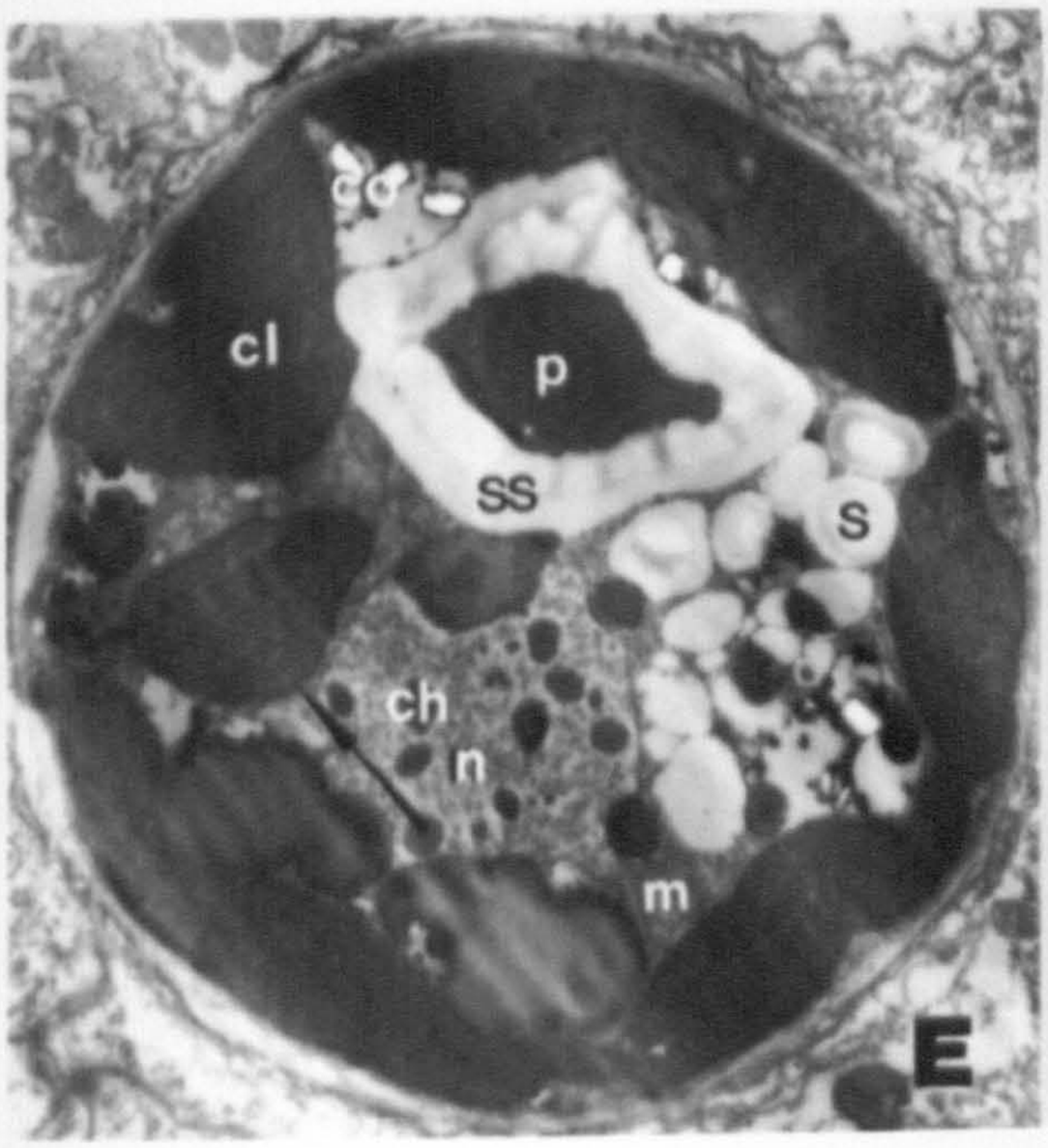
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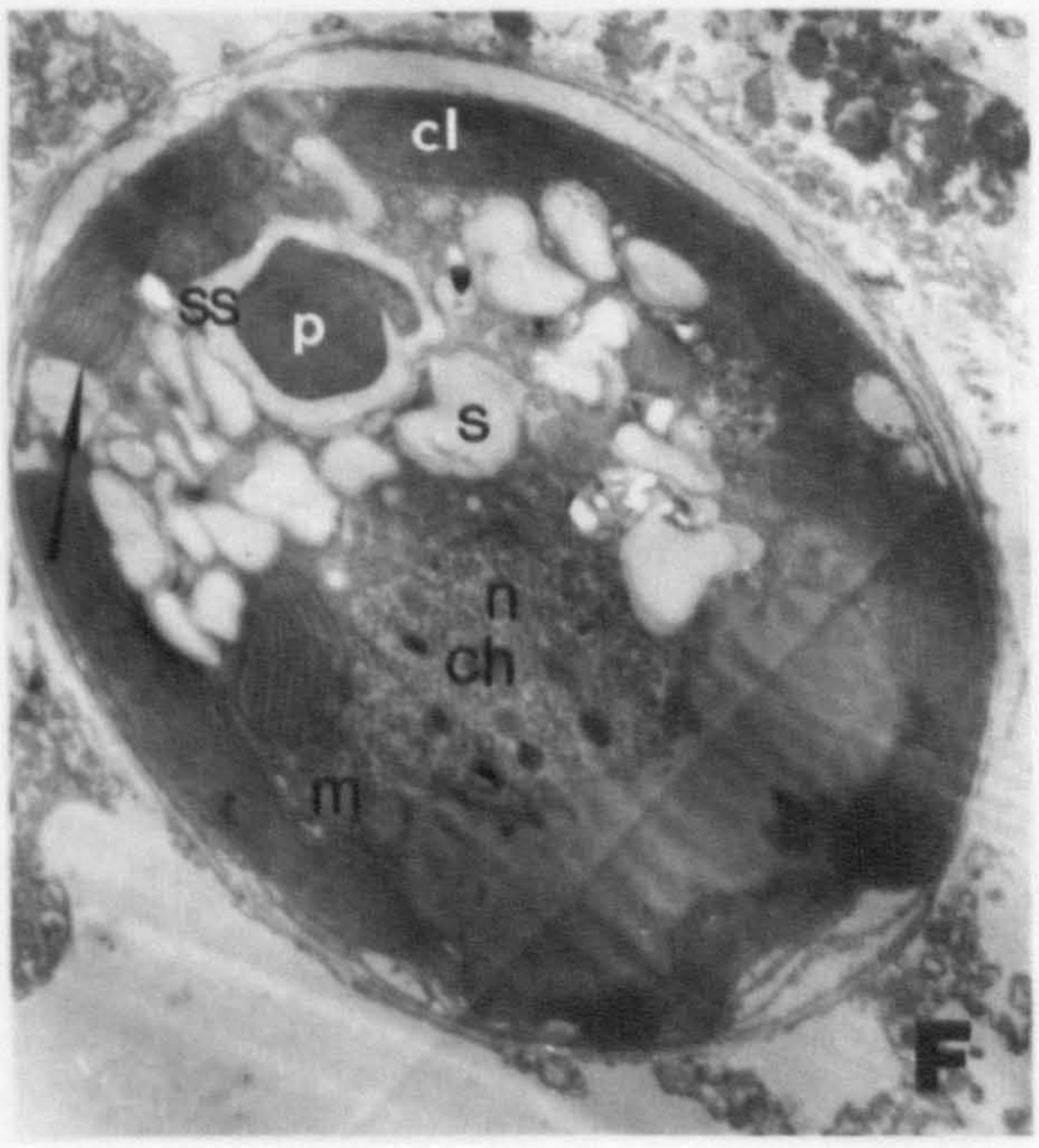
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Plate 2: Transmission electron micrographs of cultured and freshly isolated Symbiodinium sp. cells contd..

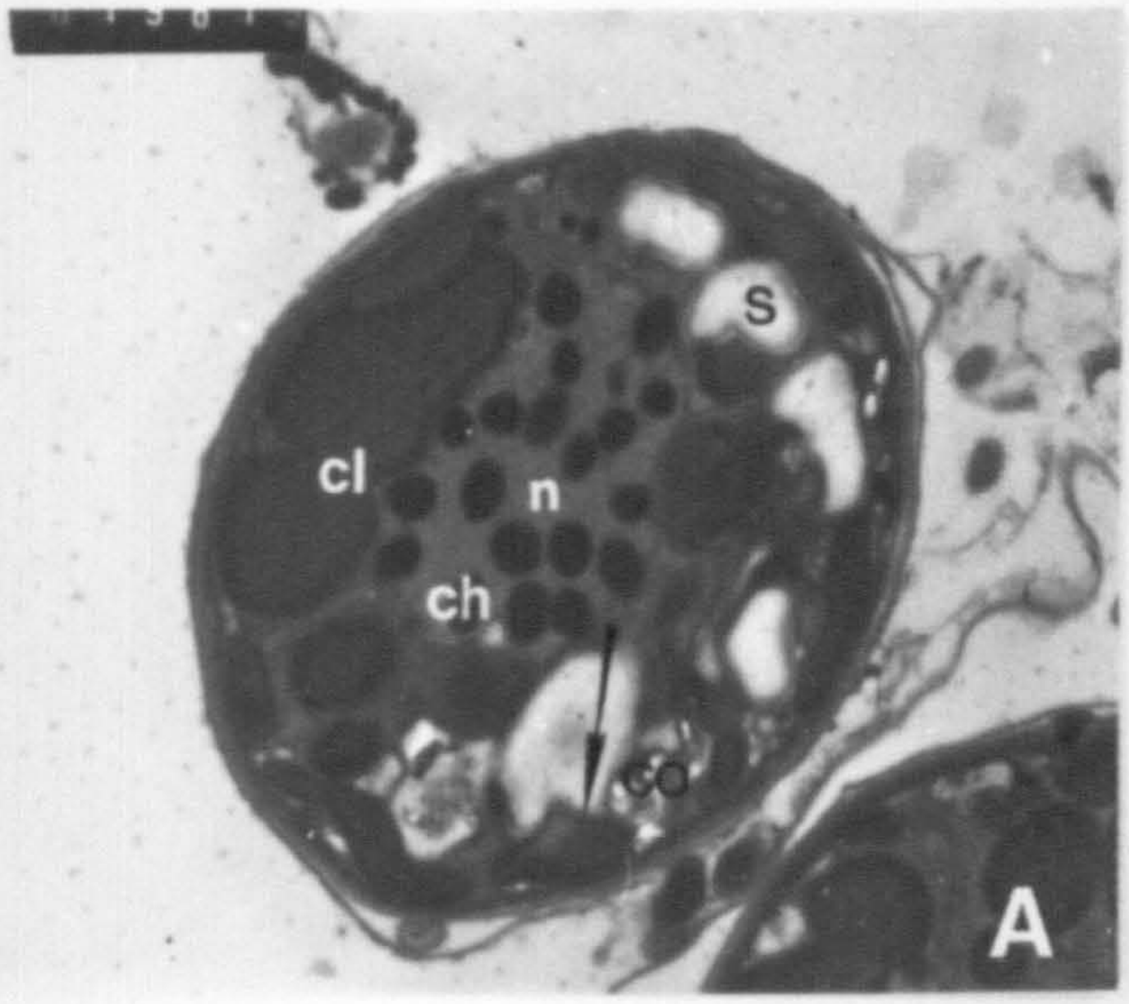
(A) Cultured *Symbiodinium* sp. from *A. viridis* (Trearddur Bay) (magnification approx. x13,000).

(B) *Symbiodinium* sp. freshly isolated from *A. viridis* (Loch Sween) (magnification approx. x13,000).

(C) *Symbiodinium* sp. freshly isolated from *A. viridis* (Bembridge) (magnification approx. x13,000).

(D) *Symbiodinium* sp. freshly isolated from *A. viridis* (Weymouth Harbour) (magnification approx. x13,000).

cl = chloroplast; m = mitochondrion; s = starch; p = stalked pyrenoid; ss = starch sheath of pyrenoid; co = calcium oxalate crystals; ch = chromosome; n = nucleus; nu = nucleolus; arrows indicate peripheral thylakoid lamellae.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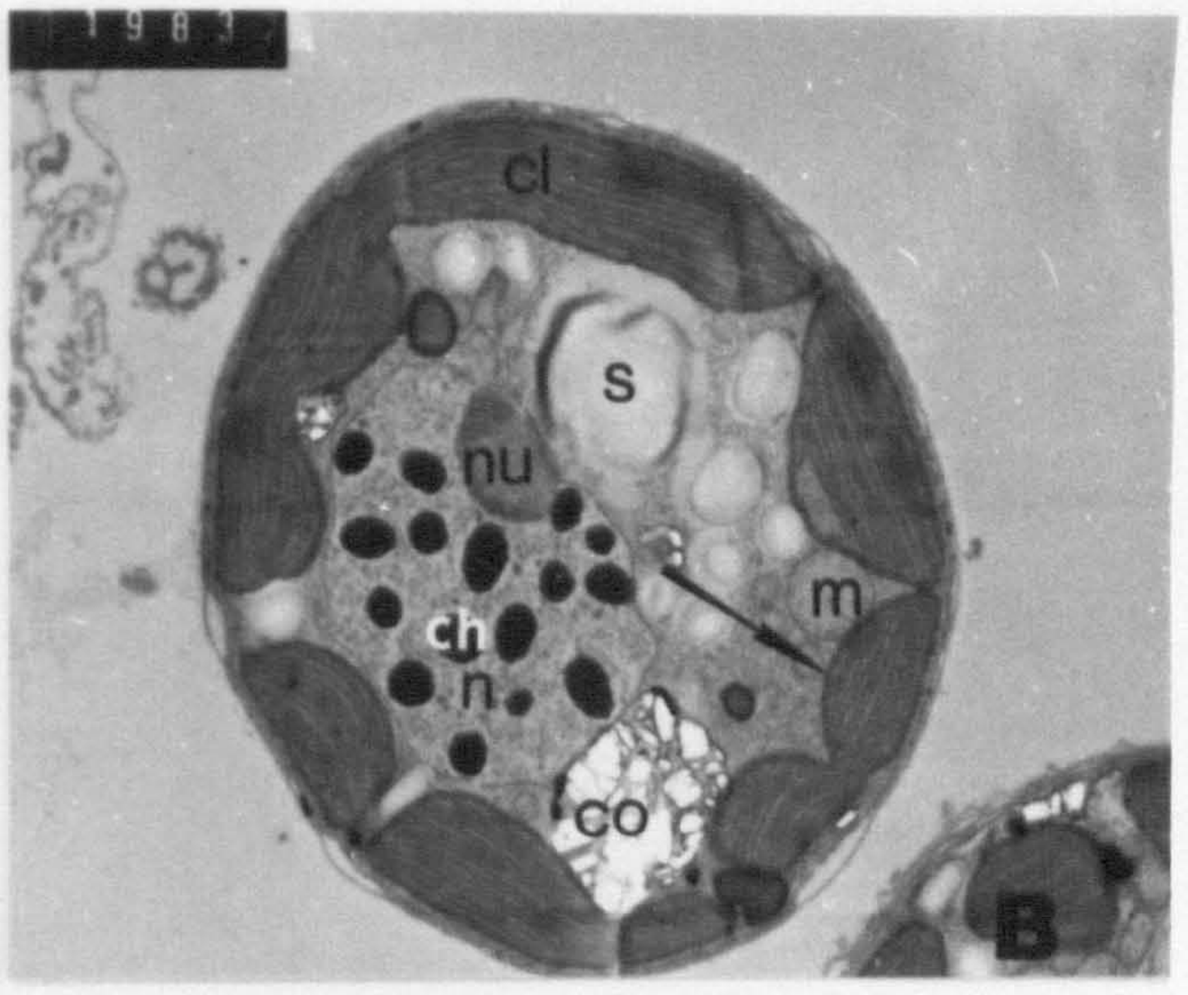
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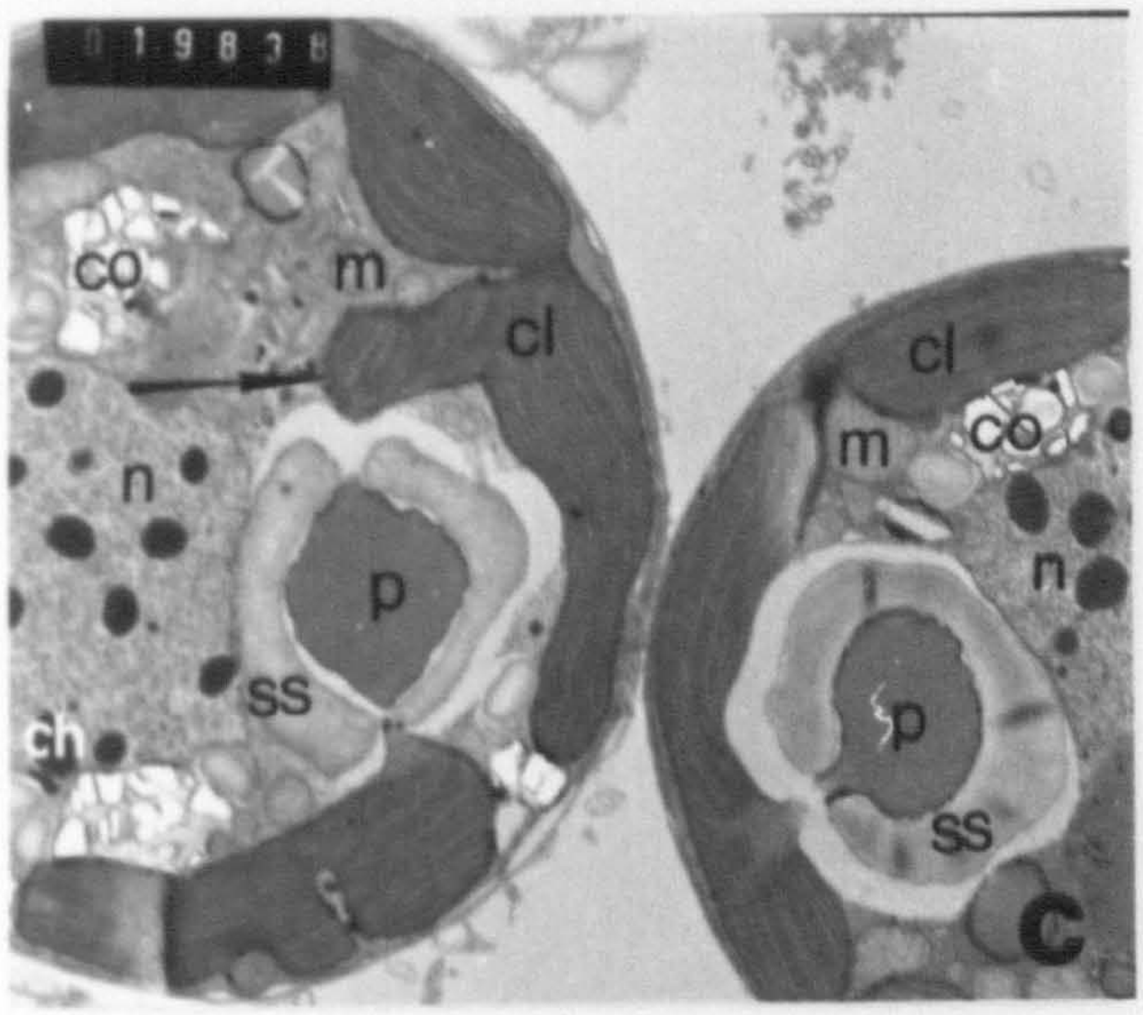
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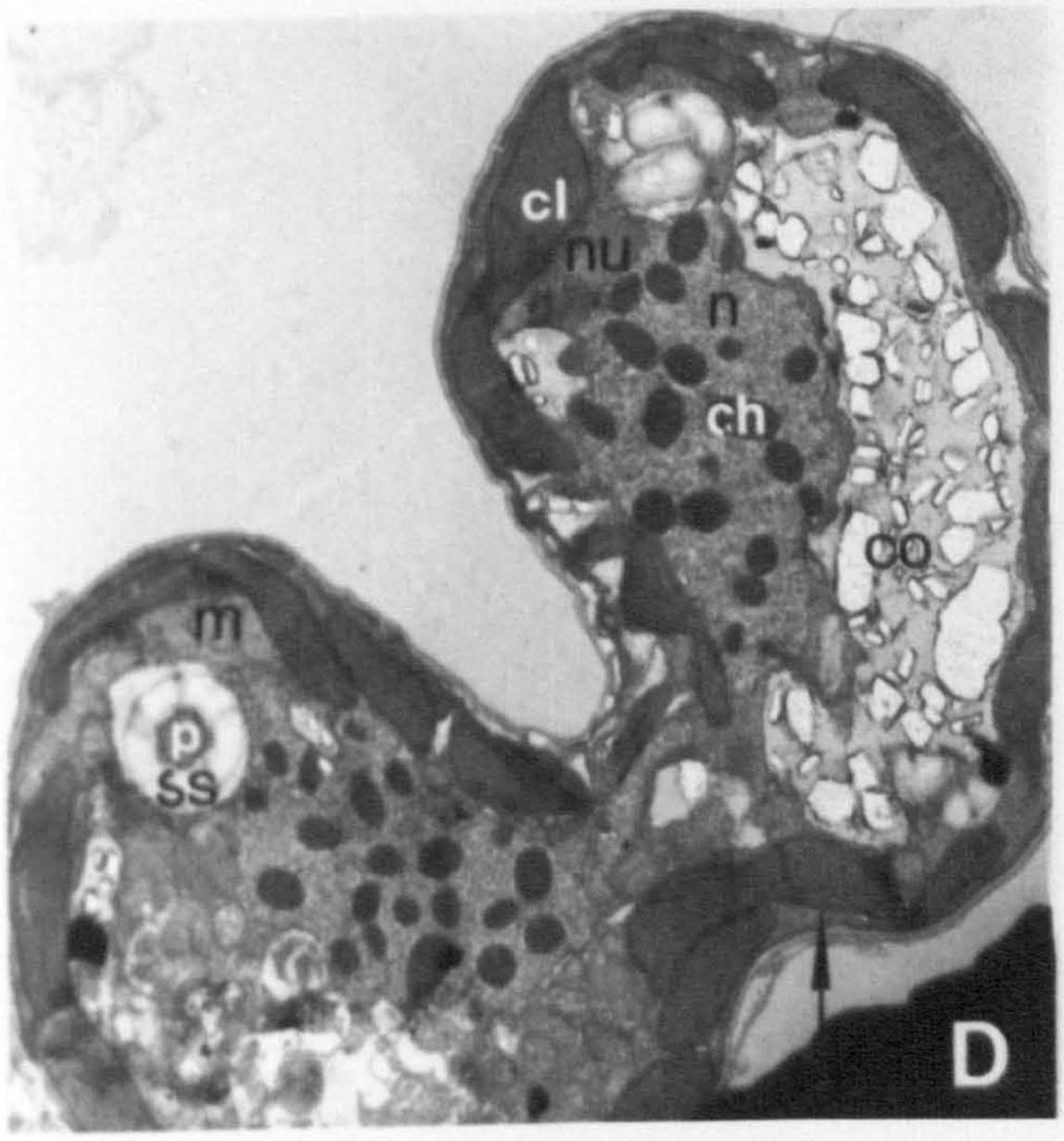
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Plate 3: Transmission electron micrographs of stalked pyrenoids in freshly isolated and cultured Symbiodinium sp. cells.

(A) Stalked pyrenoid in cultured Symbiodinium sp. from C. pedunculatus (Lough Hyne) (magnification approx. x23,400).

(B) Stalked pyrenoid in Symbiodinium sp. freshly isolated from A. ballii (magnification approx. x22,000).

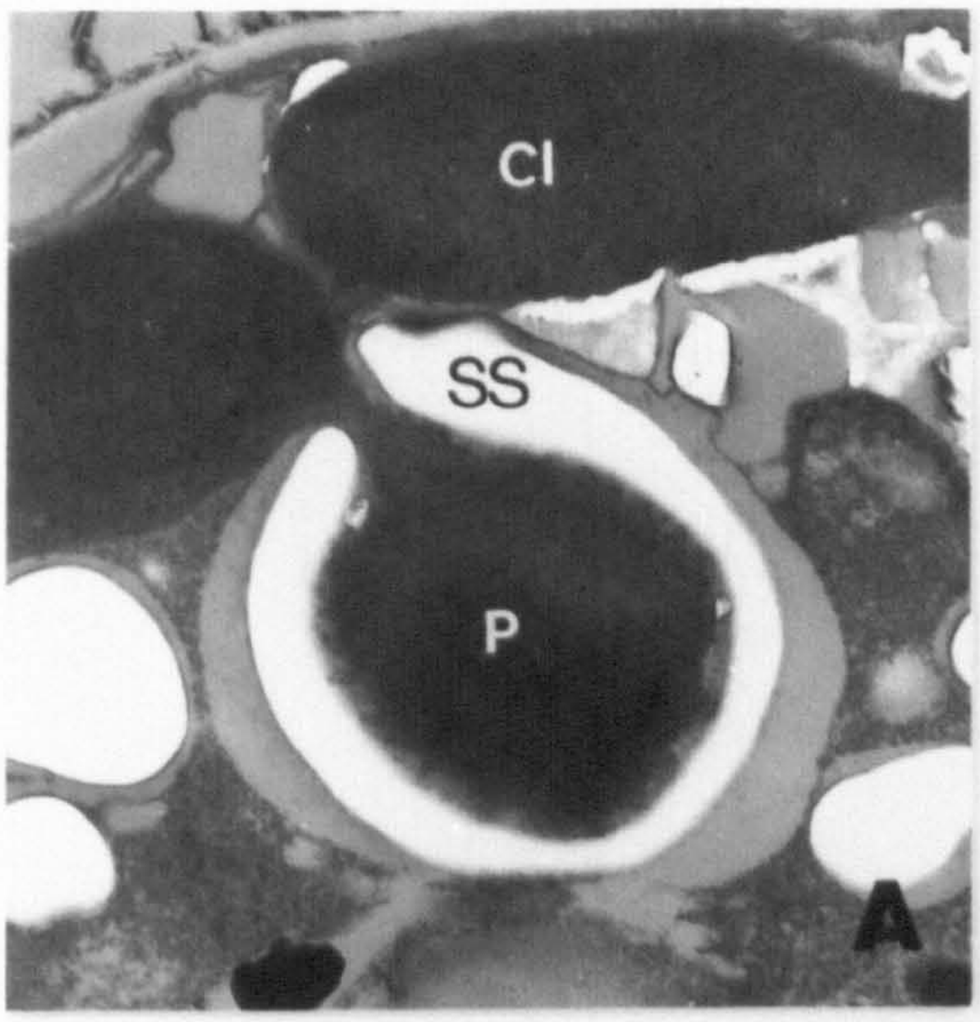
(C) Stalked pyrenoid in Symbiodinium sp. freshly isolated from I. sulcatus (magnification approx. x40,800).

(D) Stalked pyrenoid in Symbiodinium sp. freshly isolated from A. viridis (Lough Hyne) (magnification approx. x22,100).

(E) Stalked pyrenoid in cultured Symbiodinium sp. from A. viridis (Shell Island) (magnification approx. x22,000).

(F) Stalked pyrenoid in Symbiodinium sp. freshly isolated from A. viridis (Trearddur Bay) (magnification approx. x22,000).

P = stalked pyrenoid; Cl = chloroplast; SS = starch sheath of pyrenoid.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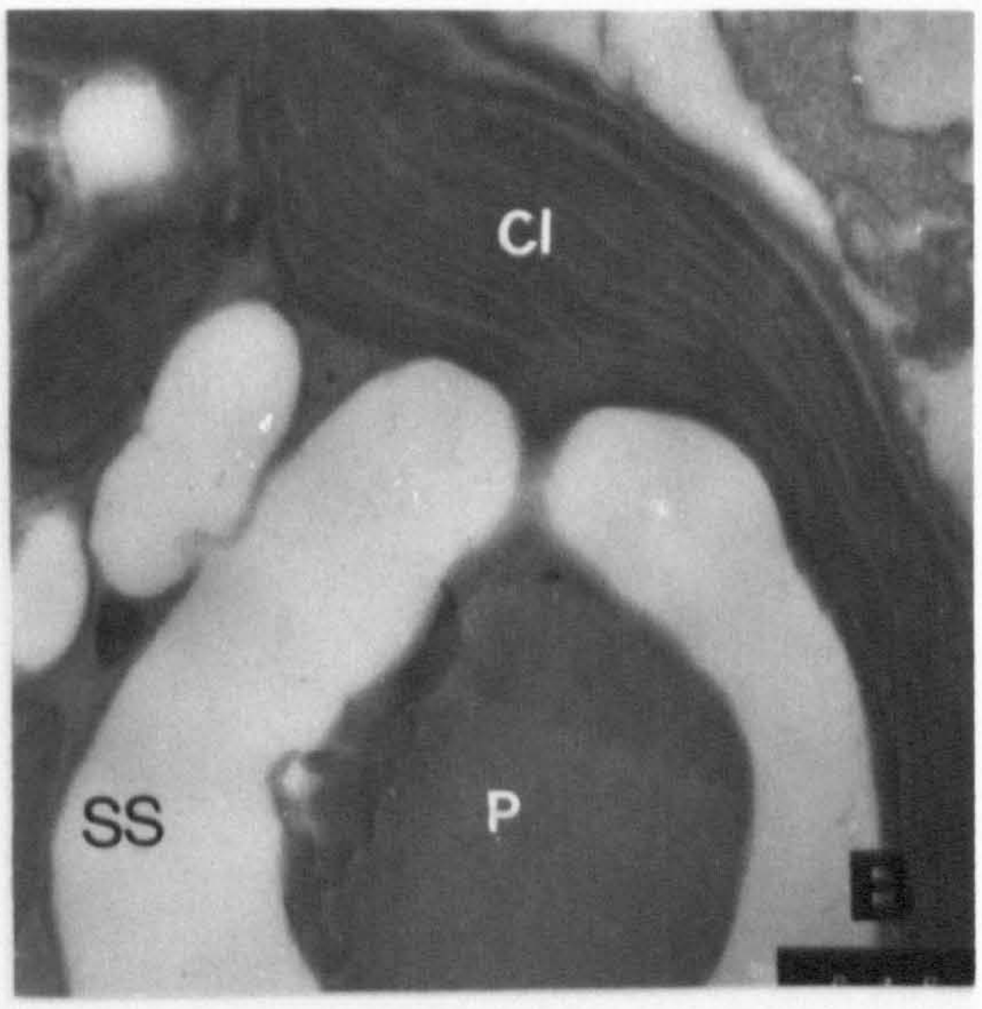
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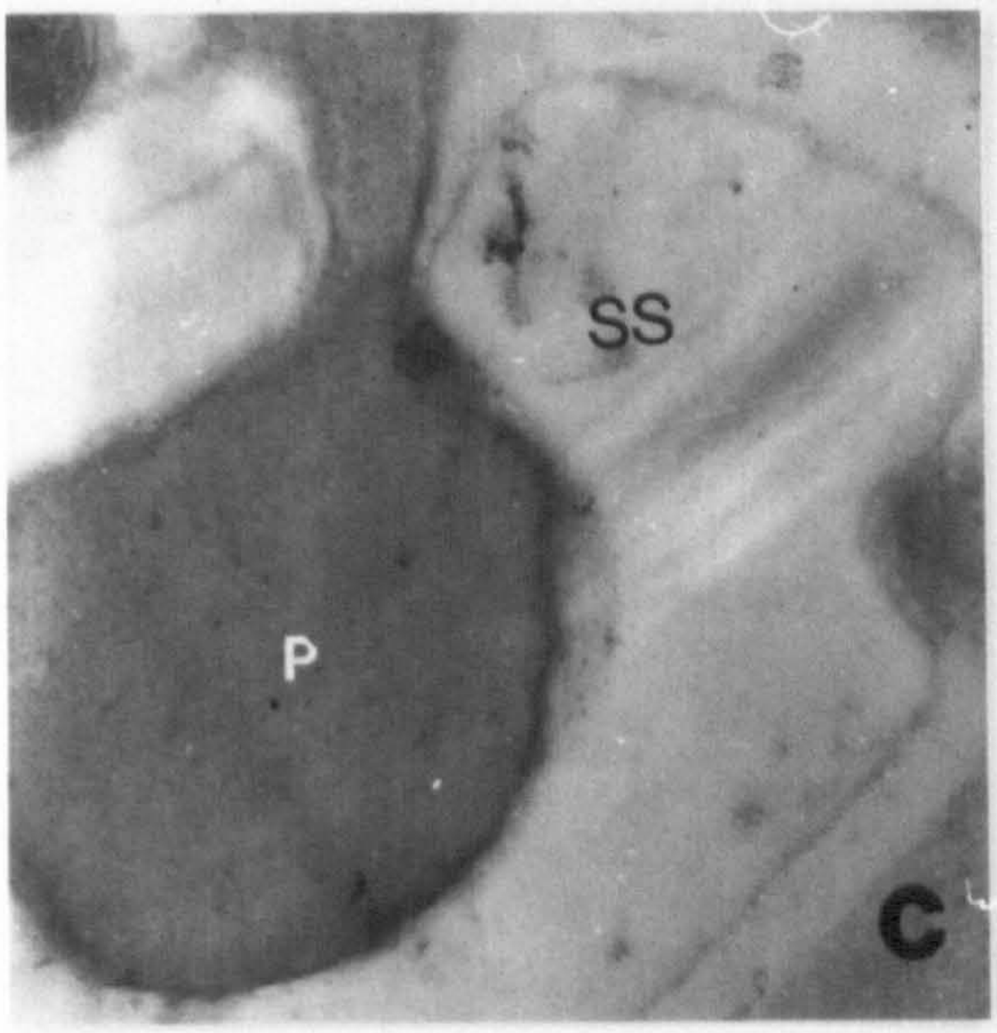
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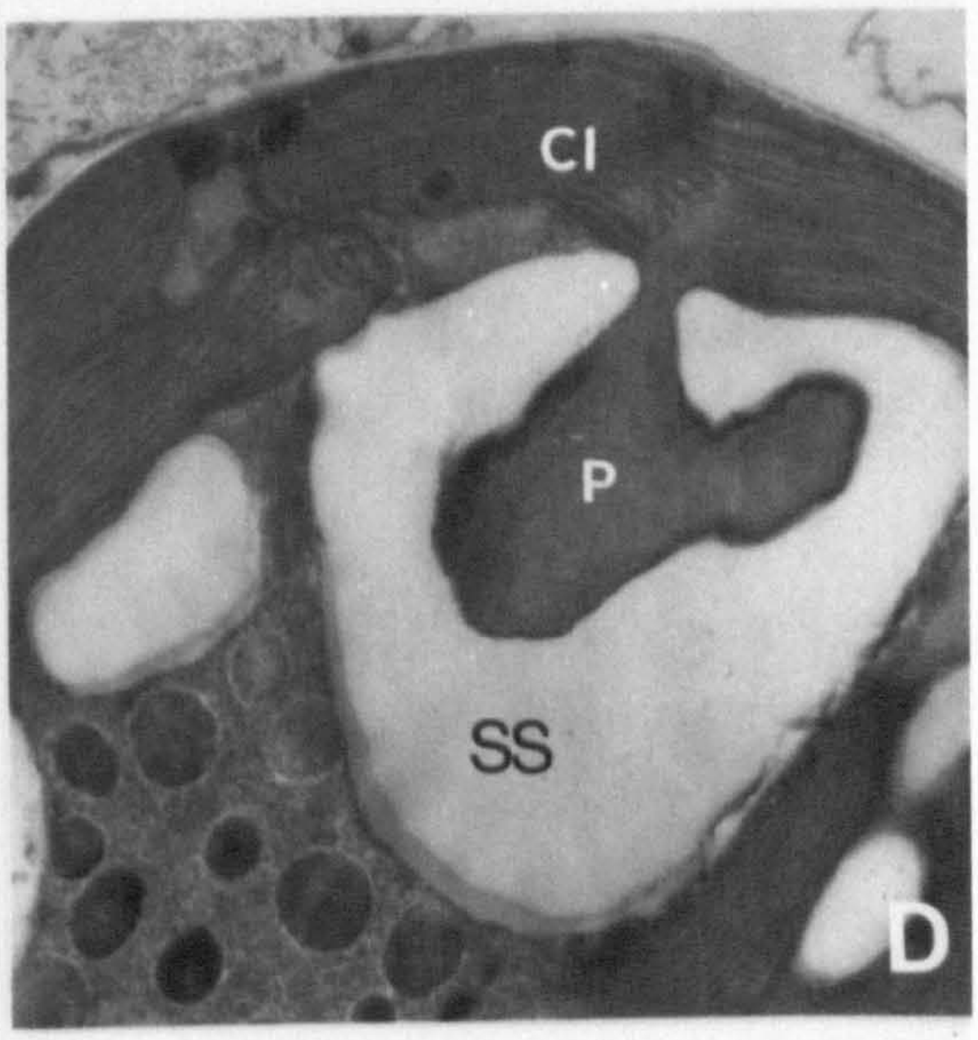
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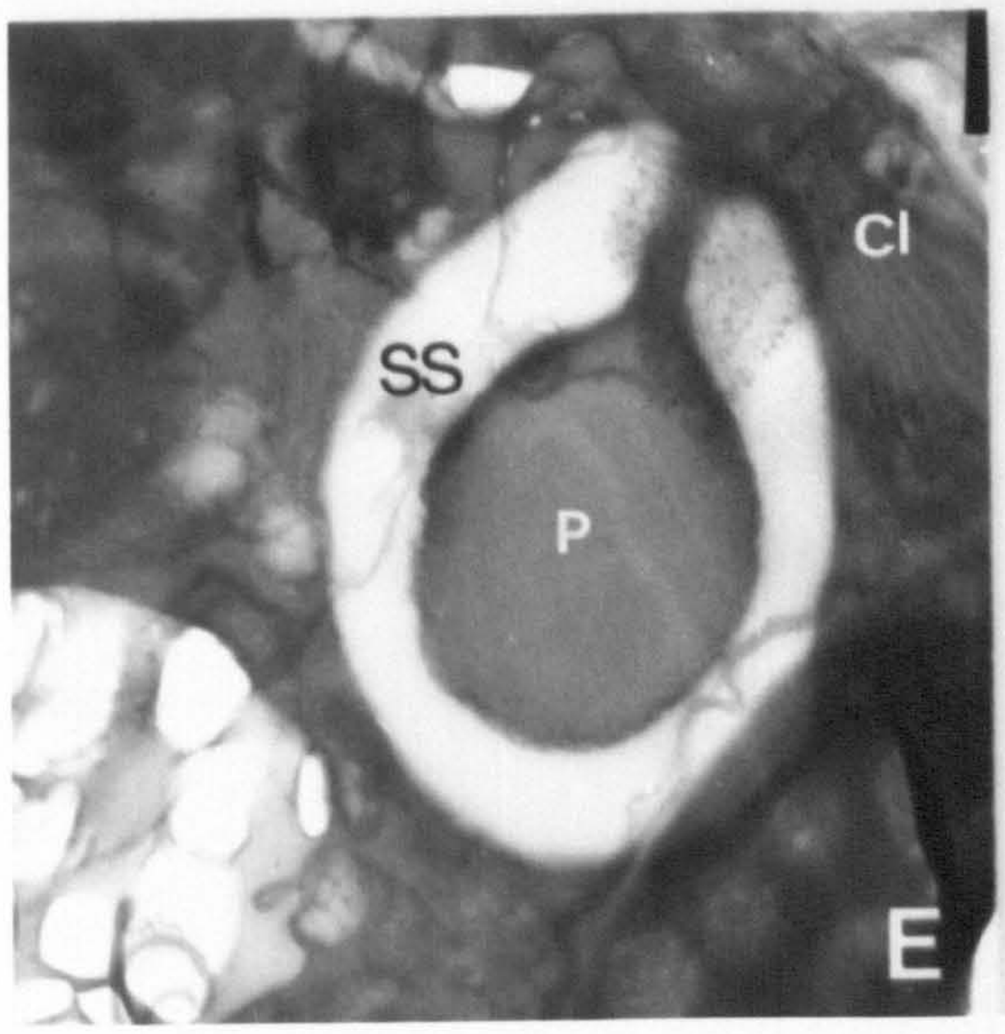

SS
Cl
P
E

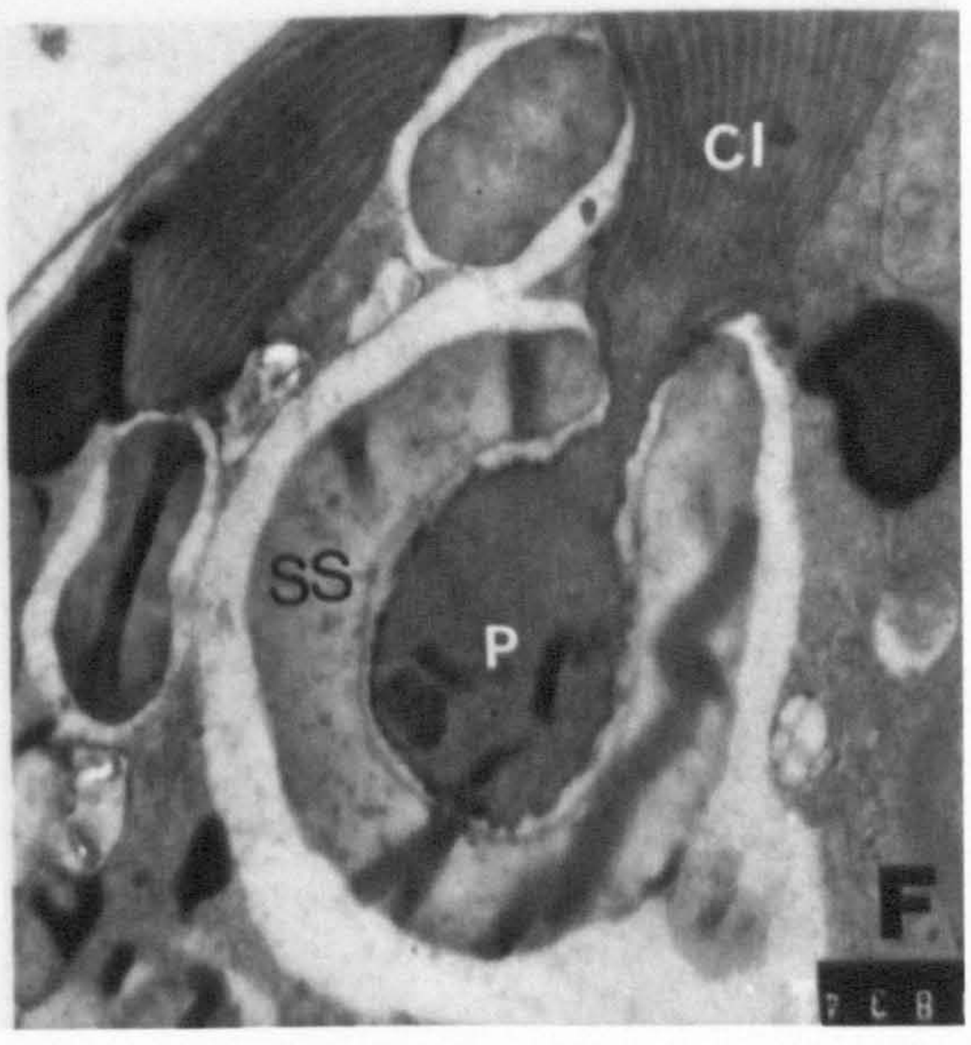

Cl
SS
P
F

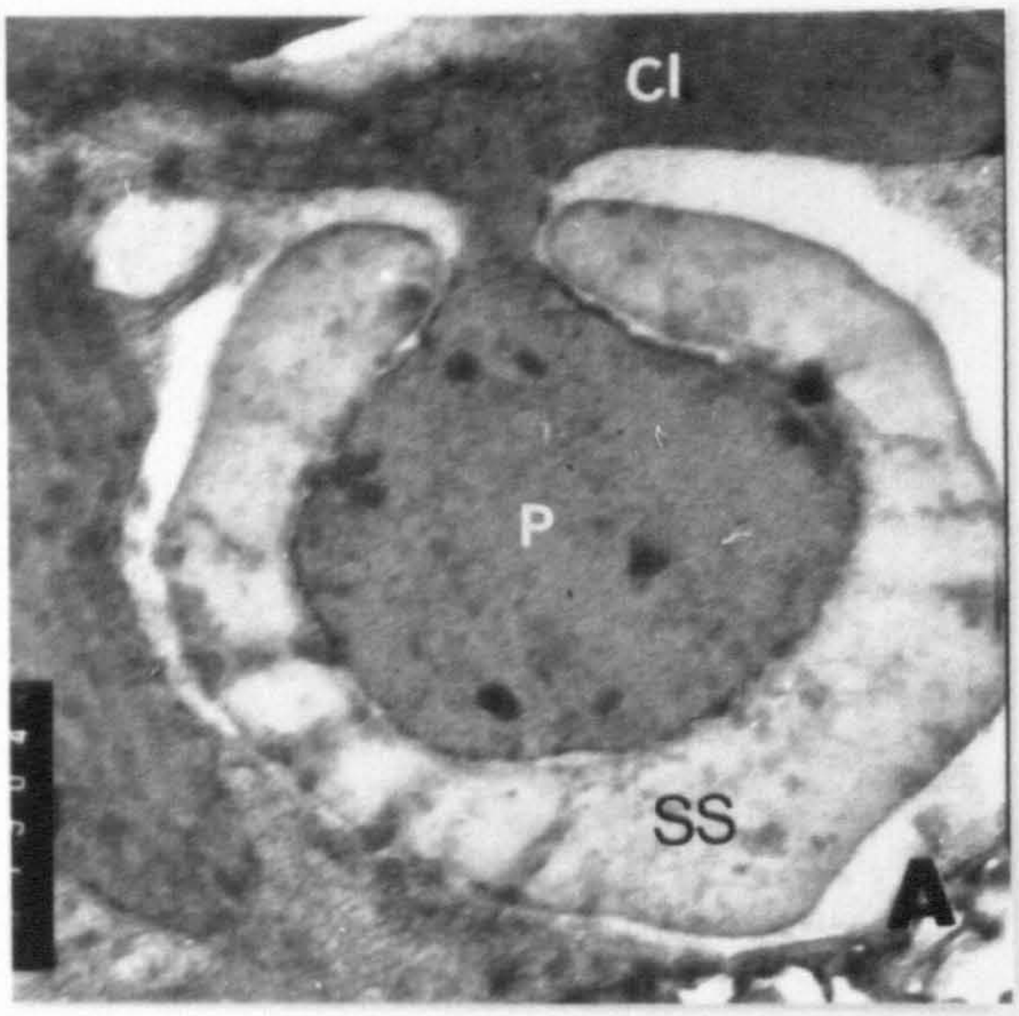
Plate 4: Transmission electron micrographs of stalked pyrenoids in freshly isolated and cultured Symbiodinium sp. cells contd..

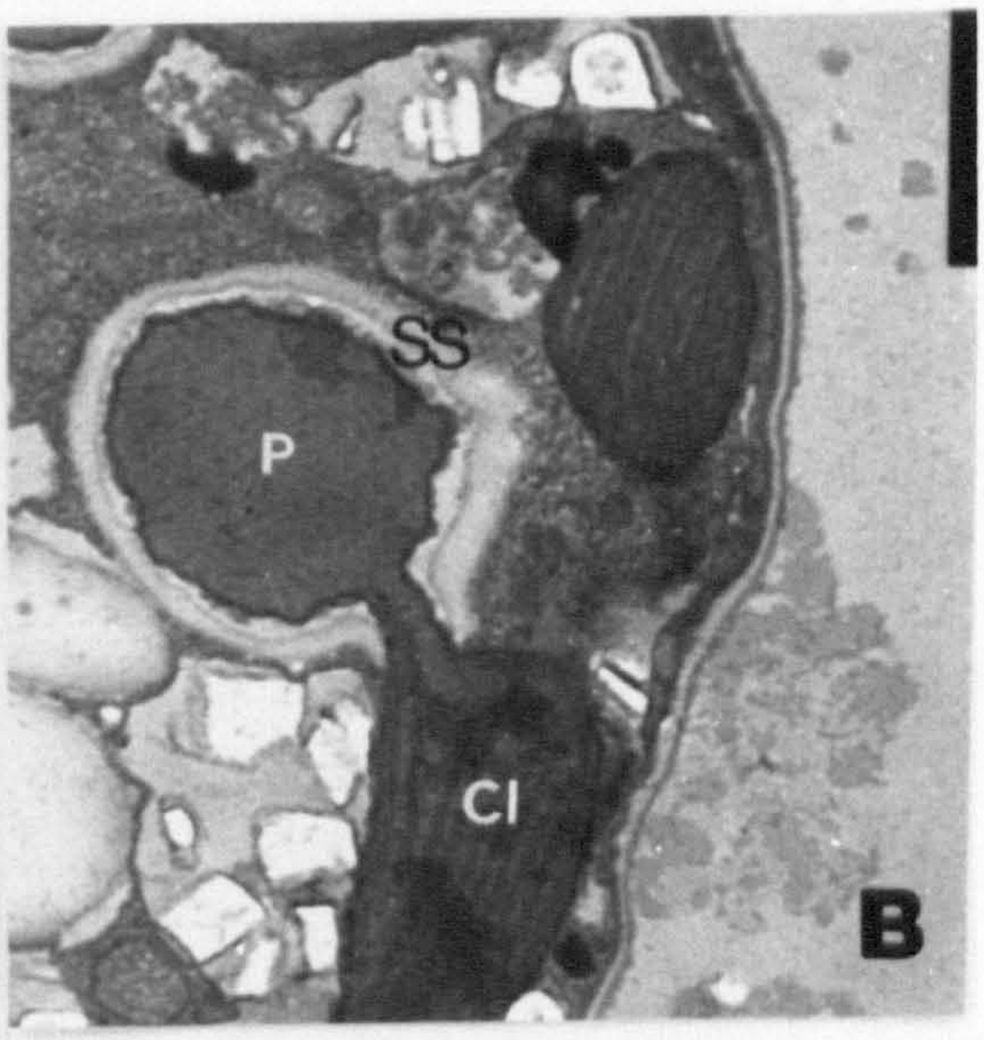
(A) Stalked pyrenoid in *Symbiodinium* sp. freshly isolated from *A. viridis* (Weymouth Harbour) (magnification approx. x36,000).

(B) Stalked pyrenoid in *Symbiodinium* sp. freshly isolated from *A. viridis* (Loch Sween) (magnification approx. x22,000).

(C) Stalked pyrenoid in *Symbiodinium* sp. freshly isolated from *A. viridis* (Bembridge) (magnification approx. x22,000).

P = stalked pyrenoid; Cl = chloroplast; SS = starch sheath of pyrenoid.


Cl
P
SS
A


SS
P
Cl
B

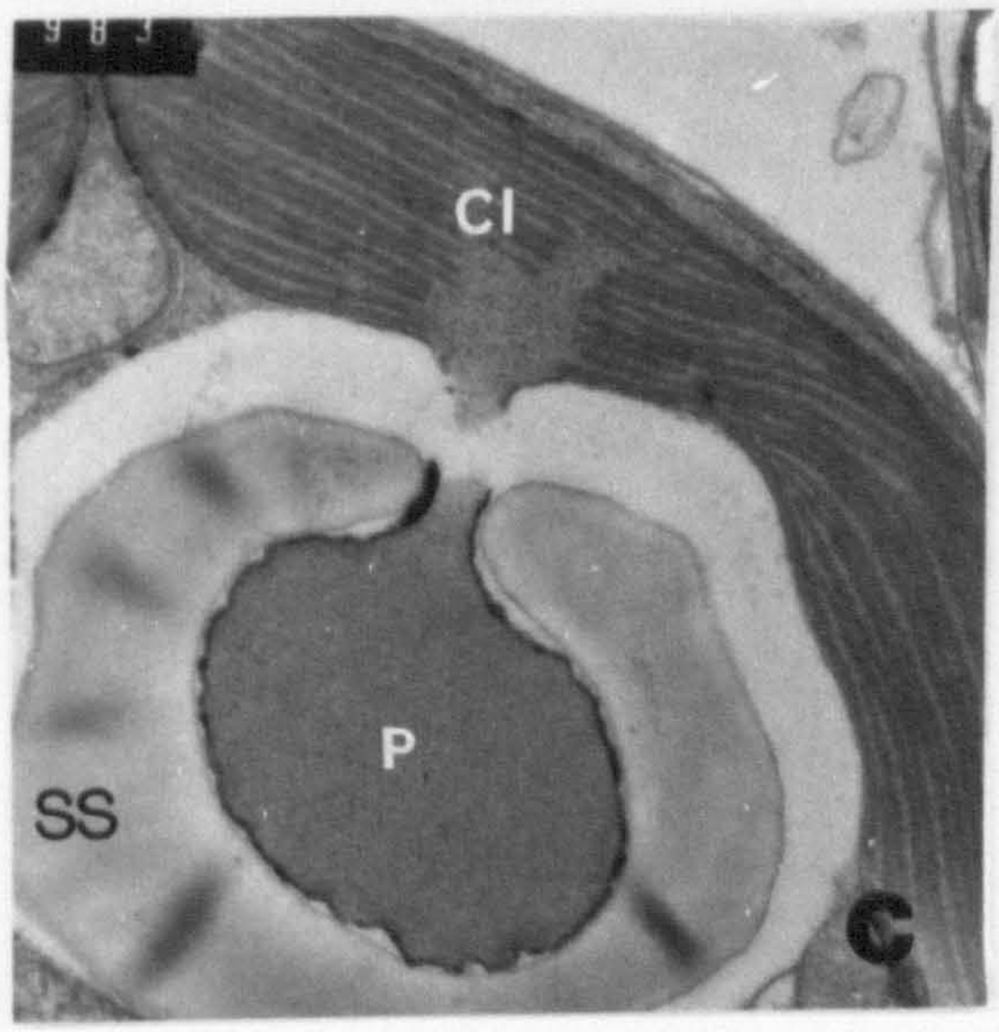

Cl
P
SS
C

Plate 5: Transmission electron micrographs showing the amphiesma of cultured and freshly isolated Symbiodinium sp. cells.

(A) Amphiesma of cultured *Symbiodinium* sp. from *C. pedunculatus* (Netley) (magnification approx. x94,500).

(B) Amphiesma of cultured *Symbiodinium* sp. from *A. ballii* (magnification approx. x165,000).

(C) Amphiesma of *Symbiodinium* sp. freshly isolated from *I. sulcatus* (magnification approx. x59,000).

(D) Amphiesma of cultured *Symbiodinium* sp. from *A. pallida* (magnification approx. x59,000).

(E) Amphiesma of cultured *Symbiodinium* sp. from *A. viridis* (Shell Is.) (magnification approx. x43,000).

(F) Amphiesma of *Symbiodinium* sp. freshly isolated from *A. viridis* (Trearddur Bay) (magnification approx. x94,500).

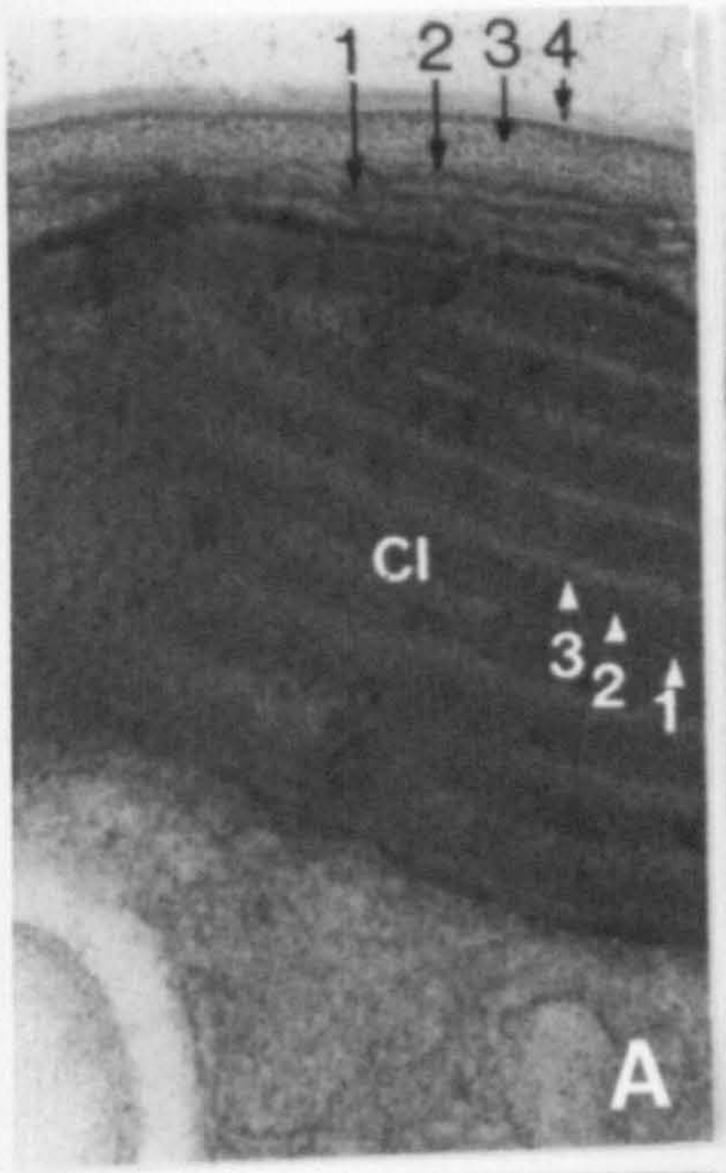
(G) Amphiesma of *Symbiodinium* sp. freshly isolated from *A. viridis* (Weymouth Harbour) (magnification approx. x59,000).

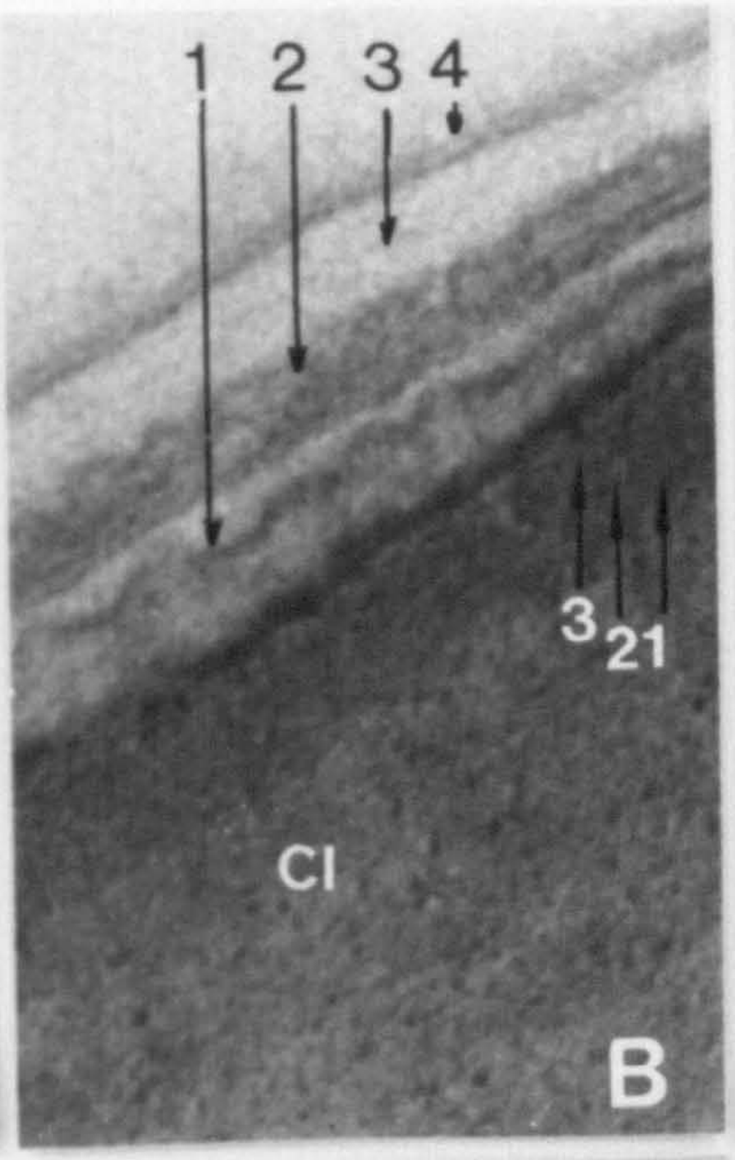
(H) Amphiesma of *Symbiodinium* sp. freshly isolated from *A. viridis* (Bembridge) (magnification approx. x36,000).

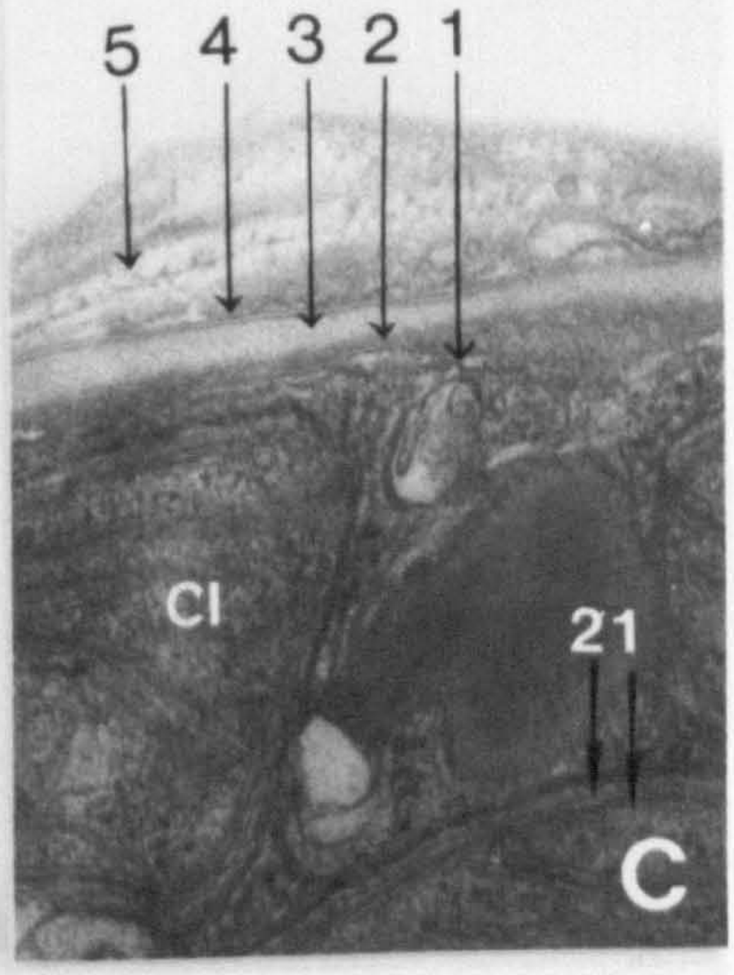
Arrows with black numbering: 1 = plasmalemma; 2 = series of wavy membranes; 3 = "pellicle"; 4 = peripheral algal membrane; 5 = residual host membranes.

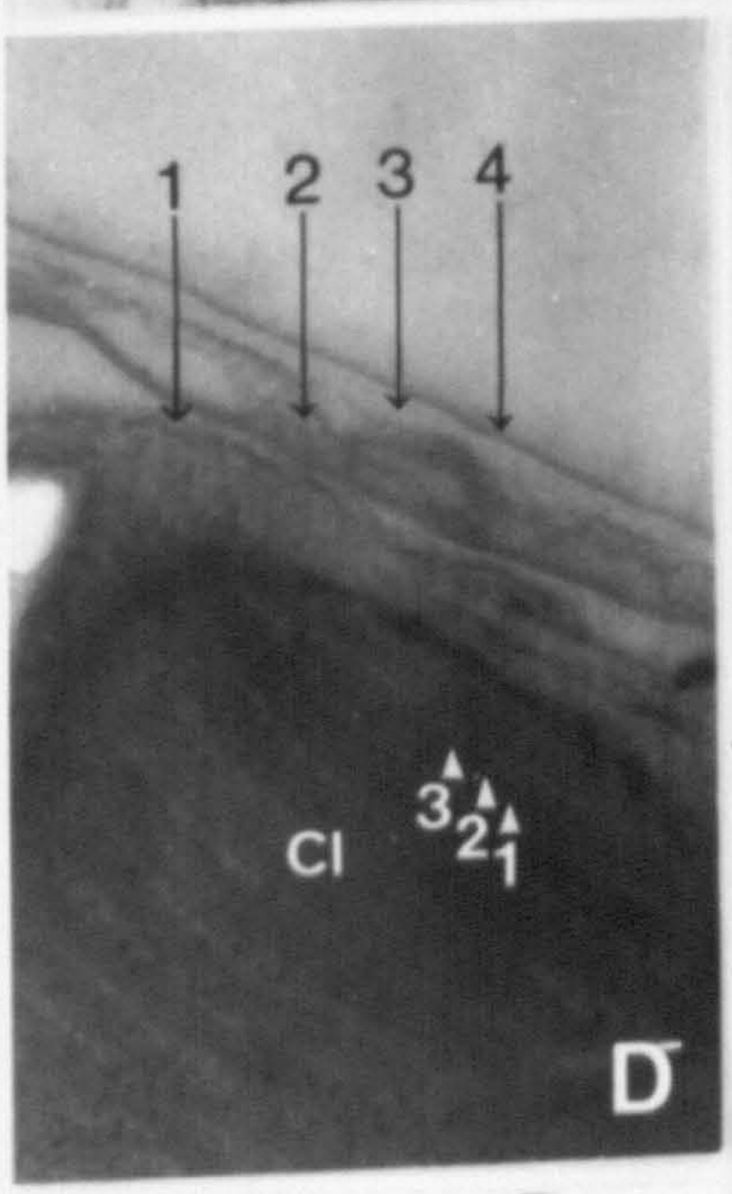
Arrows with white numbering: 1-3 indicate number and position of thylakoid lamellae in each stack.

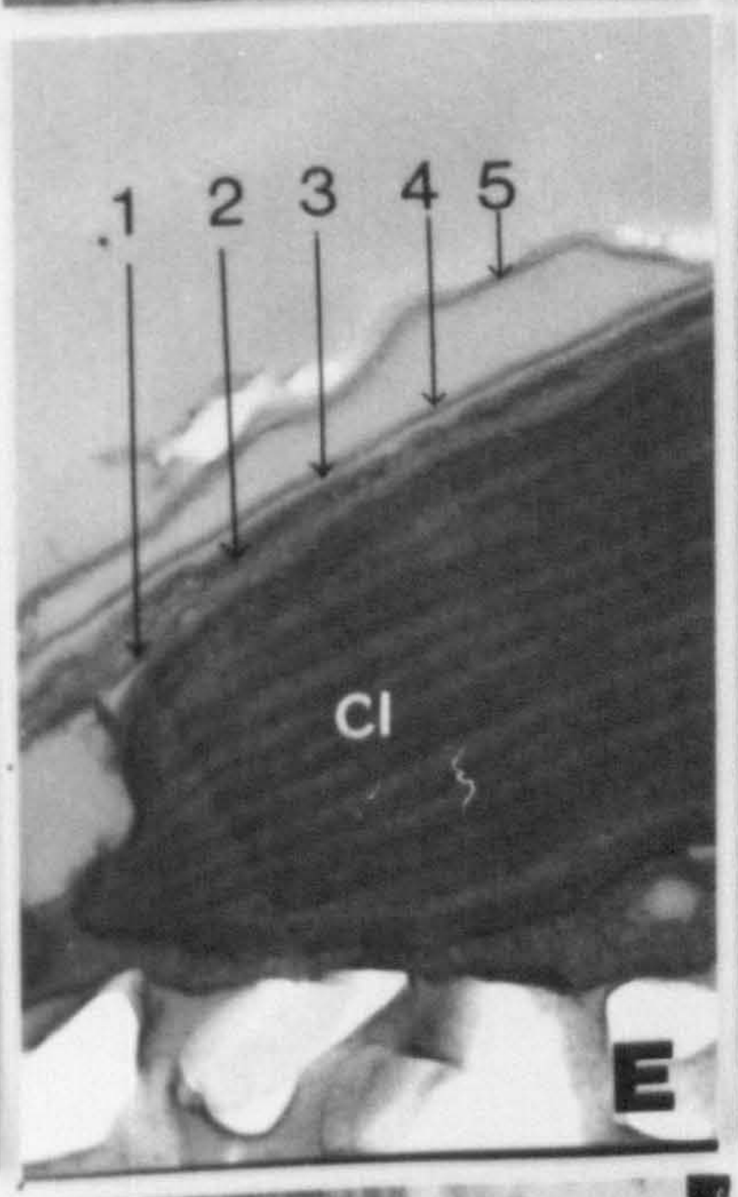
Cl = chloroplast.

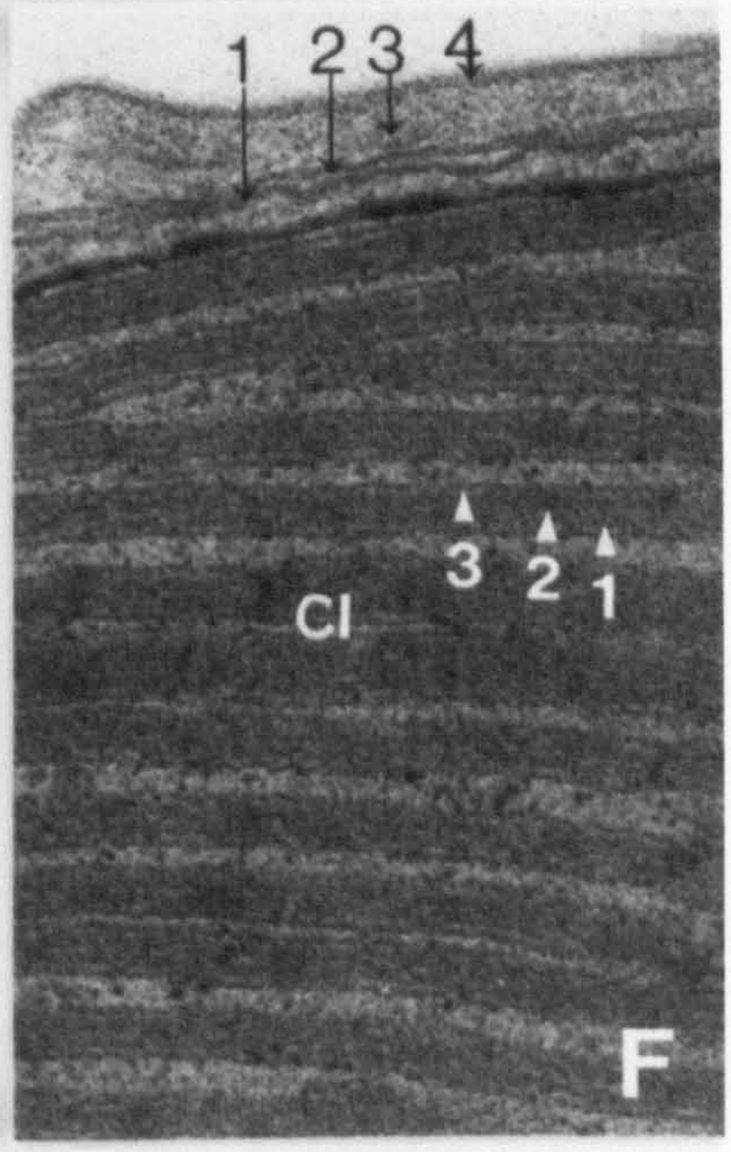

1 2 3 4
CI
3 2 1
A

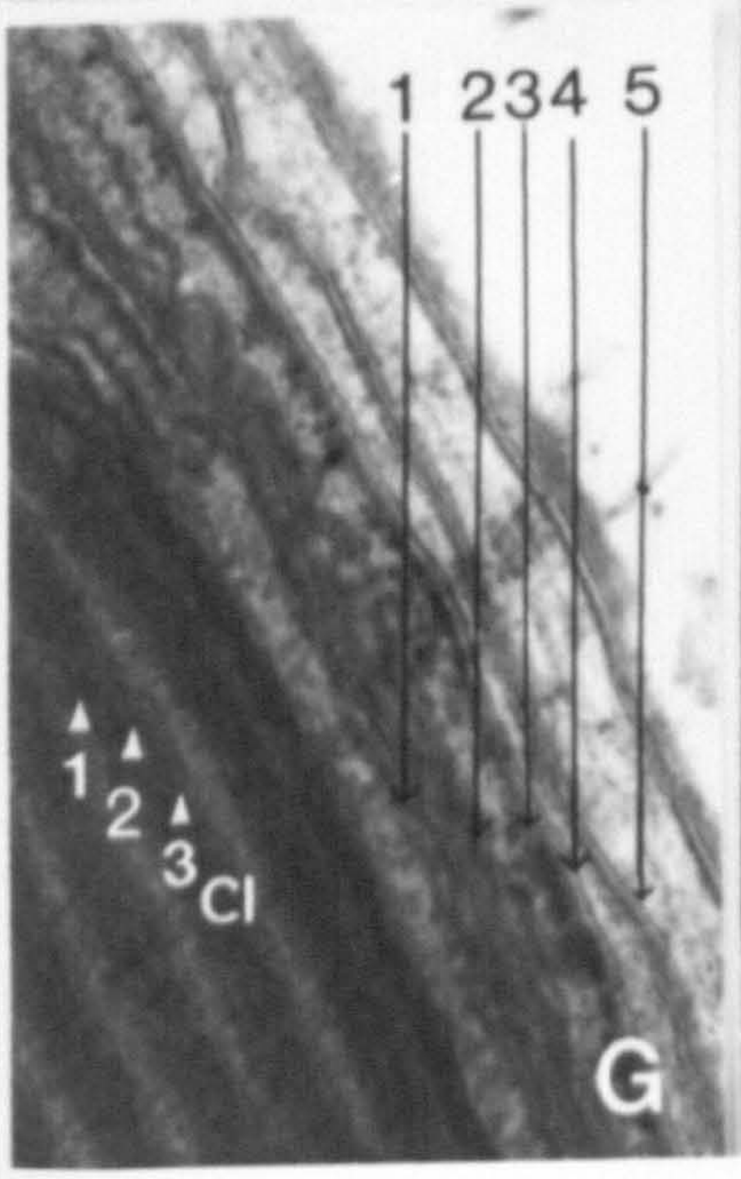

1 2 3 4
3 21
CI
B

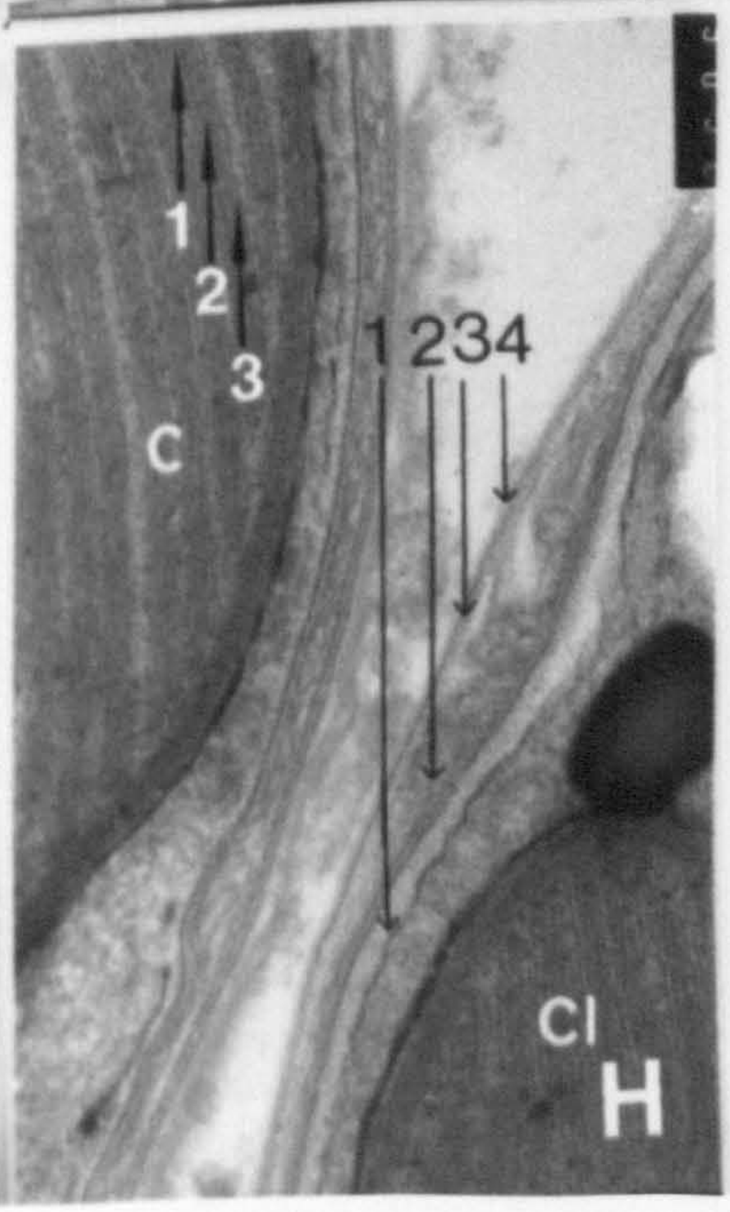

5 4 3 2 1
CI
21
C


1 2 3 4
CI
3 2 1
D


1 2 3 4 5
CI
E


1 2 3 4
3 2 1
CI
F


1 2 3 4 5
1 2 3
CI
G


1
2
3
1 2 3 4
C
CI
H

APPENDIX 5: STATISTICS RELATING TO PHOTOSYNTHETIC PIGMENT CONCENTRATIONS.

TABLE 1 RESULTS OF TUKEY'S TEST (CONFIDENCE INTERVALS) COMPARING THE CONCENTRATIONS OF CHLOROPHYLL - a IN THE SYMBIONTS OF DIFFERENT HOST SPECIES (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK) (N=3).

Host sp.	AvSI	Cp	AvLH	Is	Ab
AvSI	-	-6.6/ 17.2	-5.5/ 18.3	**-23.8/ -0.0 ***	**2.23/ 26.0 ***
Cp	-	-	-13.0/ 10.8	-18.5/ 5.3	-20.7/ 3.1
AvLH	-	-	-	-17.4/ 6.4	-4.2/ 19.6
Is	-	-	-	-	-9.7/ 14.1
Ab	-	-	-	-	-

TABLE 2 RESULTS OF TUKEY'S TEST (CONFIDENCE INTERVALS) COMPARING THE CONCENTRATIONS OF PERIDININ IN SYMBIONTS OF DIFFERENT HOST SPECIES (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK) (N=3)

Host sp.	AvSI	Ab	Cp	Is	AvLH
AvSI	-	-1.7/ 5.3	-0.5/ 6.6	**0.3/ 7.3 ***	**-10.3/ -3.3 ***
Ab	-	-	-2.3/ 4.7	-5.5/ 1.5	**-8.5/ -1.5 ***
Cp	-	-	-	-4.3/ 2.8	**-7.3/ -0.3 ***
Is	-	-	-	-	-6.5/ 0.5
AvLH	-	-	-	-	-

TABLE 3 RESULTS OF TUKEY'S TEST (CONFIDENCE INTERVALS) COMPARING THE CONCENTRATION OF DIADINOXANTHIN IN THE SYMBIONTS OF DIFFERENT HOST SPECIES (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK) (N=3).

Host sp.	AvSI	Ab	Cp	Is	AvLH
AvSI	-	-0.3/ 2.9	-0.3/ 0.3	**0.6/ 3.8 ***	**-4.2/ -1.0 ***
Ab	-	-	-1.6/ 1.6	-2.5/ 0.7	-2.9/ 0.2
Cp	-	-	-	-2.5/ 0.7	-2.9/ 0.3
Is	-	-	-	-	-2.0/ 1.1
AvLH	-	-	-	-	-

APPENDIX 6: STATISTICS RELATING TO THE MITOTIC INDEX OF THE SYMBIONTS.

TABLE 1 COMPARISON OF THE 2 REPLICATE SERIES OF DIEL DIVISION CYCLES USING STUDENT'S T-TEST (N=7) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH AN ASTERISK).

HOST SPECIES	COMPARISON OF REPLICATE DIEL DIVISION CYCLES OF FRESHLY ISOLATED SYMBIONTS	COMPARISON OF REPLICATE DIEL DIVISION CYCLES OF CULTURED SYMBIONTS
Cp	T=1.20, P=0.26	T=0.70, P=0.50
CpNt	T=1.89, P=0.09	T=1.40, P=0.19
Ab	T=0.00, P>0.99	T=1.40, P=0.19
Is	T=0.43, P=0.68	T=0.59, P=0.57
Ap	T=0.98, P=0.35	T=0.53, P=0.61
AvLH	T=0.98, P=0.35	-
AvSI	T=0.82, P=0.43	T=1.46, P=0.18
AvBm	T=1.54, P=0.15	T=0.11, P=0.91
AvLS	T=0.74, P=0.47	T=1.43, P=0.18
AvTB	T=0.00, P>0.99	T=0.75, P=0.47
AvWH	T=0.75, P=0.47	T=1.17, P=0.27

TABLE 2 COMPARISONS OF THE MITOTIC INDEX (MI) OF SYMBIONTS FROM THE SAME HOST SPECIES AND LOCATION BETWEEN WHEN IN SITU AND WHEN IN CULTURE, USING STUDENT'S T-TEST (N=14) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD AND WITH AN ASTERISK).

Host species	Values of significance test
Cp	**T=14.90, P<0.0001 ***
CpNt	**T=21.97, P<0.0001 ***
Ab	**T=16.98, P<0.0001 ***
Is	**T=19.17, P<0.0001 ***
Ap	**T=10.08, P<0.0001 ***
AvLH	-
AvSI	**T=15.08, P<0.0001 ***
AvBm	**T=21.90, P<0.0001 ***
AvLS	**T=26.15, P<0.0001 ***
AvTB	**T=20.31, P<0.0001 ***
AvWH	**T=21.06, P<0.0001 ***

TABLE 3 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) INVESTIGATING THE MITOTIC INDEX (MI) OF THE SYMBIONTS OF DIFFERENT HOST SPECIES FROM DIFFERENT LOCATIONS WHEN IN CULTURE (N=14) (CRITICAL VALUE OF Z = 2.81)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host Sp.	Ap	Is	AvSI	Ab	Cp
Ap	-	**3.72 ***	**4.46 ***	**4.58 ***	**5.46 ***
Is	-	-	0.74	0.86	1.74
AvSI	-	-	-	0.12	1.00
Ab	-	-	-	-	0.88
Cp	-	-	-	-	-

TABLE 4 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) INVESTIGATING THE MITOTIC INDEX (MI) OF THE SYMBIONTS OF DIFFERENT HOST SPECIES WHEN IN SITU (N=14) (CRITICAL VALUE OF Z = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH AN ASTERISK).

Host Sp.	Ap	Is	AvSI	Ab	AvLH	Cp
Ap	-	1.76	2.04	**3.11 ***	**3.63 ***	**5.34 ***
Is	-	-	0.28	1.36	1.88	**3.58 ***
AvSI	-	-	-	1.07	1.59	**3.30 ***
Ab	-	-	-	-	0.52	2.22
AvLH	-	-	-	-	-	1.70
Cp	-	-	-	-	-	-

TABLE 5 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) INVESTIGATING THE MITOTIC INDEX (MI) OF SYMBIONTS FROM A. VIRIDIS FROM DIFFERENT LOCATIONS WHEN IN CULTURE (N=14) (CRITICAL Z VALUE = 2.81, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host species	AvWH	AvSI	AvBm	AvLS	AvTB
AvWH	-	0.61	1.16	2.58	2.74
AvSI	-	-	0.55	1.96	2.13
AvBm	-	-	-	1.42	1.59
AvLS	-	-	-	-	0.17
AvTB	-	-	-	-	-

APPENDIX 7: INVESTIGATION OF THE RELATIONSHIP BETWEEN TENTACLE AND ORAL DISC AREA, AND PROTEIN CONTENT.

INVESTIGATION OF THE RELATIONSHIP BETWEEN TENTACLE AND ORAL DISC AREA AND PROTEIN CONTENT

Introduction

In an attempt to facilitate the measurement of *in situ* symbiont population densities, without the use of protein determination, it was proposed to relate symbiont numbers to tentacle or oral disc area. Therefore, the aim of this work was to investigate whether the relationship between these areas and the protein content of the tentacle or oral disc was linear and how the relationship changed with an increase in tissue area.

Materials and Methods

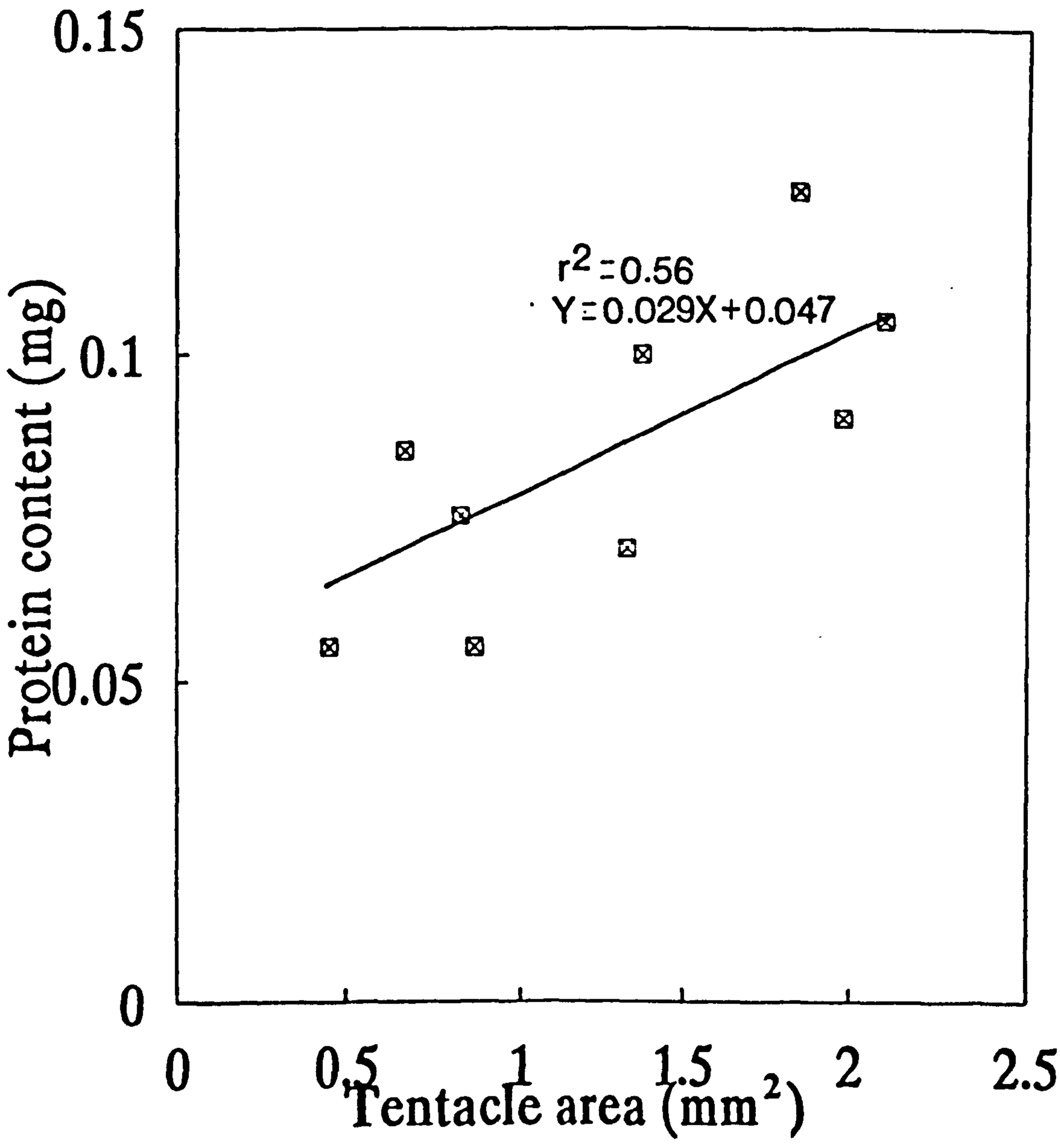
A total of 9 tentacles were excised from 3 predominantly symbiont-free *A. ballii* and 9 aposymbiotic *C. pedunculatus* were collected. The *C. pedunculatus* were settled in a petri dish, which was half-filled with filtered sea water (FSW), until fully expanded. A 7.5 % solution of magnesium chloride ($MgCl_2.6H_2O$) was then added to the petri dish until full and the animals relaxed until they did not respond to a seeker. Tentacles of *A. ballii* were relaxed on a slide in drops of the $MgCl_2.6H_2O$ solution for 10 minutes. The length of each tentacle and the width at 25, 50 and 75 % along the length of the tentacle were measured with an ocular micrometer. The area of the tentacle was the length x the average width. The diameter of the oral disc of each *C. pedunculatus* was

measured with the ocular micrometer and the area calculated by assuming the oral disc to be circular. Keeping note of each tentacle and polyp, their protein contents were determined using the Lowry method as described in chapter 5, section 5.2 (c) (pp.236-240). The tentacles and polyps were homogenized in 0.5 ml and 1.0 ml distilled water respectively. Protein content was plotted against area and the linearity of the relationship determined using correlation.

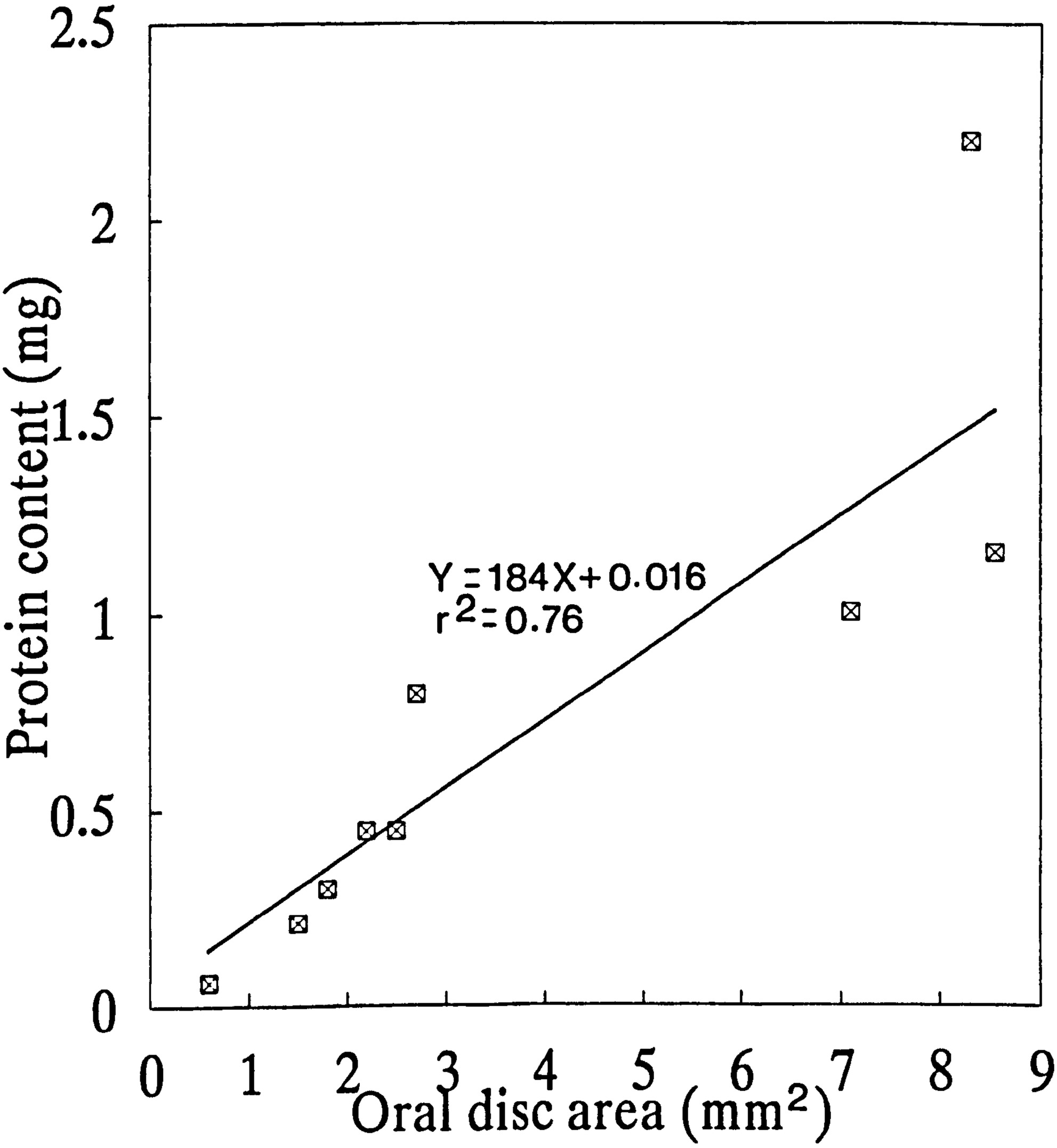
Results

The plots of protein content vs. tissue area are shown in graphs 1 (p.492) and 2 (p.493). It is evident from both plots that protein content and area are linearly related. The equations of this linearity are given on graphs 1 and 2. However, whilst oral disc diameter was accompanied by a proportional increase in animal biomass, increases in tentacle area were not. For example, a 2-fold increase in tentacle area resulted in a less than 2-fold increase in the protein content.

Graph 1: Aposymbiotic A. ballii tentacle area vs. protein content



Graph 2: Oral disc area of aposymbiotic C. pedunculatus vs. protein content



Discussion

Cnidarian dimensions have been used previously to express symbiont population densities, as symbionts per mm tentacle (Schoenberg and Trench 1980c), per cm medusa bell diameter (Muscatine et al. 1986) or per cm^2 coral surface area (Schonwald et al. 1987; Muscatine et al. 1989a). Alternatively, polyp sizes have been standardized using oral disc diameters prior to experimental use (Trench 1981; Trench et al. 1981a; Colley and Trench 1983; Fitt and Trench 1983b; Cook et al. 1988). The plots shown here suggest that oral disc diameter is a satisfactory indicator of anthozoan biomass. However, tentacle area appears to be less reliable. If such a dimension is to be used then it should be ensured that all the tentacles are of similar sizes to limit error.

APPENDIX 8: STATISTICS, VALUES AND GRAPHS RELATING TO CHAPTER 4.

TABLE 1 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DENSITIES (SYMBIONTS/MM ORAL DISC^2) OF 'HOMOLOGOUS' SYMBIONTS ENDOCYTOSED WHEN DIFFERENT DENSITIES OF ALGAL CELLS WERE AVAILABLE TO APOSYMBIOTIC C.PEDUNCULATUS (N=5) (CRITICAL Z VALUE=2.635)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Available density of symbionts (cells/ ml)	10^5	10^6	10^7	10^8
10^5	-	0.05	2.51	1.60
10^6	-	-	2.46	1.55
10^7	-	-	-	0.91
10^8	-	-	-	-

TABLE 2 THE DENSITIES OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 4 HOURS, 2 DAYS AND 4 DAYS FOLLOWING ENDOCYTOSIS (N=5) (+/- S.E.).

Cp + symbionts from named host sp.	Symbiont density after 4 hours (cells/mm oral disc^2).	Symbiont density after 2 days (cells/mm oral disc^2).	Symbiont density after 4 days (cells/mm oral disc^2).
Cp	5227 +/- 500	4077 +/- 347	2512 +/- 351
Ab	2053 +/- 396	1399 +/- 385	1179 +/- 162
AvLH	3907 +/- 859	1484 +/- 488	1450 +/- 310
AvSI	5499 +/- 1287	1056 +/- 258	1605 +/- 307
Is	6326 +/- 990	879 +/- 90	1484 +/- 460
Ap	3435 +/- 421	2023 +/- 472	1162 +/- 361
Control	97 +/- 48	68 +/- 44	106 +/- 36

TABLE 3 RESULTS OF TUKEY TESTS (CONFIDENCE LIMITS) COMPARING THE DENSITIES (SYMBIONTS/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS PRESENT IN REINFECTED C. PEDUNCULATUS 4 HOURS AFTER INOCULATION (N=5) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Cp + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-376.5/ 6722.5	-2229.5 / 4869.5	-3822.5 / 3276.5	-4649.5 / 2449.5	- 1758.5 / 5340.5
Ab	-	-	-5402.5 / 1696.5	-6995.5 / 103.5	-7822.5 / -723.6 *	- 4931.5 / 2167.5
AvLH	-	-	-	-5142.5 / 1956.5	-5969.5 / 1129.5	- 3078.5 4020.5 /
AvSI	-	-	-	-	-4376.5 / 2722.5	- 1485.5 / 5613.5
Is	-	-	-	-	-	-658.5 / 6440.5
Ap	-	-	-	-	-	-

TABLE 4 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DENSITIES (SYMBIONTS/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 2 DAYS AFTER INOCULATION (N=5) (CRITICAL Z VALUE = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	2.48	2.59	3.07 *	3.39 *	1.51
Ab	-	-	0.11	0.59	0.92	0.97
AvLH	-	-	-	0.49	0.81	1.08
AvSI	-	-	-	-	0.32	1.56
Is	-	-	-	-	-	1.89
Ap	-	-	-	-	-	-

TABLE 5 THE BIOMASS OF THE SYMBIONT POPULATION IN REINFECTED C. PEDUNCULATUS 4 HOURS, 2 DAYS AND 4 DAYS FOLLOWING ENDOCYTOSIS (N=5) (+/- S.E.).

Cp + symbionts from named host sp.	Symbiont biomass after 4 hours (pg carbon/mm oral disc^2).	Symbiont biomass after 2 days (pg carbon/mm oral disc^2).	Symbiont biomass after 4 days (pg carbon/mm oral disc^2).
Cp	270470 +/- 25859	210971 +/- 17975	130007 +/- 18167
Ab	195449 +/- 37745	133139 +/- 36609	112195 +/- 15469
AvLH	204662 +/- 44985	77742 +/- 25586	75981 +/- 16226
AvSI	456542 +/- 106877	87666 +/- 21408	133244 +/- 25457
Is	643364 +/- 100656	89415 +/- 9122	150959 +/- 46743
Ap	141332 +/- 17326	83247 +/- 19436	47807 +/- 14855
Control	5041 +/- 2459	3512 +/- 2269	5471 +/- 1878

TABLE 6 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 4 HOURS AFTER INOCULATION (N=5) (CRITICAL Z VALUE = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.04	0.90	0.61	1.55	1.83
Ab	-	-	0.14	1.65	2.59	0.79
AvLH	-	-	-	1.51	2.44	0.93
AvSI	-	-	-	-	0.93	2.44
Is	-	-	-	-	-	**3.82 ***
Ap	-	-	-	-	-	-

TABLE 7 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 2 DAYS AFTER INOCULATION (N=5) (CRITICAL Z VALUE = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbi-onts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.36	**3.16 ***	2.66	2.19	2.80
Ab	-	-	1.80	1.29	0.83	1.44
AvLH	-	-	-	0.50	0.97	0.36
AvSI	-	-	-	-	0.47	0.14
Is	-	-	-	-	-	0.61
Ap	-	-	-	-	-	-

TABLE 8 THE DENSITIES OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 2, 4, 6 AND 8 WEEKS, AND 9 MONTHS FOLLOWING ENDOCYTOSIS. (+/- S.E.) (N=5, EXCEPT N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. BALLII AND I. SULCATUS AFTER 9 MONTHS).

Cp + symbionts from named host sp.	Density after 2 weeks (cells/ mm oral disc²)	Density after 4 weeks (cells/ mm oral disc²)	Density after 6 weeks (cells/ mm oral disc²)	Density after 8 weeks (cells/ mm oral disc²)	Density after 9 months (cells/ mm oral disc²)
Cp	10984 +/- 2870	106744 +/- 28145	109104 +/- 8432	135579 +/- 24708	45810 +/- 6398
Ab	1987 +/- 649	22429 +/- 18495	47351 +/- 16337	47646 +/- 14969	50410 +/- 5999
AvLH	969 +/- 68	64442 +/- 11856	41589 +/- 15381	57742 +/- 7674	61394 +/- 8989
AvSI	262 +/- 147	5487 +/- 2667	11623 +/- 4695	25638 +/- 2203	67726 +/- 8154
Is	2360 +/- 313	44174 +/- 6828	38523 +/- 12597	30520 +/- 6057	58657 +/- 10000
Ap	1147 +/- 287	15184 +/- 2254	28017 +/- 8249	63488 +/- 15579	77317 +/- 10445
Control	0	0	0	0	0

TABLE 9 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DENSITIES (CELLS/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS 2 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.98	**3.05 ***	**4.45 ***	1.26	2.73
Ab	-	-	1.08	2.48	0.72	0.75
AvLH	-	-	-	1.40	1.80	0.32
AvSI	-	-	-	-	**3.20 ***	1.72
Is	-	-	-	-	-	1.47
Ap	-	-	-	-	-	-

TABLE 10 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DENSITIES (CELLS/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 4 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE=2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	2.80	0.90	**4.20 ***	1.58	**3.13 ***
Ab	-	-	1.90	1.40	1.22	0.32
AvLH	-	-	-	**3.31 ***	0.68	2.23
AvSI	-	-	-	-	2.62	1.08
Is	-	-	-	-	-	1.55
Ap	-	-	-	-	-	-

TABLE 11 RESULTS OF TUKEY TESTS (CONFIDENCE LIMITS) BETWEEN THE DENSITIES (CELLS/MM ORAL DISC^2) OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 6 WEEKS AFTER INOCULATION (N=5) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	**25166/98340 ***	**30929/104103 ***	**60894/134068 ***	**33994/107168 ***	**44499.9/117674 ***
Ab	-	-	-30824/42350	-859/72315	-27759/45415	-17253/55921
AvLH	-	-	-	-6622/66552	-33522/39652	-23016/50158
AvSI	-	-	-	-	-63487/9687	-52981/20193
Is	-	-	-	-	-	-26081/47093
Ap	-	-	-	-	-	-

TABLE 12 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DENSITIES (CELLS/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 8 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE = 2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.98	1.44	**3.70 ***	**3.34 ***	1.51
Ab	-	-	0.54	1.72	1.37	0.47
AvLH	-	-	-	2.26	1.90	0.07
AvSI	-	-	-	-	0.36	2.19
Is	-	-	-	-	-	1.83
Ap	-	-	-	-	-	-

TABLE 13 THE BIOMASS OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 2, 4, 6 AND 8 WEEKS, AND 9 MONTHS FOLLOWING REINFECTION (+/- S.E.) (N=5, EXCEPT N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. BALLII AND I. SULCATUS AFTER 9 MONTHS).

Cp + symbionts from named host sp.	Biomass after 2 weeks (pg C/ mm oral disc²)	Biomass after 4 weeks (pg C/ mm oral disc²)	Biomass after 6 weeks (pg C/ mm oral disc²)	Biomass after 8 weeks (pg C/ mm oral disc²)	Biomass after 9 months (pg C/ mm oral disc²)
Cp	568422 +/- 148546	5523987 +/- 651367	5646109 +/- 436381	7016195 +/- 1278662	1519963 +/- 212300
Ab	189145 +/- 61819	2135245 +/- 787404	4507823 +/- 695528	4535888 +/- 637317	2657095 +/- 316184
AvLH	50783 +/- 3564	3376100 +/- 621147	2178821 +/- 360363	3025082 +/- 402056	2459460 +/- 360093
AvSI	21781 +/- 5470	455504 +/- 221447	964948 +/- 389814	2128460 +/- 182910	3244095 +/- 390586
Is	240012 +/- 31815	4492475 +/- 694372	3917809 +/- 1281085	3103835 +/- 616019	3272480 +/- 557889
Ap	47186 +/- 11802	624804 +/- 92748	1152906 +/- 339466	2612516 +/- 641085	2685735 +/- 363055
Control	0	0	0	0	0

TABLE 14 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 2 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE=2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.29	2.84	**4.13 ***	0.79	**3.13 ***
Ab	-	-	1.55	2.84	0.50	1.83
AvLH	-	-	-	1.29	2.05	0.29
AvSI	-	-	-	-	**3.34 ***	1.01
Is	-	-	-	-	-	2.34
Ap	-	-	-	-	-	-

TABLE 15 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 4 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE=2.93) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.80	1.08	**3.70 ***	0.79	**3.13 ***
Ab	-	-	1.55	2.84	0.50	1.83
AvLH	-	-	-	1.29	2.05	0.29
AvSI	-	-	-	-	**3.34 ***	1.01
Is	-	-	-	-	-	2.34
Ap	-	-	-	-	-	-

TABLE 16 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 6 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE=2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.79	2.26	**3.59 ***	1.33	**3.34 ***
Ab	-	-	1.47	2.80	0.54	2.55
AvLH	-	-	-	1.33	0.93	1.08
AvSI	-	-	-	-	2.26	0.25
Is	-	-	-	-	-	2.01
Ap	-	-	-	-	-	-

TABLE 17 MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE BIOMASSES (PG CARBON/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN REINFECTED C. PEDUNCULATUS 8 WEEKS AFTER INOCULATION (N=5) (CRITICAL Z VALUE=2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.86	2.12	**3.31 ***	2.37	2.62
Ab	-	-	1.29	2.44	1.51	1.76
AvLH	-	-	-	1.15	0.22	0.47
AvSI	-	-	-	-	0.93	0.68
Is	-	-	-	-	-	0.25
Ap	-	-	-	-	-	-

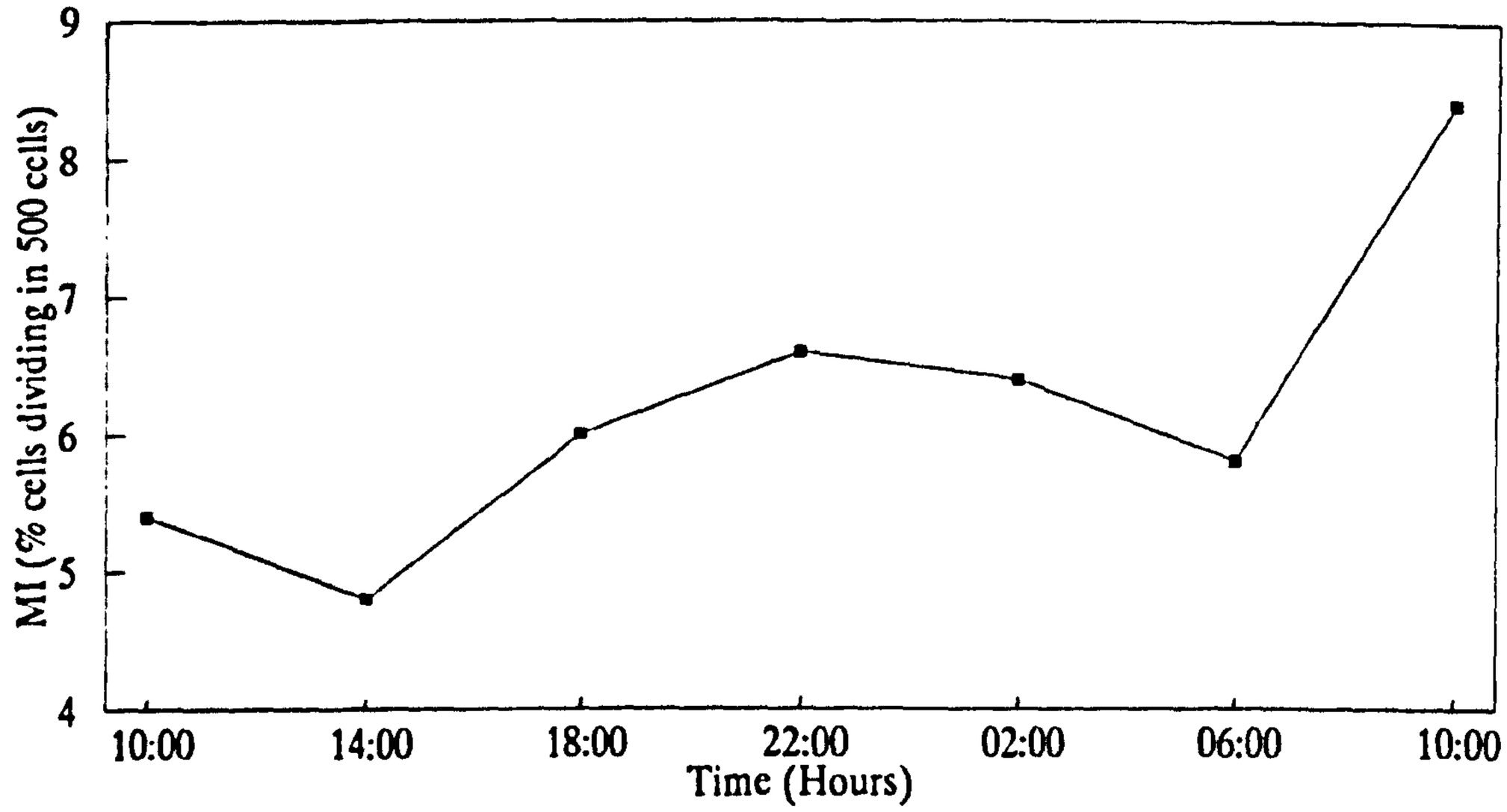
TABLE 18 RESULTS OF SCHEFFE'S TEST (CONFIDENCE LIMITS) COMPARING THE BIOMASSES (PG C/MM ORAL DISC^2) OF DIFFERENT 'STRAINS' OF SYMBIONTS IN C. PEDUNCULATUS 9 MONTHS AFTER INOCULATION (N=5, EXCEPT N=4 FOR REINFECTIONS WITH SYMBIONTS FROM I. SULCATUS AND A. BALLII) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	- 2626604 /352335	- 2343783 /464787	- 3128418 /- 319847 *	- 3241988 /- 263049 *	- 257186 0/2367 10
Ab	-	-	- 1291833 /168710 6	- 2076468 /902471	- 2185423 /954654	- 151991 0/1459 028
AvLH	-	-	-	- 2188920 /619650	- 2302490 /676449	- 163236 2/1176 208
AvSI	-	-	-	-	- 1517855 /146108 4	- 847728 /19608 42
Is	-	-	-	-	-	- 904526 /20744 13
Ap	-	-	-	-	-	-

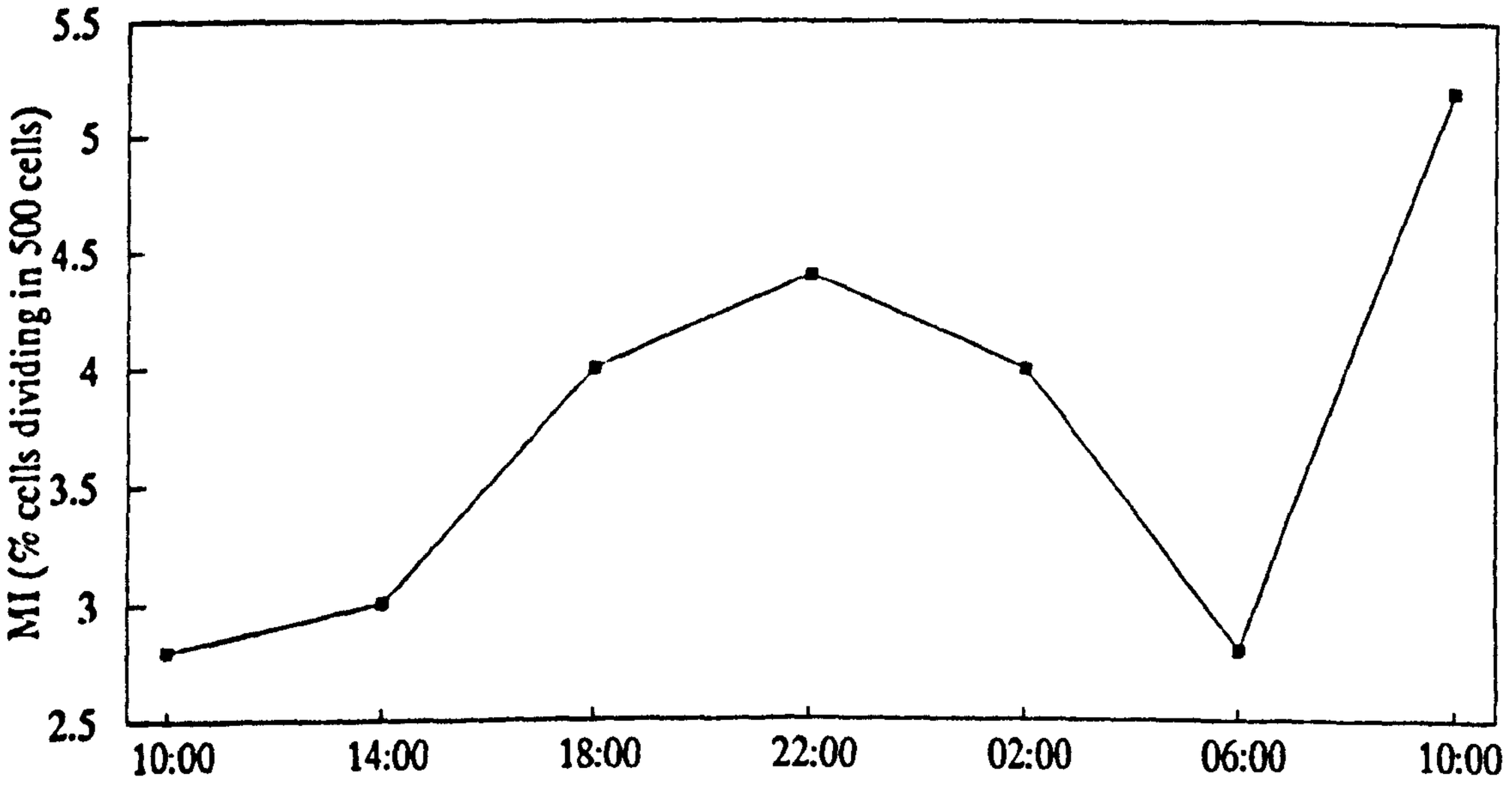
TABLE 19 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE DIAMETERS OF DIFFERENT 'STRAINS' OF SYMBIONT CELLS 9 MONTHS AFTER INOCULATION INTO APOSYMBIOTIC C. PEDUNCULATUS (N=100) (CRITICAL Z VALUE=2.93)(SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Original host sp. of symbiont	Cp	Ap	AvLH	AvSI	Ab	Is
Cp	-	1.24	3.44 *	6.87 *	8.53 *	9.66 *
Ap	-	-	2.20	5.63 *	7.23 *	8.42 *
AvLH	-	-	-	3.43 *	3.44 *	6.22 *
AvSI	-	-	-	-	1.66	2.79
Ab	-	-	-	-	-	1.13
Is	-	-	-	-	-	-

Graph 1: Diel division cycle of symbionts originally isolated from C. pedunculatus, 3 weeks after the establishment of a symbiosis with aposymbiotic C. pedunculatus (N = 1).



Graph 2: Diel division cycle of symbionts originally isolated from A. viridis (Shell Is.), 3 weeks after the establishment of a symbiosis with aposymbiotic C. pedunculatus (N = 1).



APPENDIX 9: INVESTIGATION OF THE INFLUENCE OF NaOH ON THE LOWRY PROTEIN TEST.

INVESTIGATION OF THE INFLUENCE OF NaOH ON THE LOWRY PROTEIN TEST

Introduction

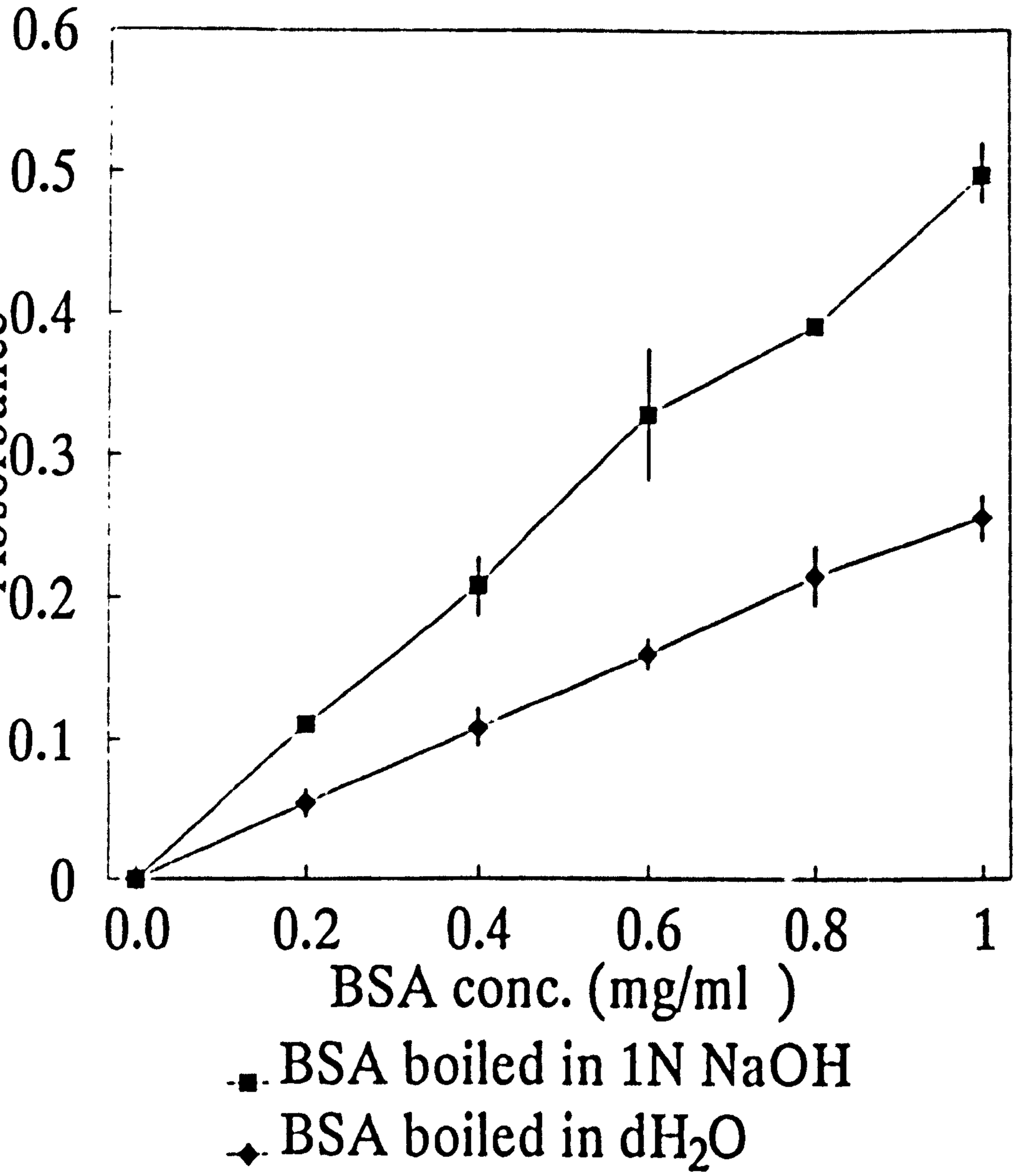
This experiment aimed to determine whether the use of 1N sodium hydroxide (NaOH) in the solubilization of proteins influenced the effectiveness of the Lowry protein test (Lowry et al. 1951), as had been suggested by Zamer et al. (1989). These authors suggested that solubilizing sea anemone proteins by boiling in deionized water (dH_2O) risks incomplete protein extraction. However, Zamer et al. (1989) also showed that the use of 10 % NaOH interferes with the Lowry test, probably by destroying the folin reagent before completion of its reaction with the protein. These authors therefore suggested the use of 10 % NaOH to solubilize low concentrations of sea anemone proteins, quantifying the protein content against a bovine serum albumen (BSA) in dH2O standard. Consequently, the addition of 1N NaOH to the BSA standards, as suggested by R. Day (University of the West Indies, Jamaica), was considered.

Materials and Methods

The Lowry protein test and analysis was as described in chapter 5, section 5.2 (c) (pp.236-240), except that 3 replicates of BSA standard were boiled in either 1N NaOH or in dH_2O only. The 2 standard curves were plotted and compared.

Graph 1: Absorbance vs. protein concentration using the Lowry Test when BSA boiled in dH_20 and NaOH

(N=3) (+/- S.E.).



Results

The standard curves are illustrated in graph 1 (p.510). The slope when the protein was solubilized in NaOH was greater than that when only dH_2O was used. When dH_2O was used, the standard plot appeared linear to concentrations of at least 0.8 mg protein/ml. When NaOH was used, the standard plot appeared to be linear to concentrations of 1.0 mg/ml.

Discussion

The results indicated that protein extraction was more incomplete when boiled in dH_2O than in 1N NaOH. The maintenance of linearity until 1.0 mg BSA/ml when 1N NaOH was used for solubilization was contrary to the findings of Zamer *et al*. (1989). These authors showed a deviation from linearity at above 0.2 mg BSA/ml when 10 % NaOH was used. This could suggest that a lower concentration of NaOH more successfully solubilizes protein than dH_2O, but does not prevent the completion of the folin reaction. These results supported the view that, in addition to the samples, the BSA standards should also be treated with NaOH. It should be noted, however, that sea anemone proteins may be underestimated by the Lowry protein determination method when BSA is used as a standard (Fitt and Pardy 1981; Zamer *et al*. 1989). The limitations of the Lowry method should therefore be noted, although these limitations are of more importance during the quantification of energetic values

directly from the protein content than during comparative work (Zamer et al. 1989).

APPENDIX 10: INVESTIGATION OF THE RELATIONSHIP BETWEEN SYMBIONT NUMBER AND SYMBIOSIS BIOMASS.

INVESTIGATION OF THE RELATIONSHIP BETWEEN SYMBIONT NUMBER AND SYMBIOSIS BIOMASS

Introduction

The aim of this investigation was to establish whether the total number of symbionts in a host was linearly related to the biomass of the association. Due to the relatively small contribution of symbiont biomass to the total symbiosis biomass, the biomass of the association could be assumed to be equivalent to host biomass. Changes in the ratio of symbiont : host biomass with host size may alter the total net photosynthetic carbon fixation and hence the amount of carbon available to the host. Therefore, host ontogenetic differences in symbiont density were investigated.

Materials and Methods

In addition to the Anthozoa used in the respirometry work, 5 further individuals of C. pedunculatus, A. ballii, A. viridis (Lough Hyne and Shell Is.) and A. pallida, which had been maintained at 21 ^{o}C and 34 ^{o}/oo under a 12 hour light:dark cycle at 80 $\mu E/m^2/s$, were collected. These animals were selected to cover a wide size range. The anthozoans were homogenized in a hand held glass tissue grinder and their symbiont numbers and total protein contents determined as described in chapter 5, section 5.2 (c) (pp.236-240). The total number of symbionts was then

plotted against the protein content of the association and the linearity investigated using correlation. The relationship in I. sulcatus could not be investigated because of the similarity of the biomass of the available polyps.

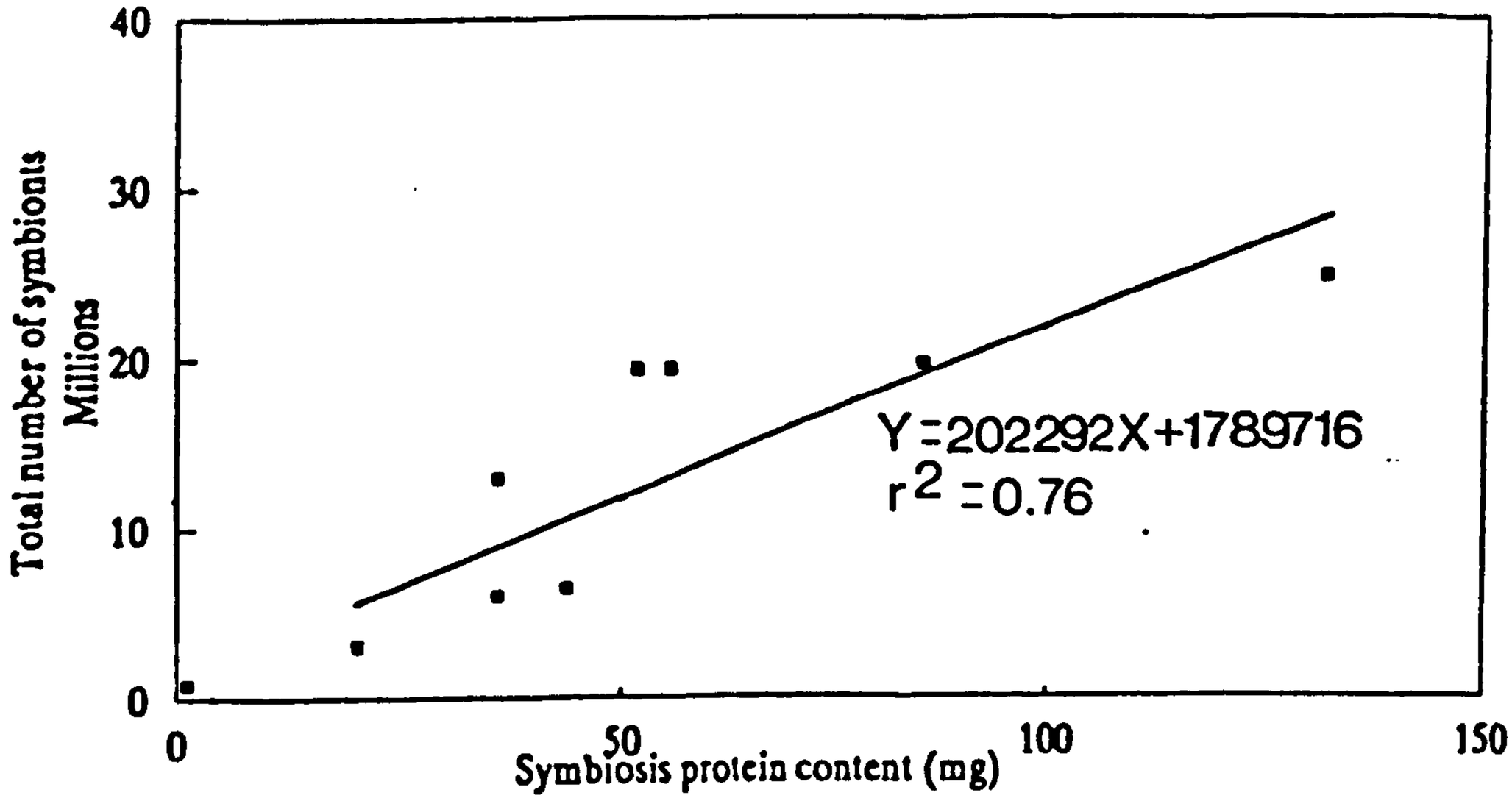
Results

The plots, linear equations and r^2 values of symbiont number vs. symbiosis protein content are shown in graphs 1-5 (pp.516-518). From these graphs it is evident that symbiont number is linearly related to association biomass. The density of symbionts (number/mg association protein) was approximately constant with changes in symbiosis biomass in all of the host species.

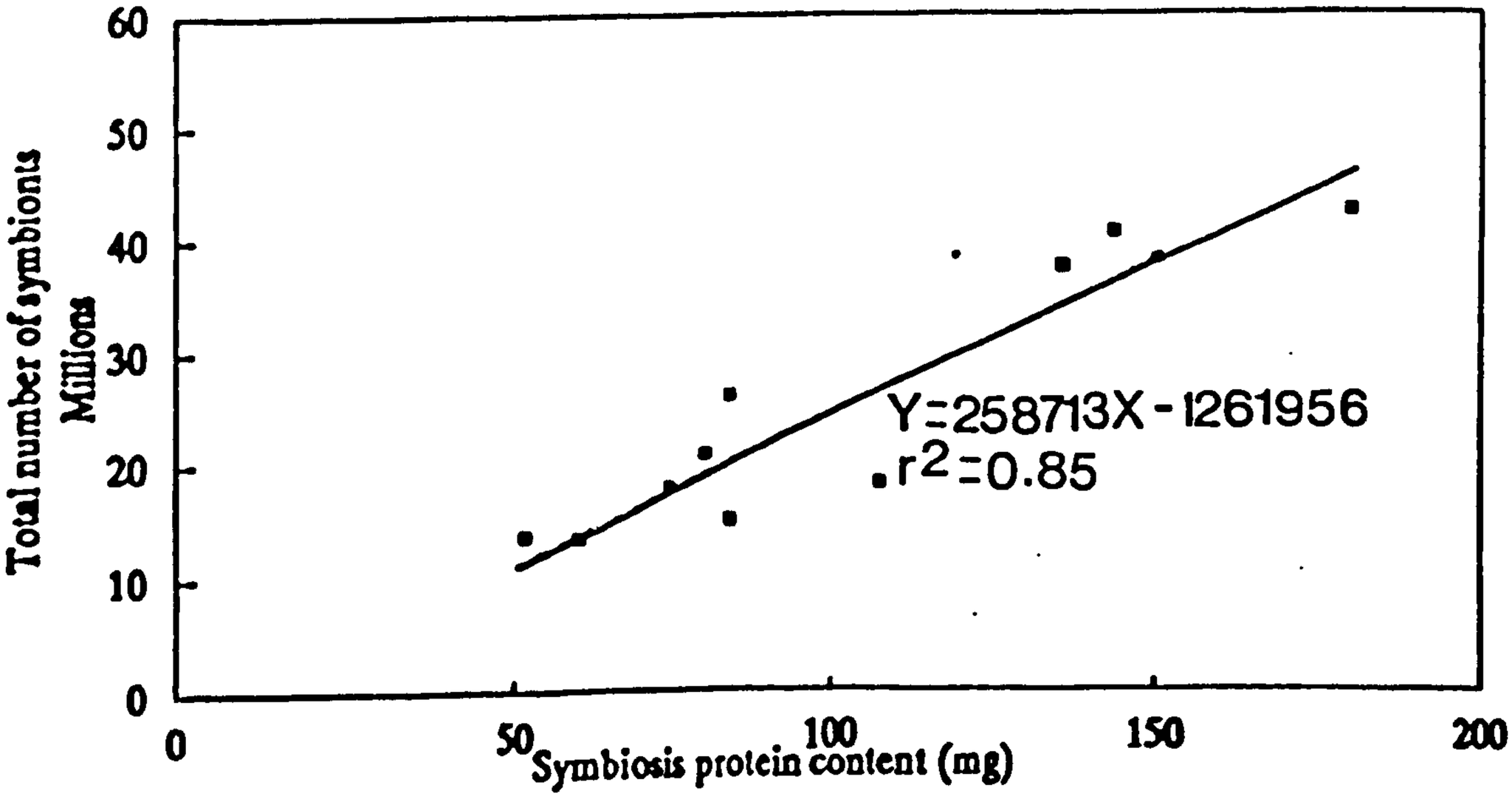
Discussion

The constant density of symbionts in all the symbioses, except that involving A. viridis (Lough Hyne), indicates that the ratio of symbiont to total protein is constant in conspecific anthozoans of different sizes, assuming that symbiont size is not variable between differently sized adult animals. Thus, according to equation 5.4 (p.242), the contribution of the symbiont and host fractions to the association respiration may remain constant in differently sized conspecific hosts under identical conditions. If host biomass specific respiration, biomass specific symbiont respiration and photosynthesis, and the percentage

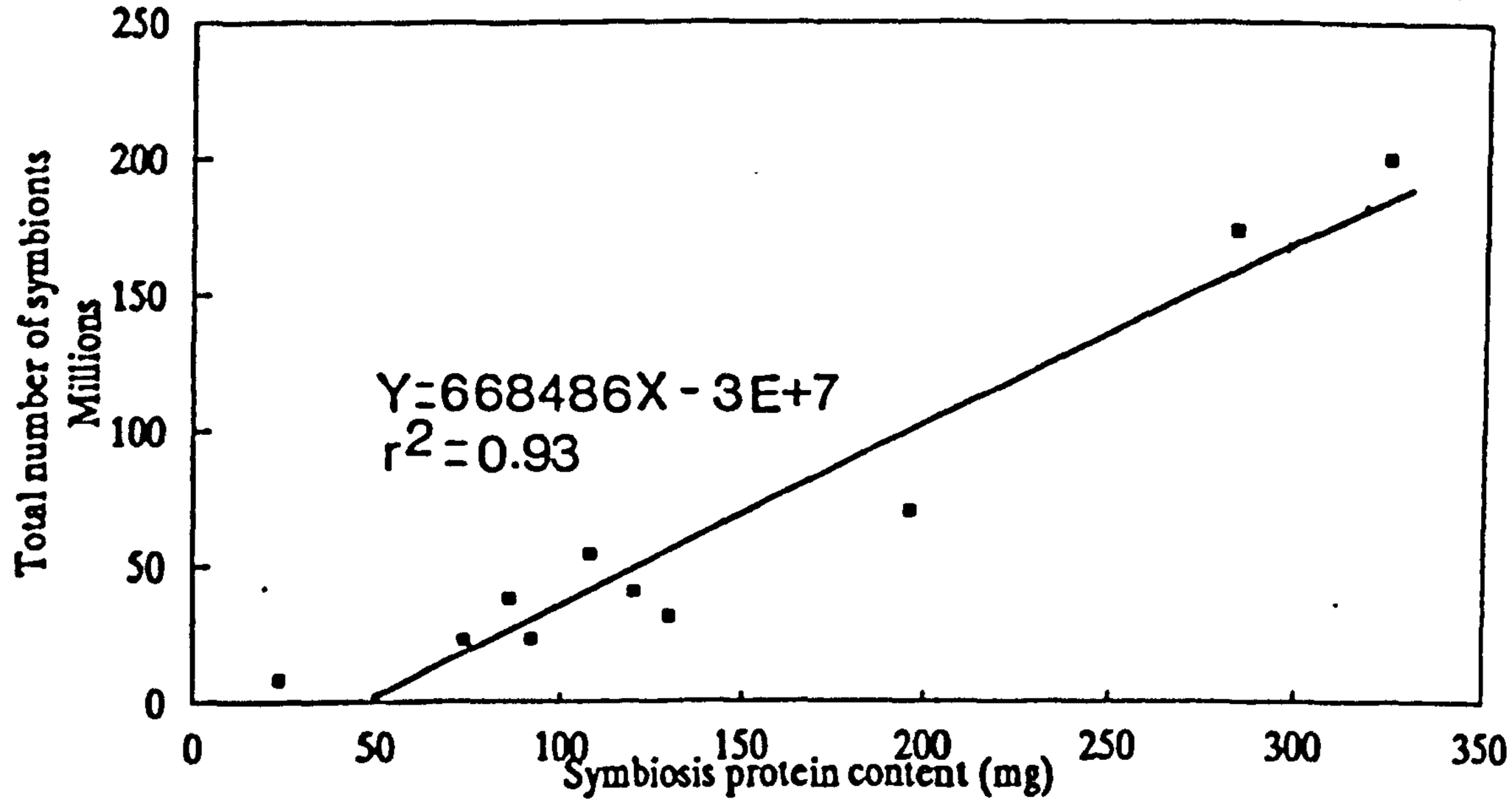
Graph 1: Symbiont number vs. symbiosis protein content in C. pedunculatus.



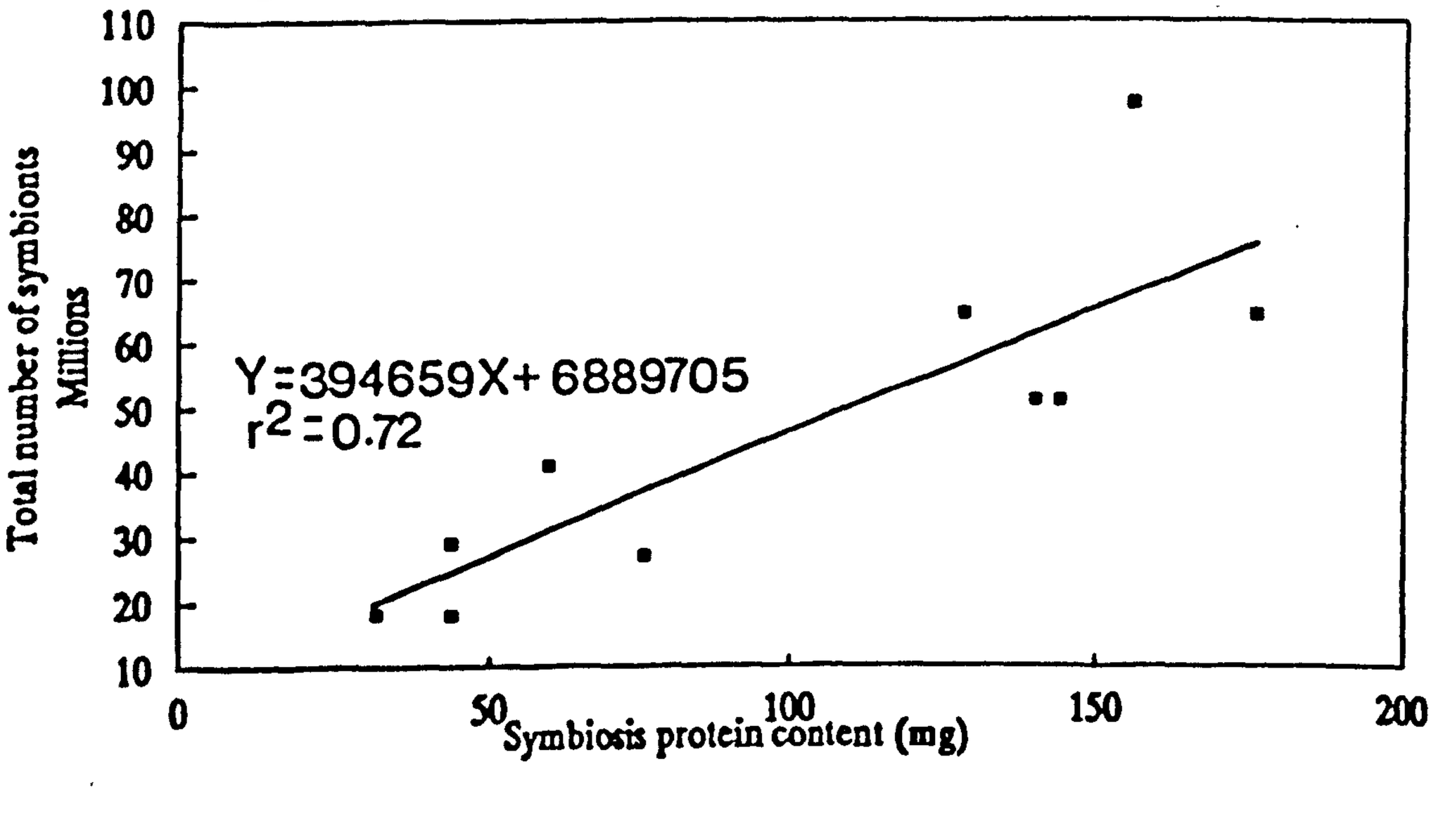
Graph 2: Symbiont number vs.symbiosis protein content in A. ballii



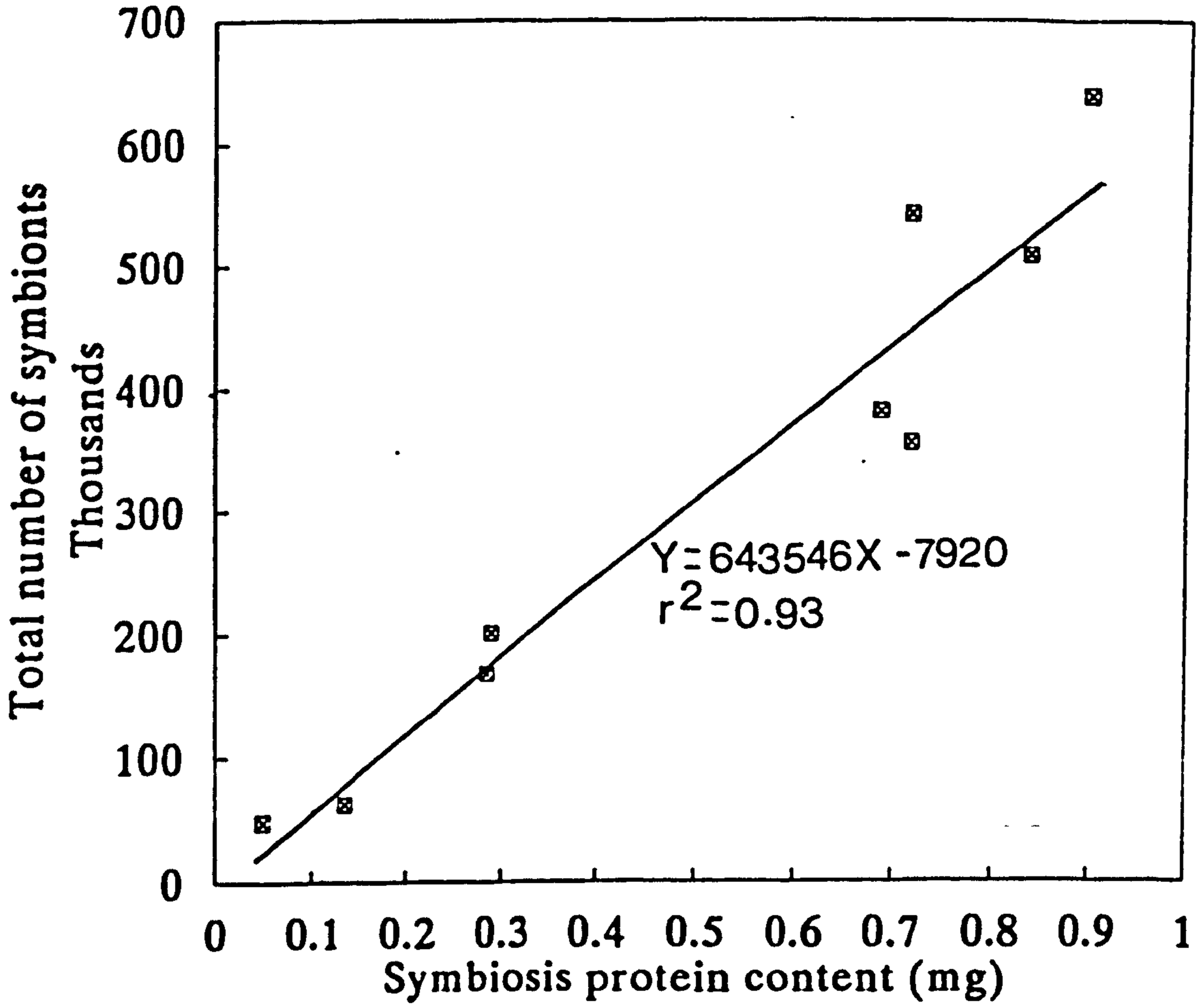
Graph 3: Symbiont density vs. symbiosis protein content in A. viridis (Lough Hyne).



Graph 4: Symbiont number vs. symbiosis protein content in A. viridis (Shell Is.).



Graph 5: Symbiont number vs. symbiosis protein content in A. pallida.



translocation remain constant between symbioses involving differently sized conspecific anthozoans, then the constant symbiont densities suggest that the CZAR would be the same in a range of differently sized conspecific hosts. However, ontogenetic changes in respiration, photosynthesis and translocation have not been investigated in any of the symbioses discussed here, resulting in any conclusions regarding the CZAR in differently sized hosts impossible to draw simply from data on symbiont densities.

APPENDIX 11: STATISTICS AND VALUES RELATING TO THE RESPIROMETRY WORK.

TABLE 1 GROSS PHOTOSYNTHESIS (ml O_2/h/mg association protein) VS. IRRADIANCE (I) IN 'NATURAL' SYMBIOSES +/- S.E..

I (µE/ m^2/s)	Cp (N=5)	Ab (N=5)	AvLH (N=5)	AvSI (N=5)	Is (N=3)	Ap (N=5)
0	0	0	0	0	0	0
10	-	-	-	-	0.0101 +/- 0.0041	-
40	0.0008 +/- 0.0003	0.0021 +/- 0.0008	0.0027 +/- 0.0004	0.0013 +/- 0.0004	0.0125 +/- 0.0031	0.0158 +/- 0.0048
80	0.0017 +/- 0.0005	0.0040 +/- 0.0016	0.0055 +/- 0.0007	0.0026 +/- 0.0005	0.0181 +/- 0.0043	0.0242 +/- 0.0071
120	0.0028 +/- 0.0005	0.0050 +/- 0.0021	0.0069 +/- 0.0010	0.0037 +/- 0.0007	0.0193 +/- 0.0034	0.0280 +/- 0.0064
160	0.0042 +/- 0.0008	0.0058 +/- 0.0022	0.0072 +/- 0.0010	0.0044 +/- 0.0008	0.0211 +/- 0.0054	0.0254 +/- 0.0054
200	0.0044 +/- 0.0009	0.0062 +/- 0.0022	0.0083 +/- 0.0014	0.0054 +/- 0.0009	0.0207 +/- 0.0033	0.0270 +/- 0.0049
240	0.0047 +/- 0.0008	0.0060 +/- 0.0024	0.0090 +/- 0.0015	0.0060 +/- 0.0010	0.0226 +/- 0.0028	0.0293 +/- 0.0061
320	0.0057 +/- 0.0013	0.0062 +/- 0.0024	0.0109 +/- 0.0022	0.0060 +/- 0.0011	0.0213 +/- 0.0029	0.0330 +/- 0.0105
400	0.0060 +/- 0.0014	0.0060 +/- 0.0022	0.0103 +/- 0.0018	0.0060 +/- 0.0012	0.0212 +/- 0.0042	0.0243 +/- 0.0070
440	0.0063 +/- 0.0015	-	0.0103 +/- 0.0018	-	-	-
480	0.0068 +/- 0.0014	-	-	-	-	-
520	0.0069 +/- 0.0016	-	-	-	-	-
600	0.0073 +/- 0.0019	-	-	-	-	-
720	0.0071 +/- 0.0019	-	-	-	-	-

TABLE 2 GROSS PHOTOSYNTHESIS (ml O_2/h/mg association protein) VS.IRRADIANCE (I) IN C. PEDUNCULATUS REINFECTED WITH SYMBIONTS FROM THE NAMED HOST SPECIES +/- S.E..

I (µE/ m^2/s)	Cp (N=3)	Ab (N=3)	AvLH (N=3)	AvSI (N=3)	Is (N=3)	Ap (N=3)
0	0	0	0	0	0	0
40	0.0021 +/- 0.0009	0.0039 +/- 0.0010	0.0021 +/- 0.0005	0.0023 +/- 0.0007	0.0015 +/- 0.0006	0.0011 +/- 0.0004
80	0.0038 +/- 0.0005	0.0066 +/- 0.0003	0.0038 +/- 0.0007	0.0036 +/- 0.0002	0.0033 +/- 0.0007	0.0032 +/- 0.0010
120	0.0048 +/- 0.0008	0.0078 +/- 0.0004	0.0029 +/- 0.0003	0.0040 +/- 0.0006	0.0030 +/- 0.0005	0.0042 +/- 0.0010
160	0.0065 +/- 0.0004	0.0072 +/- 0.0008	0.0043 +/- 0.0007	0.0037 +/- 0.0005	0.0033 +/- 0.0003	0.0050 +/- 0.0005
200	0.0059 +/- 0.0005	0.0075 +/- 0.0006	0.0040 +/- 0.0005	0.0037 +/- 0.0011	0.0040 +/- 0.0010	0.0043 +/- 0.0008
240	0.0056 +/- 0.0006	0.0062 +/- 0.0003	0.0045 +/- 0.0006	0.0047 +/- 0.0005	0.0040 +/- 0.0010	0.0047 +/- 0.0009
320	0.0064 +/- 0.0005	0.0070 +/- 0.0006	0.0049 +/- 0.0006	0.0047 +/- 0.0004	0.0040 +/- 0.0010	0.0054 +/- 0.0002
400	0.0055 +/- 0.0006	0.0072 +/- 0.0008	0.0040 +/- 0.0003	0.0046 +/- 0.0004	0.0040 +/- 0.0010	0.0049 +/- 0.0007

TABLE 3 THE AVERAGE SYMBIONT CELL NUMBER PER POLYP, SYMBIONT POPULATION PROTEIN CONTENT, TOTAL POLYP PROTEIN CONTENT AND SYMBIONT : ASSOCIATION PROTEIN RATIO IN THE 'NATURAL' SYMBIOSES (+/- S.E.) (N=5, EXCEPT N=3 FOR I. SULCATUS).

Host sp.	Total symbiont no.(cells/ polyp)	Total symbiont protein (mg/ polyp)	Total polyp protein (mg)	Symbiont : association protein ratio
Cp	19211112 +/- 1875722	1.02 +/- 0.10	72.40 +/- 17.0	1:68.1 +/- 9.9
Ab	33044444 +/- 4749379	3.22 +/- 0.46	123.60 +/- 19.70	1:38.4 +/- 1.9
AvLH	45166668 +/- 7968011	2.43 +/- 0.43	116.80 +/- 21.40	1:49.3 +/- 4.3
AvSI	66066668 +/- 8416023	5.62 +/- 0.72	148.80 +/- 8.14	1:27.7 +/- 2.9
Is (10 polyps)	577778 +/- 67586	0.06 +/- 0.01	2.53 +/- 0.25	1:42.6 +/- 2.7
Ap	486333 +/- 52531	0.02 +/- 0.002	0.77 +/- 0.04	1:39.0 +/- 3.0

TABLE 4 THE AVERAGE SYMBIONT CELL NUMBER PER POLYP, SYMBIONT POPULATION PROTEIN CONTENT, TOTAL POLYP PROTEIN CONTENT AND SYMBIONT : ASSOCIATION PROTEIN RATIO FOR THE REESTABLISHED SYMBIOSES (+/- S.E.) (N=5, EXCEPT N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. BALLII AND I. SULCATUS).

Cp + symbionts from named host sp.	Total symbiont no.(cells/ polyp)	Total symbiont protein (mg/ polyp)	Total polyp protein (mg)	Symbiont : associaton protein ratio
Cp	225333 +/- 55930	0.0077 +/- 0.0019	0.56 +/- 0.12	1:74.4 +/- 2.2
Ab	201111 +/- 8240	0.0109 +/- 0.0005	0.48 +/- 0.08	1:44.0 +/- 6.1
AvLH	273333 +/- 59213	0.0112 +/- 0.0024	0.60 +/- 0.13	1:55.1 +/- 5.9
AvSI	295111 +/- 50486	0.0145 +/- 0.0025	0.64 +/- 0.13	1:45.6 +/- 6.8
Is	293333 +/- 51312	0.0168 +/- 0.0029	0.78 +/- 0.07	1:53.7 +/- 13.7
Ap	339111 +/- 43967	0.0121 +/- 0.0016	0.63 +/- 0.07	1:55.1 +/- 8.2

TABLE 5 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE RATE OF NET ALGAL PHOTOSYNTHESIS IN THE 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS) (CRITICAL Z VALUE =2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.35	0.88	0.15	2.38	2.81
Ab	-	-	1.23	0.19	2.68	**3.15 ***
AvLH	-	-	-	1.04	1.62	1.92
AvSI	-	-	-	-	2.51	**2.96 ***
Is	-	-	-	-	-	0.05
Ap	-	-	-	-	-	-

TABLE 6 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE ANIMAL RESPIRATION RATES IN 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.00	0.08	1.12	1.22	2.46
Ab	-	-	0.08	1.12	1.22	2.46
AvLH	-	-	-	1.19	1.49	2.38
AvSI	-	-	-	-	2.35	**3.58 ***
Is	-	-	-	-	-	0.92
Ap	-	-	-	-	-	-

TABLE 7 RESULTS OF SCHEFFE'S TEST COMPARING THE RATES OF GROSS PHOTOSYNTHESIS IN THE REESTABLISHED SYMBIOSES (N=7, EXCEPT N=5 FOR SYMBIONTS FROM C. PEDUNCULATUS AND N=6 FOR SYMBIONTS FROM A. PALLIDA) (SCHEFFE CONFIDENCE INTERVALS GIVEN AND SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	- 0.0022/ 0.0000	0.0008/ 0.0030 *	0.0007/ 0.0030 *	0.0012/ 0.0034 *	0.0001 / 0.0024 *
Ab	-	-	0.0020/ 0.0041 *	0.0019/ 0.0040 *	0.0024/ 0.0045 *	0.0013 / 0.0034 *
AvLH	-	-	-	- 0.0011/ 0.0010	- 0.0006/ 0.0014	- 0.0017 6/ 0.0003 98
AvSI	-	-	-	-	- 0/0006/ 0.0015	- 0.0017 / 0.0005
Is	-	-	-	-	-	- 0.0022 / - 0.0000 *
Ap	-	-	-	-	-	-

TABLE 8 RESULTS OF SCHEFFE'S TEST COMPARING THE CELL DENSITIES OF SYMBIONTS IN THE 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS) (SCHEFFE CONFIDENCE INTERVALS GIVEN, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-226411 /168411	-289352 /106470	-51283 /344539	-157877 /299179	-523541 /-27719*
Ab	-	-	-317852 /77970	-22783 /373039	-186377 /270679	-552041 /-156219 *
AvLH	-	-	-	-142724 /253098	-66436 /390620	-432100 / -36278*
AvSI	-	-	-	-	-11249 /445807	-376913 /18909
Is	-	-	-	-	-	-624809 /-167753 *
Ap	-	-	-	-	-	-

TABLE 9 RESULTS OF SCHEFFE'S TEST COMPARING THE ALGAL CARBON STANDING STOCK (C')/MG ASSOCIATION PROTEIN IN THE 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS)(SCHEFFE CONFIDENCE INTERVALS GIVEN, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-0.0014 /0.0228	-0.0176 /0.0076	**0.0089 /0.0341 ***	-0.0223 /0.0068	-0.0229 /0.0024
Ab	-	-	-0.0073 /0.0179	-0.0013 /0.0239	-0.0121 /0.0171	-0.0126 /0.0126
AvLH	-	-	-	**0.0039 /0.029 ***	-0.0173 /0.0118	-0.0179 /0.0073
AvSI	-	-	-	-	-0.0008 /0.0284	-0/0014 /0.0239
Is	-	-	-	-	-	-0.0171 /0.0121
Ap	-	-	-	-	-	-

TABLE 10 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE RATE OF NET ALGAL PHOTOSYNTHESIS/CELL IN 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.15	0.62	1.00	2.41	1.77
Ab	-	-	0.77	0.85	2.55	1.92
AvLH	-	-	-	1.61	1.91	1.15
AvSI	-	-	-	-	**3.25 ***	2.77
Is	-	-	-	-	-	0.88
Ap	-	-	-	-	-	-

TABLE 11 RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE RATES OF NET ALGAL PHOTOSYNTHESIS/MG SYMBIONT CARBON IN THE 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.31	0.46	1.58	1.67	1.81
Ab	-	-	1.81	0.27	2.83	**3.15 ***
AvLH	-	-	-	2.04	1.27	1.35
AvSI	-	-	-	-	**3.00 ***	**3.38 ***
Is	-	-	-	-	-	0.10
Ap	-	-	-	-	-	-

TABLE 12 RESULTS OF SCHEFFE'S TEST COMPARING THE RATES OF NET ALGAL PHOTOSYNTHESIS/CELL IN THE REESTABLISHED SYMBIOSES (N=5, EXCEPT N=4 FOR SYMBIONTS ORIGINALLY FROM A.BALLII AND I.SULCATUS) (SCHEFFE CONFIDENCE INTERVALS GIVEN, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-1.008/ 0.680	-0.205/ 1.387	-0.211/ 1.381	-0.453/ 1.235	-0.22/ 1.376
Ab	-	-	-0.089/ 1.599	-0.095/ 1.593	-0.335/ 1.445	-0.10/ 1.588
AvLH	-	-	-	-0.802/ 0.790	-1.044/ 0.644	-0.81/ 0.785
AvSI	-	-	-	-	-1.038/ 0.650	-0.80/ 0.791
Is	-	-	-	-	-	-0.66/ 1.033
Ap	-	-	-	-	-	-

TABLE 13 RESULTS OF SCHEFFE'S TEST COMPARING THE RATES OF NET ALGAL PHOTOSYNTHESIS/MG SYMBIONT C IN THE REESTABLISHED SYMBIOSES (N=5, EXCEPT N=4 FOR SYMBIONTS ORIGINALLY FROM A. BALLI AND I. SULCATUS) (SCHEFFE CONFIDENCE INTERVALS GIVEN, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-0.043/ 0.316	**0.056/ 0.395 ***	**0.093/ 0.431 ***	**0.074/ 0.433 ***	**0.018/ 0.36 ***
Ab	-	-	-0.091/ 0.268	-0.054/ 0.305	-0.073/ 0.306	-0.129/ 0.231
AvLH	-	-	-	-0.133/ 0.206	-0.152/ 0.208	-0.21/ 0.132
AvSI	-	-	-	-	-0.188/ 0.171	-0.24/ 0.095
Is	-	-	-	-	-	-0.25/ 0.114
Ap	-	-	-	-	-	-

TABLE 14 RESULTS OF STUDENT'S T-TEST OR MANN-WHITNEY TEST COMPARING THE RATE OF NET ALGAL PHOTOSYNTHESIS/ALGAL CELL BY SYMBIONTS IN 'NATURAL' SYMBIOSES WITH THAT BY THE SAME SYMBIONT 'STRAIN' IN THE REESTABLISHED SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS AND N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. BALLII AND I. SULCATUS) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Test statistic and probability
Cp	T=1.29, P=0.27
Ab	T=0.63, P=0.56
AvLH	**T=3.24, P=0.032 ***
AvSI	T=1.60, P=0.16
Is	**T=5.98, P=0.03 ***
Ap	**Mann-Whitney statistic=40.0, P=0.01 ***

TABLE 15 STUDENT'S T-TESTS OR MANN-WHITNEY TESTS COMPARING THE RATE OF NET ALGAL PHOTOSYNTHESIS/MG SYMBIONT C BY THE SYMBIONTS IN THE 'NATURAL' SYMBIOSES WITH THAT BY THE SAME SYMBIONT 'STRAINS' IN THE REESTABLISHED SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS AND N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. BALLII AND I. SULCATUS) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Test statistic and probability
Cp	T=-0.35, P=0.74
Ab	T=-1.12, P=0.31
AvLH	**T=2.80, P=0.05 ***
AvSI	T=-0.68, P=0.52
Is	**T=5.06, P=0.04 ***
Ap	**Mann-Whitney statistic=40.0, P=0.01 ***

APPENDIX 12: STATISTICS AND VALUES RELATING TO THE ESTIMATION OF THE PERCENTAGE TRANSLOCATION.

TABLE 1 THE EFFICIENCY OF THE ACIDIFICATION OF ^{14}C

Volume of ^{14}C stock solution (µl).	No acidification (d.p.m.)	Acidification (d.p.m.)
100	2259169.7	283.1
500	11295848.3	1166.5

TABLE 2 AVERAGE D.P.M. IN EACH FRACTION OF IN VIVO LABELLED, DARK INCUBATED A. VIRIDIS (SHELL IS.), WHEN THE INFLUENCE OF THE INCUBATION PERIOD ON THE PERCENTAGE TRANSLOCATION WAS BEING INVESTIGATED (N=3, EXCEPT N=5 FOR 3 HOUR INCUBATION).

Incubation period (hours)	Symbiont fraction (d.p.m.) +/- S.E.	Host fraction (d.p.m.) +/- S.E.	Sea water fraction (d.p.m.) +/- S.E.
1	5489 +/- 605	35970 +/- 6187	161920 +/- 48218
2	15631 +/- 2652	61090 +/- 9625	119915 +/- 19316
3	5218 +/- 1118	11179 +/- 1357	23287 +/- 1994
4	17034 +/- 5777	59962 +/- 15040	91740 +/- 34009
5	17328 +/- 3559	57699 +/- 3194	62807 +/- 21757

TABLE 3 AVERAGE DARK CORRECTED D.P.M. IN EACH FRACTION OF IN VIVO LABELLED, LIGHT INCUBATED A. VIRIDIS (SHELL IS.), WHEN INVESTIGATING THE INFLUENCE OF INCUBATION PERIOD ON PERCENTAGE TRANSLOCATION (N=3, EXCEPT N=5 FOR 3 HOUR INCUBATION).

Incubation period (Hours)	Symbiont fraction (d.p.m.) +/- S.E.	Host fraction (d.p.m.) +/- S.E.	Sea water fraction (d.p.m.) +/- S.E.
1	197717 +/- 26279	87166 +/- 15246	0.0 +/- 0.0
2	337468 +/- 104656	207467 +/- 43018	0.0 +/- 0.0
3	409259 +/- 78281	274751 +/- 51746	14396 +/- 8797
4	884699 +/- 168507	473142 +/- 39797	2316 +/- 2316
5	1136694 +/- 310319	867932 +/- 232681	20939 +/- 5393

TABLE 4 PERCENTAGE OF ^{14}C FIXED CARBON IN EACH FRACTION OF A. VIRIDIS (SHELL IS.) AFTER INCUBATION FOR DIFFERENT TIME PERIODS, AND THE PERCENTAGE TRANSLOCATION (N=3, EXCEPT N=5 FOR 3 HOUR INCUBATION).

Incubation period (Hours)	Symbiont fraction (%) +/- S.E.	Host fraction (%) +/- S.E.	Sea water fraction (%) +/- S.E.	Percentage translocation (T)(%) +/- S.E.
1	69.5 +/- 2.9	30.5 +/- 2.9	0.0 +/- 0.0	30.5 +/- 2.9
2	59.5 +/- 6.8	40.5 +/- 6.8	0.0 +/- 0.0	40.5 +/- 6.8
3	58.1 +/- 2.6	40.0 +/- 2.9	1.9 +/- 1.3	41.9 +/- 2.6
4	64.2 +/- 3.2	35.7 +/- 3.3	0.1 +/- 0.1	35.8 +/- 3.2
5	56.2 +/- 8.8	42.8 +/- 8.8	1.0 +/- 0.1	43.8 +/- 8.7

TABLE 5 AVERAGE D.P.M. OF EACH FRACTION OF THE IN VIVO ^{14}C CONTROL INCUBATIONS, USING 'NATURAL'AND REESTABLISHED SYMBIOSES (N=3).

Host sp.	Symbiont fraction (d.p.m.) +/- S.E.	Host fraction (d.p.m.) +/- S.E.	Sea water fraction (d.p.m.) +/- S.E.
Cp	4165 +/- 929	33710 +/-9637	33348 +/-6920
Ab	2308 +/- 478	12877 +/-3564	18316 +/-1946
AvLH	3198 +/- 642	9672 +/- 2510	45086 +/13322
AvSI	5218 +/- 1118	11179 +/-1357	23287 +/-1994
Is	769 +/- 97	2184 +/- 216	100731 +/- 38439
Ap	1711 +/- 603	1766 +/- 409	27321 +/-2047
Reinfected Cp (+ symbionts from Cp)	546 +/- 30	2496 +/- 477	66256 +/-5903

TABLE 6 AVERAGE D.P.M. OF EACH FRACTION OF IN VIVO ^{14}C LIGHT INCUBATIONS, AFTER CORRECTION FOR DARK FIXATION, USING 'NATURAL' SYMBIOSES (N=5, EXCEPT N=3 FOR I. SULCATUS).

Host sp.	symbiont fraction (d.p.m.) +/- S.E.	Host fraction (d.p.m.) +/- S.E.	Sea water fraction (d.p.m.) +/- S.E.
Cp	244061 +/- 730043	285735 +/- 98573	5875 +/- 5875
Ab	385594 +/- 67886	178007 +/- 19800	10046 +/- 6426
AvLH	334129 +/- 65837	263722 +/- 26943	904 +/- 904
AvSI	409259 +/- 78281	274751 +/- 51746	14396 +/- 8797
Is	29658 +/- 2443	45614 +/- 10243	0.0 +/- 0.0
Ap	92992 +/- 29867	26727 +/- 5080	5944 +/- 3660

TABLE 7 AVERAGE D.P.M. OF EACH FRACTION OF IN VIVO ^{14}C LIGHT INCUBATIONS, AFTER CORRECTION FOR DARK FIXATION, FOR REESTABLISHED SYMBIOSES (N=5, EXCEPT N=4 FOR REINFECTIONS WITH SYMBIONTS FROM A. VIRIDIS (SHELL IS. AND LOUGH HYNE)).

Cp + symbionts from named host sp.	Symbiont fraction (d.p.m.) +/- S.E.	Host fraction (d.p.m.) +/- S.E.	Sea water fraction (d.p.m.) +/- S.E.
Cp	4400 +/- 1125	6118 +/- 1126	0.0 +/- 0.0
Ab	6109 +/- 1413	4879 +/- 1116	0.0 +/- 0.0
AvLH	5003 +/- 1028	5755 +/- 1298	14.0 +/- 14.0
AvSI	4305 +/- 1051	5509 +/- 1435	0.0 +/- 0.0
Is	3351 +/- 805	4570 +/- 1250	0.0 +/- 0.0
Ap	4438 +/- 1427	6113 +/- 1645	0.0 +/- 0.0

TABLE 8 THE RESULTS OF SCHEFFE'S TEST COMPARING THE PERCENTAGE TRANSLOCATION IN THE 'NATURAL' SYMBIOSES WHEN INCUBATED IN VIVO WITH ^{14}C (N=5, EXCEPT N=3 FOR I. SULCATUS)(SCHEFFE CONFIDENCE INTERVALS GIVEN, SIGNIFICANT DIFFERENCES ($p<0.05$) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-2.98/ 32.46	-14.56/ 20/88	-11.04/ 24.40	-31.22/ 9.71	**2.50/ 37.94 ***
Ab	-	-	-29.3/ 6.14	-25.78/ 9.66	**-45.96/ -5.03 ***	- 12.24/ 23.20
AvLH	-	-	-	-14.20/ 21.24	-34.38/ 6.55	-0.66/ 34.78
AvSI	-	-	-	-	-37.90/ 3.03	-4.18/ 31.26
Is	-	-	-	-	-	**10.51/ 51.44 ***
Ap	-	-	-	-	-	-

TABLE 9 AVERAGE D.P.M. OF BOTH FRACTIONS OF THE IN VITRO ^{14}C CONTROL AND LIGHT INCUBATIONS (CORRECTED FOR DARK CONTROLS), INVESTIGATING DIFFERENCES BETWEEN TRANSLOCATION IN DIFFERENT HOST SPECIES (N=3 FOR ALL DARK AND SEA WATER INCUBATIONS, N=5 FOR ALL OTHER INCUBATIONS) (Hom. = Origin of host homogenate, Sym = Origin of algal symbionts, Sw = sea water only)

Incubation	Symbiont fraction (d.p.m.) +/- S.E.	Host or sea water fraction (d.p.m.) +/- S.E.
Cp(sym)+Cp(hom) in dark	49089 +/- 943	39997 +/- 2003
Cp(sym)+ Sw in dark	49620 +/- 3018	19593 +/- 1525
Cp(sym) + Sw	3140400 +/- 44532	414136 +/- 23972
Ab(sym) + Sw	2091063 +/- 26284	525073 +/- 13464
AvLH(sym) + Sw	165345 +/- 213787	91521 +/- 8289
AvSI(sym) + Sw	145158 +/- 44555	97128 +/- 7518
Is(sym) + Sw	2431534 +/- 699043	56812 +/- 9905
Ap(sym) + Sw	648428 +/- 104122	38624 +/- 14269
Cp(Sym)+Cp(hom)	3450831 +/- 636298	28490361+/- 544076
Ab(sym)+Ab(hom)	209930 +/- 37777	92409 +/- 21391
AvLH(sym)+AvLH(hom	8896 +/- 2291	47799 +/- 3267
AvSI(sym)+AvSI(hom	2101135 +/- 312663	158519 +/- 20456
Is(sym)+Is(hom)	268937 +/- 60373	34708 +/- 4027
Ap(sym)+Ap(hom)	952747 +/- 124843	179294 +/- 16767
Ab(sym)+Cp(hom)	2546205 +/- 195834	841219 +/- 85478
AvLH(sym)+Cp(hom)	2161027 +/- 250288	445033 +/- 145065
AvSI(sym)+Cp(hom)	2843956 +/- 79387	420251 +/- 46780
Is(sym)+Cp(hom)	3953376 +/- 562822	671650 +/- 89264
Ap(sym)+Cp(hom)	659940 +/- 64343	83621 +/- 8967
Cp(sym)+Ab(hom)	705384 +/- 50248	114885 +/- 14869
Cp(sym)+AvLH(hom)	977887 +/- 99946	201370 +/- 23447
Cp(sym)+AvSI(hom)	415590 +/- 18225	37268 +/- 3092
Cp(sym)+Is(hom)	1508363 +/- 59258	114287 +/- 6626
Cp(sym)+Ap(hom)	574418 +/- 56773	65156 +/- 17373

TABLE 10 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE PERCENTAGE TRANSLOCATION IN 'HOMOLOGOUS' IN VITRO ^{14}C INCUBATIONS (N=5) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.90	0.90	**3.56 ***	1.80	1.72
Ab	-	-	2.80	1.65	0.11	0.18
AvLH	-	-	-	**4.45 ***	2.69	2.62
AvSI	-	-	-	-	1.76	1.83
Is	-	-	-	-	-	0.07
Ap	-	-	-	-	-	-

TABLE 11 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE PERCENTAGE TRANSLOCATION BY SYMBIONTS FROM DIFFERENT HOST SPECIES, IN RESPONSE TO HOMOGENATE OF C. PEDUNCULATUS IN VITRO (N=5) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp homog. + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	**3.31 ***	2.23	**3.23 ***	1.19	**3.52 ***
Ab	-	-	1.08	0.07	2.12	0.22
AvLH	-	-	-	1.01	1.04	1.29
AvSI	-	-	-	-	2.05	0.29
Is	-	-	-	-	-	2.34
Ap	-	-	-	-	-	-

TABLE 12 TUKEY CONFIDENCE INTERVALS COMPARING PERCENTAGE TRANSLOCATION BY C. PEDUNCULATUS SYMBIONTS IN RESPONSE TO HOMOGENATES OF DIFFERENT HOST SPECIES (N=5) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp symbionts + homog. from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	28.0/ 35.2 *	25.1/ 32.4 *	30.4/ 37.7 *	30.4/ 37.7 *	29.2/ 36.4 *
Ab	-	-	-6.5/ 0.8	-1.2/ 6.1	-1.2/ 6.1	-2.4/ 4.8
AvLH	-	-	-	1.6/ 8.9 *	1.6/ 8.9 *	0.4/ 7.7 *
AvSI	-	-	-	-	-3.6/ 3.6	-4.9/ 2.4
Is	-	-	-	-	-	-4.9/ 2.4
Ap	-	-	-	-	-	-

TABLE 13 AVERAGE D.P.M. OF BOTH FRACTIONS OF THE IN VITRO ^{14}C DARK AND LIGHT INCUBATIONS INVESTIGATING GEOGRAPHICAL INTRA HOST SPECIFIC DIFFERENCES IN PHOTOSYNTHATE TRANSLOCATION (N=3 FOR ALL SEA WATER CONTROLS, N=5 FOR ALL OTHER INCUBATIONS)(KEY AS FOR TABLE 9).

Incubation	Symbiont fraction (d.p.m.) +/- S.E.	Homogenate or sea water fraction (d.p.m.) +/- S.E.
CpNt(sym) + Sw	2352627 +/- 65641	202647 +/- 39191
AvLS(sym) + Sw	2310828 +/- 308576	192244 +/- 49992
AvBm(sym) + Sw	3245270 +/- 236663	191036 +/- 27536
AvWH(sym) + Sw	2622099 +/- 217028	206344 +/- 44424
AvTB(sym) + Sw	3999020 +/- 443577	266533 +/- 38162
CpNt(sym)+CpNt(hom	1322846 +/- 65455	767771 +/- 49149
AvLS(sym)+AvLS(hom	2759833 +/- 312383	143753 +/- 28274
AvBm(sym)+AvBm(hom	2847700 +/- 598059	247320 +/- 75854
AvWH(sym)+AvWH(hom	1502299 +/- 1922203	270293 +/- 1502299
AvTB(sym)+AvTB(hom	1309624 +/- 170204	392200 +/- 55235
Cp(sym)+CpNt(hom)	167042 +/- 17621	126856 +/- 14497
CpNt(sym)+Cp(hom)	1972185 +/- 66070	880118 +/- 66312
AvLS(sym)+AvLH(hom	2720875 +/- 75636	153294 +/- 20694
AvBm(sym)+AvLH(hom	3994780 +/- 74835	263825 +/- 14301
AvWH(sym)+AvLH(hom	1575993 +/- 292468	321075 +/- 59339
AvTB(sym)+AvLH(hom	1609200 +/- 220091	225807 +/- 31910
AvSI(sym)+AvLH(hom	2171600 +/- 185394	237413 +/- 19380
AvLH(sym)+AvLS(hom	227983 +/- 30010	446640 +/- 59775
AvLH(sym)+AvBm(hom	170779 +/- 40565	94167 +/- 24172
AvLH(sym)+AvWH(hom	146428 +/- 18287	144266 +/- 19023
AvLH(sym)+AvTB(hom	224677 +/- 62510	495612 +/- 95204
AvLH(sym)+AvSI(hom	115320 +/- 23524	98303 +/- 7477

TABLE 14 THE RESULTS OF TUKEY'S TEST (CONFIDENCE LIMITS) COMPARING THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF C. PEDUNCULATUS FROM DIFFERENT LOCATIONS WHEN STIMULATED BY 'HOMOLOGOUS' AND 'HETEROLOGOUS' C. PEDUNCULATUS HOMOGENATES (N=5) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Combination of homogenate and symbiont	Cp(hom) + Cp(sym)	CpNt(hom) + CpNt(sym)	Cp(hom) + CpNt(sym)	CpNt(hom) + Cp(sym)
Cp(hom) + Cp(sym)	-	-1.6/ 12.2	**4.3/ 18.1 ***	-4.2/ 9.6
CpNt(hom) + CpNt(sym)	-	-	-1.0/ 12.8	-9.5/ 4.4
Cp(hom) + CpNt(sym)	-	-	-	**-15.4/ -1.5 ***
CpNt(hom) + Cp(sym)	-	-	-	-

TABLE 15 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF A. VIRIDIS FROM DIFFERENT GEOGRAPHICAL LOCATIONS WHEN SUSPENDED IN 'HOMOLOGOUS' HOMOGENATES (N=5) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	AvLH	AvSI	AvLS	AvBm	AvWH	AvTB
AvLH	-	**3.32 ***	**4.22 ***	**3.23 ***	1.80	0.90
AvSI	-	-	0.90	0.09	1.53	2.43
AvLS	-	-	-	1.0	2.43	**3.32 ***
AvBm	-	-	-	-	1.44	2.34
AvWH	-	-	-	-	-	0.90
AvTB	-	-	-	-	-	-

TABLE 16 STUDENT'S T-TEST AND MANN-WHITNEY TEST VALUES COMPARING THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF A. VIRIDIS FROM DIFFERENT LOCATIONS WHEN IN THEIR 'HOMOLOGOUS' HOMOGENATES WITH THE PERCENTAGE TRANSLOCATION BY THE SAME SYMBIONTS WHEN IN HOMOGENATE OF A. VIRIDIS (LOUGH HYNE) (N=5) (SIGNIFICANT DIFFERENCES IN BOLD WITH ASTERISK).

Host sp.	t-test and Mann-Whitney statistics and probability
AvSI	**T=-3.26, P=0.022 ***
AvLS	Values identical, therefore T could not be calculated, P>0.999
AvBm	T=1.35, P=0.25
AvWH	T=-1.13, P=0.29
AvTB	**Mann-Whitney statistic=40.0, P=0.01 ***

TABLE 17 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) COMPARING THE PERCENTAGE TRANSLOCATION BY SYMBIONTS OF A. VIRIDIS FROM THE NAMED LOCATIONS WHEN IN THE PRESENCE OF A HOMOGENATE OF A. VIRIDIS (LOUGH HYNE) (N=5) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	AvLH	AvLS	AvBm	AvWH	AvTB	AvSI
AvLH	-	**4.40 ***	**3.68 ***	1.02	1.85	2.51
AvLS	-	-	0.72	**3.38 ***	2.55	1.89
AvBm	-	-	-	2.66	1.83	1.17
AvWH	-	-	-	-	0.83	1.49
AvTB	-	-	-	-	-	0.67
AvSI	-	-	-	-	-	-

TABLE 18 THE RESULTS OF TUKEY'S TEST (CONFIDENCE INTERVALS) COMPARING THE PERCENTAGE TRANSLOCATION BY SYMBIONTS FROM A. VIRIDIS (LOUGH HYNE) WHEN INCUBATED IN HOMOGENATES OF A. VIRIDIS FROM THE NAMED LOCATIONS (N=5) (SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	AvLH	AvLS	AvBm	AvWH	AvTB	AvSI
AvLH	-	**-0.3/ 38.9**	**32.3/ 71.5 ***	**16.0/ 55.2 ***	**-5.0/ 34.3**	**17.0/ 56.3 ***
AvLS	-	-	**12.9/ 52.2 ***	**-3.4/ 35.9**	**-24.3/ 15.0**	**-2.3/ 37.0**
AvBm	-	-	-	**-35.9/ 3.4**	**-56.9/ -17.6 ***	**-34.9/ 4.4**
AvWH	-	-	-	-	**-40.6/ -1.3**	**-18.6/ 20.7**
AvTB	-	-	-	-	-	**2.4/ 41.6 ***
AvSI	-	-	-	-	-	-

TABLE 19 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) OF THE PERCENTAGE TRANSLOCATION IN 'NATURAL' SYMBIOSES, AS ESTIMATED USING THE 'GROWTH RATE METHOD' (N=5, EXCEPT N=3 FOR I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.81	0.77	0.81	1.93	2.63
Ab	-	-	1.58	0.0	2.63	**3.44 ***
AvLH	-	-	-	1,58	1.27	1.86
AvSI	-	-	-	-	2.63	**3.44 ***
Is	-	-	-	-	-	0.35
Ap	-	-	-	-	-	-

TABLE 20 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) OF THE PERCENTAGE TRANSLOCATION, AS ESTIMATED USING THE 'GROWTH RATE METHOD', IN THE REESTABLISHED SYMBIOSES (N=5, EXCEPT N=4 FOR SYMBIOSES INVOLVING SYMBIONTS FROM A. BALLII AND I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.76	2.44	**3.21 ***	**3.24 ***	2.29
Ab	-	-	0.54	1.45	1.41	0.40
AvLH	-	-	-	0.96	0.94	0.15
AvSI	-	-	-	-	0.04	1.12
Is	-	-	-	-	-	1.09
Ap	-	-	-	-	-	-

APPENDIX 13: STATISTICS RELATING TO THE CZAR.

TABLE 1 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE CZAR IN THE 'NATURAL' SYMBIOSES, WHEN CALCULATED USING ^{14}C (N=5, EXCEPT N=3 FOR I. SULCATUS)(CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	1.81	1.27	1.08	2.31	0.31
Ab	-	-	3.08 *	2.88	3.88 *	1.50
AvLH	-	-	-	0.19	1.22	1.58
AvSI	-	-	-	-	1.38	1.38
Is	-	-	-	-	-	2.58
Ap	-	-	-	-	-	-

TABLE 2 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE CZAR IN THE 'NATURAL' SYMBIOSES, WHEN CALCULATED USING THE 'GROWTH RATE METHOD' (N=5, EXCEPT N=3 FOR I. SULCATUS)(CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK)

Host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.85	2.00	1.73	2.65	1.85
Ab	-	-	2.85	2.58	3.38 *	2.69
AvLH	-	-	-	0.27	0.92	0.15
AvSI	-	-	-	-	1.15	0.12
Is	-	-	-	-	-	1.05
Ap	-	-	-	-	-	-

TABLE 3 THE RESULTS OF MULTIPLE COMPARISONS (Z VALUES) BETWEEN THE CZAR IN THE REESTABLISHED SYMBIOSES, WHEN CALCULATED USING THE 'GROWTH RATE METHOD' (N=5, EXCEPT N=4 FOR SYMBIOSES INVOLVING A. BALLII AND I. SULCATUS) (CRITICAL Z VALUE = 2.93, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named host sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.82	1.08	3.31 *	2.74	2.06
Ab	-	-	1.83	3.93 *	3.37 *	2.76
AvLH	-	-	-	2.23	1.72	0.98
AvSI	-	-	-	-	0.38	1.18
Is	-	-	-	-	-	0.80
Ap	-	-	-	-	-	-

TABLE 4 THE RESULTS OF SCHEFFE'S TEST (CONFIDENCE LIMITS) COMPARING THE CZAR/ MG ALGAL C/ MG ASSOCIATION PROTEIN, WHEN CALCULATED USING ^{14}C, BETWEEN THE DIFFERENT REESTABLISHED SYMBIOSES (N=5, EXCEPT N=4 FOR SYMBIOSES INVOLVING SYMBIONTS FROM A. BALLII AND I. SULCATUS, SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	0.16/ 5.18 *	-0.02/ 4.71	0.77/ 5.50 *	-0.01/ 5.01	-0.13/ 4.59
Ab	-	-	-2.83/ 2.18	-2.04/ 2.97	-2.81/ 2.47	-2.95/ 2.07
AvLH	-	-	-	-1.57/ 3.16	-2.35/ 2.66	-2.48/ 2.25
AvSI	-	-	-	-	-3.14/ 1.87	-3.27/ 1.46
Is	-	-	-	-	-	-2.78/ 2.24
Ap	-	-	-	-	-	-

TABLE 5 THE RESULTS OF SCHEFFE'S TEST (CONFIDENCE INTERVALS) COMPARING THE CZAR/MG ALGAL C/ MG ASSOCIATION PROTEIN IN THE REESTABLISHED SYMBIOSES, WHEN CALCULATED USING THE 'GROWTH RATE METHOD' (N=5, EXCEPT N=4 FOR SYMBIOSES INVOLVING SYMBIONTS FROM A. BALLII AND I. SULCATUS. SIGNIFICANT DIFFERENCES (P<0.05) IN BOLD WITH ASTERISK).

Cp + symbionts from named sp.	Cp	Ab	AvLH	AvSI	Is	Ap
Cp	-	-1.68/ 7.13	-0.74/ 7.56	1.47/ 9.78 *	-0.25/ 8.56	-0.32/ 7.98
Ab	-	-	-3.72/ 5.09	-1.51/ 7.30	-3.21/ 6.08	-3.30/ 5.51
AvLH	-	-	-	-1.94/ 6.37	-3.66/ 5.15	-3.73/ 4.57
AvSI	-	-	-	-	-5.87/ 2.94	-5.95/ 2.36
Is	-	-	-	-	-	-4.73/ 4.08
Ap	-	-	-	-	-	-