Regulation of the population of symbionts in *Anemonia viridis*.

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DECLARATION

I declare that the research described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It has not, in whole or in part, been submitted for any other degree.

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SUMMARY

This study was undertaken to investigate the effects of different environmental variables on the association between the temperate anemone, *Anemonia viridis* and its symbionts. The effects of exposure to ammonium enrichment, changes in light intensity, feeding and starvation were studied. Many studies involving tropical associations have addressed this question by monitoring changes in the symbiont population density. However, the symbiont population density can change as a result of changes in the zooxanthella population or changes in the host biomass or host surface area. In addition, the zooxanthella population is determined by the rate of division and the rate at which cells are lost from the population.

Anemones maintained at light intensities of 20 and 300μE.m^{-2}s^{-1} had similar population densities measured as cells.μg host protein^{-1} in tentacles, however the zooxanthella division rate, measured as the mitotic index, increased from 2 to 4% with increasing light intensity within the range 20 to 300μE.m^{-2}s^{-1} after 3 weeks exposure. The specific expulsion rate also increased with light intensity over the range 50 to 300μE.m^{-2}s^{-1} from 0.0003 to 0.002 cells.cell^{-1}.d^{-1}.

Although the mitotic index of zooxanthellae increased with increasing light intensity, the increase was much larger in animals receiving ammonium enrichment for 4 weeks. Under ammonium enrichment, the symbiont mitotic index increased from 2% at 20μE.m^{-2}.s^{-1} to 9% at 300μE.m^{-2}.s^{-1}. The rate of ammonia uptake in *A. viridis* has previously been shown to be determined by the level of illumination and therefore the above observations indicate that the rate of cell division may be limited by low light intensity through limitation of ammonium uptake even under ammonium enrichment.

Gastroderm cells of the tentacles of *A. viridis* were found to contain between 1 and 7 symbiotic algae. Host cells with one symbiont made up the highest proportion of gastroderm cells from control animals while in the ammonium enriched anemones, host cells with two symbionts made up the highest proportion of gastroderm cells.

The mitotic index of zooxanthellae *in hospite* at 300μE.m^{-2}s^{-1} increased with increasing ammonium concentration from 4% in control sea water to 8% at 50μM ammonium. This ammonium concentration is far greater than that which is found in temperate coastal waters.

When anemones were maintained in sea water enriched to 20μM ammonium for 80 days, the symbiont population density normalised to host protein did not change significantly from 1120

cells.μg protein^{-1}. The total zooxanthella population increased only temporarily by 14% from 2 to 2.3 x 10^8 cells before decreasing to the pre-treatment level. Overall, the zooxanthella population had increased by 4% while the host weight had increased by 11% and host protein content had increased by 19%.

Ammonium enrichment over 80 days led to a significant increase in host weight. These results show for the first time in symbiotic anthozoans that the flux of nitrogen compounds from zooxanthellae to host is sufficiently high to permit host growth.

Host feeding over 80 days led to a 46% increase in host weight but only a 7% increase in the total zooxanthella population. The percentage of animal biomass made up of protein had not changed after 80 days with weekly feeding. This resulted in a significant decrease in the zooxanthella population density.

The slight decrease in the symbiont population density, measured as cells.μg protein^{-1} and cells.μg dry weight^{-1} in starved animals after 80 days was due to a 28% decrease in host biomass, a substantial loss of zooxanthellae (35% of the population) and a 37% decrease in animal protein content. During starvation the mitotic index of zooxanthellae remained at 4% over 80 days. Since there was no change in the mitotic index it was assumed that the fall in the population was brought about by an increase in the rate of loss of zooxanthellae. From results obtained in this study, expulsion of symbionts in recognisable form probably accounts for only a small proportion of the decrease in the population.

Enrichment of sea water to 20μM ammonium increased the zooxanthella mitotic index to a maximum of 8% for 35 days. After that time, the initially rapid peak in mitotic index began to subside, but division rate still remained elevated for a further 45 days. Feeding stimulated the zooxanthella mitotic index, compared to starved animals, suggesting that some of the ingested nitrogen could be made available to the symbionts. The increase in mitotic index with weekly feeding was less rapid than the increase under ammonium enrichment, however the long term increase in the mitotic index of zooxanthellae from fed *Anemonia viridis* was similar to the effect of 20μM ammonium enrichment.

The zooxanthella mitotic index was inversely related to body mass. This may be interpreted in relation to host growth leading to the creation of space for symbionts to occupy, assuming that small anemones have a higher relative growth rate than larger anemones.

The zooxanthella mitotic index was found to be inversely proportional to zooxanthella population density in anemones maintained in control sea water. In addition, zooxanthellae

from partially aposymbiotic *A. viridis*, containing 50% of the original symbiont population had higher mitotic index values than those of naturally symbiotic anemones when maintained under similar light intensities. These observations could result from competition between zooxanthellae for available nitrogen for cell division. Alternatively, they may also be interpreted in relation to availability of intra-cellular "space" for the symbionts to occupy. The increase in mitotic index and regrowth in the population of zooxanthellae in the light in partially aposymbiotic anemones, containing 50% of the initial zooxanthella population, was enhanced by the addition of ammonium to the sea water. This suggests that competition for nitrogen is an important factor in limiting growth rate at high symbiont densities. However, zooxanthella growth rates in partially aposymbiotic anemones were greater than those in symbiotic anemones when both groups were maintained with ammonium enrichment and these results suggest that nitrogen is not the only factor limiting to zooxanthella growth at high symbiont population densities but that intra-cellular "space" available to the zooxanthellae may limit symbiont growth rates.

When animals were maintained in continuous darkness, division rates of symbionts, measured as the mitotic index, from fed and starved animals, had not changed significantly over 66 days. It is suggested that the zooxanthellae were deriving nutrients from the host to support this division in darkness.

For animals maintained in control sea water, the percentage of symbionts added through division that were expelled ranged from 0.8 to 5.5%, with an average value of 3.4%. In animals maintained in sea water enriched with ammonium, this percentage was higher and ranged from 7 to 15% with a mean of 11%. A positive correlation was found between the expulsion rate normalised to animal weight and the mitotic index of zooxanthellae within *A. viridis*, however the above values of 3.4 and 11% would appear to be insignificant for expulsion to be a population control mechanism. Therefore, there must be an additional, more effective mechanism for removing symbionts. The possibility that cells are digested either intra-cellularly or within the coelenteron or mesenteries has not been ruled out. In addition, specific growth rates may be overestimated using the current measure of the mitotic index. As mentioned in Chapter 7 accurate estimation of specific growth rates relies on being able to determine the duration of particular phases in the cell cycle and being able to predict how the duration of each phase changes under different environmental conditions. Preliminary observations suggest that the duration of different phases of cell division of symbionts in *A. viridis* changes under different environmental conditions and these data question the accuracy

of the doublet technique for measuring the mitotic index and specific growth rate of symbionts.

CHAPTER 1

INTRODUCTION

1.1 The Distribution of symbioses between anthozoans and dinoflagellates.

Symbiotic associations exist between micro-algae and invertebrates of the phyla Cnidaria, Mollusca, Platyhelminthes, Protozoa and Porifera (Table 1.1). The benefits of such mutualistic relationships are best seen in the coral reef ecosystems of the shallow coastal waters of the tropics. It is commonly believed that the ecological success in terms of richness and diversity of coral reef ecosystems is due to the association between the corals and their dinoflagellate symbionts.

Most of the known host species of the Cnidaria which contain symbiotic algae are tropical, although symbioses also exist in the temperate anthozoans of the orders Actiniaria, Zoanthidea and Hydroida. Marine species found around Britain include the actinians *Anemonia viridis*, *Anthopleura ballii*, *Cereus pedunculatus*, (Manuel, 1981) and the zoanthid, *Isozoanthus sulcatus* (Manuel, 1979). These species have only been found on the West coast, ranging from the north of Scotland to the English Channel. For most temperate European species, Britain lies at the northern extreme of their geographical distribution.

Despite the high genetic diversity of host species in invertebrate symbioses, there are relatively few genera of algal symbionts. Algae of the genus *Chlorella* of the class Chlorophyceae are found in animals from 5 different orders. The dinoflagellates, *Symbiodinium* and *Amphidinium* occur in symbioses with animals from 7 different orders (Table 1.1). Dinoflagellates found in association with invertebrates were originally called "zooxanthellae" by Brandt (1881) and are still referred to as such today. Zooxanthellae from cnidarian host species appear morphologically very similar. Due to observed similarities of symbionts from different species, dinoflagellates from many cnidarian hosts were originally identified as a single species, *Symbiodinium microadriaticum* (Freudenthal, 1962). The idea that the one species, *S. microadriaticum*, exists in a wide range of host taxa has gradually been eroded through research in the last ten years which has shown that there is a higher genetic diversity amongst symbionts than was originally thought. In the last 6 years, 9 new species of *Symbiodinium* have been distinguished (Table 1.1). Dinoflagellates from 5 genera have now been recognised based on differences in characteristics such as chromosome number, the number of pyrenoids and pyrenoid stalks per cell and the nature of the photosynthetic pigments (Blank, 1990).

In anthozoans, zooxanthellae are located in the gastroderm layer throughout the animal's body, whilst in the zoanthid genus *Palythoa*, they are found in the mesoglea and in epidermal cells (Trench, 1971a). In symbiotic rhizostome scyphozoans, the zooxanthellae are located in the mesoglea, as in *Cassiopeia xamachana* (Kevin *et al*, 1969) and *Mastigias* sp., while in the

hydroid *Myrionema amboinense*, the zooxanthellae are found mostly in the digestive cells displaced towards the basal end of each gastrodermal cell (Fitt & Cook, 1989). Electron microscopical studies by Taylor (1968) and Glider *et al* (1980) showed that the zooxanthellae of *Anemonia sulcata* (= *viridis*) and *Aiptasia pallida*, are located in vacuoles within the host gastrodermal cells. In contrast, symbionts in associations with animals from the phylum Mollusca, are extracellular.

Table 1.1 Symbiotic Chlorophyceae and Dinophyceae found in a range of hosts.

Symbiont	Original host sp. identified	Order	Reference
Chlorophyceae			
Chlorella sp.	*Hydra viridis*	Hydrozoa	Oschman, 1967
Chlorella sp.	*Stentor polymorphus*	Ciliata	Reisser, 1981
Chlorella sp.	*Spongilla sp.*	Porifera	Reisser, 1984
Chlorella sp.	*Anodonta sp.*	Bivalvia	Pardy, 1980
Chlorella sp.	*Anthopleura xanthogrammica*	Actiniaria	O'Brien, 1978
Dinophyceae			
Symbiodinium spp.			
S. microadriaticum	*Cassiopeia xamachana*	Scyphozoa	Freudenthal, 1962
S. microadriaticum	*Cassiopeia frondosa*	Scyphozoa	Blank & Trench, 1986
S. goreauii	*Ragactis lucida*	Actiniaria	Trench & Blank, 1987
S. kawagutii	*Montipora verrucosa*	Scleractinia	Trench & Blank, 1987
S. pilosum	*Zoanthus sociatus*	Zoanthidea	Trench & Blank, 1987
S. corculorum	*Corculum cardissa*	Bivalvia	Banaszak *et al*, 1993
S. meandrinae	*Meandrina meandrites*	Scleractinia	Banaszak *et al*, 1993
S. pulchrorum	*Aiptasia pulchella*	Actiniaria	Banaszak *et al*, 1993
S. cariborum	*Condylactis gigantea*	Actiniaria	Banaszak *et al*, 1993
S. bermudense	*Aiptasia tagetes*	Actiniaria	Banaszak *et al*, 1993
S. californium	*Anthopleura elegantissima*	Actiniaria	Banaszak *et al*, 1993
Symbiodinium sp.	*Pteraeolidea ianthina*	Gastropoda	Rudman, 1981
Amphidinium spp.			
A. klebsii	*Amphiscolops langerhansi*	Turbellaria	Taylor, 1971
A. belauense	*Haplodiscus sp.*	Turbellaria	Banaszak *et al*, 1993
Scrippsiella velellae	*Velella velella* (Pacific)	Hydrozoa	Banaszak *et al*, 1993
S. challonii	*V. velella* (Mediterranean)	Hydrozoa	Banaszak *et al*, 1993
Gloeodinium viscum	*Millepora dichotoma*	Hydrozoa	Banaszak *et al*, 1993
Prorocentrum concavum	*Amphiscolops sp.*	Turbellaria	Yamasu, 1988

1.2 The importance of symbionts to the host.

It has been well established that the host obtains nutritional benefits from the association (Muscatine & Cernichiari, 1969; Smith, 1979), although the exact nature of the nutritional benefits received by the host is controversial and still not completely understood. Indeed, the benefits to host and symbiont vary greatly according to environmental conditions of irradiance and feeding.

1.2.1 Carbon translocation.

Yonge & Nicholls (1930a) considered the importance of the symbionts to their host by looking at the effects of feeding and starvation in the light and dark on 8 species of Madreporarian corals. When the corals were fed in the light, all corals remained healthy for 218 days. In contrast, starvation in the light led to massive extrusion of zooxanthellae, a paling of the host tissues, loss in weight, and the appearance of degenerate zooxanthellae within the tissues, especially within the mesenterial filaments. In addition, all corals fed in the dark lost their zooxanthellae but remained healthy for up to 150 days. These observations led the authors to conclude that these species obtained no nourishment from their zooxanthellae and that survival of the coral did not appear to be enhanced by the presence of symbionts. However, studies by Franzisket (1970) showed that 4 species of hermatypic corals kept in the light in zooplankton-free sea water grew as fast as those in unfiltered sea water. Corals kept in darkness died despite being supplied with unfiltered sea water. This study showed that the host must depend on the supply of translocated carbon to supplement its nutritional requirements. The above studies and, indeed, more recent studies, can serve to illustrate that different species of anthozoan depend on autotrophy of the symbionts and heterotrophy to different degrees for nutritional input. Direct evidence for carbon translocation to the host came from studies using ^{14}C-labelled precursors. Muscatine & Cernichiari (1969) showed that when $H^{14}CO_3^-$ was added to the incubation medium of corals, the label was subsequently incorporated into host lipid and protein. Later quantitative studies, showed that symbionts can potentially fix and translocate more than enough organic carbon during photosynthesis for host respiration as well as their own respiration. In corals living in shallow water on a sunny day, between 90 and 95% of photosynthetically derived carbon was predicted to pass out of the symbionts and made available to the host for respiration (Davies, 1984). Energy budgets calculated for corals living at 3m and 10m on a sunny day showed that between 91% and 78% respectively, of photosynthetically derived energy passes out of the algae and is made available for host respiration (Edmunds & Davies, 1986; Davies, 1991). Around 42% of the total fixed carbon of symbionts of *Anemonia viridis* is translocated to the host (Tytler & Davies, 1986). Thus, symbiotic hosts have a competitive advantage over

non symbiotic hosts in that they have access to an additional source of carbon to that obtained by heterotrophic feeding.

1.2.2 Nutrient uptake, translocation and assimilation.

Zooxanthellae may obtain inorganic nutrients from the products of the host's catabolism and by diffusion from the sea water. Studies have shown that symbiotic Cnidaria take up ammonium from the surrounding medium in contrast to aposymbiotic and non-symbiotic cnidarians, which excrete ammonium. This supports the view that the symbionts are responsible for ammonium uptake and assimilation (Muscatine & D'Elia, 1978; Muscatine *et al,* 1979; Burris, 1983; Wilkerson & Muscatine, 1984). Ammonium uptake has been shown to be light dependent, which also implies that the process is mediated by the symbionts. At low light levels and after prolonged darkness, symbiotic anemones excrete ammonium, while, at high light intensities, uptake is enhanced (Wilkerson & Muscatine, 1984; Davies, 1988). It has been proposed that the zooxanthellae bring about a net ammonium uptake by active depletion and assimilation of the host excretory ammonium, promoting diffusion of ambient ammonium into the host cytoplasm along a concentration gradient (D'Elia *et al,* 1983).

Further work investigating the nitrogen assimilation enzyme pathways and their relative activities in each component of the associations supports the idea of nitrogen assimilation by the symbionts. The two potential pathways by which ammonium may be incorporated into amino acids in host and symbiont involve:

1. Glutamine synthetase (GS). This catalyses the reaction between ammonium and glutamate which is irreversible (Summons & Osmond, 1981). The formation of glutamine is followed by the transfer of the amido nitrogen to α-ketoglutarate by the enzyme glutamine:α-ketoglutarate aminotransferase (GOGAT). For each mole of ammonium and glutamate assimilated by GS, two moles of glutamate are produced by GOGAT, one of which can be recycled by GS. GS is present in both animal and plant components of the symbiosis but the enzyme GOGAT is thought to be unique to plants (Falkowski, 1983).

2. NADH specific glutamate dehydrogenase (GDH) which is found in animal and plant cells (Rees, 1987).

A number of authors have shown GS to be present in the algal component of cnidarian symbioses (Wilkerson & Muscatine, 1984; Summons *et al,* 1986; Gunnersen *et al,* 1988). GOGAT has not been found in symbiotic anemones, however some evidence exists for its presence in corals. Rahav *et al* (1989) found that when *Stylophora pistillata* was incubated with azaserine, a GOGAT inhibitor, the corals excreted ammonium. They concluded from this that the symbionts were the main ammonium assimilators via the GS / GOGAT pathway.

Rees (1987) suggested that the host was responsible for nitrogen assimilation in the *Hydra-Chlorella* symbiosis. He attributed the observation that symbiotic hosts, in contrast to aposymbiotic hosts, do not excrete ammonium, to the possibility that the host shows an increased capacity to assimilate ammonium when symbionts are present in order to control their numbers. This could explain why aposymbiotic hosts showed very low levels of GS activity in comparison to symbiotic host tissue (Rees, 1987). Further evidence for host assimilation of ammonium in the *Hydra-Chlorella* symbiosis came from the observations that DCMU, a photosynthetic inhibitor, did not stimulate release of ammonium from symbiotic *Hydra* (Rees, 1987).

Miller & Yellowlees (1989) found that levels of GDH were much higher in host tissue than in symbionts of marine cnidarians, and did not find GS in either host or symbiont. They concluded that the host was responsible for ammonia assimilation via GDH. However, Muscatine *et al* (1979) found that aposymbiotic corals do not significantly assimilate external ammonium directly implying that host tissue could not assimilate inorganic nitrogen. The reverse reaction involving GDH has been implicated in ammonium excretion rather than assimilation in a variety of marine invertebrates (Rahav *et al*, 1989).

In tropical symbioses, it is thought that most of the symbionts' nutrient requirements are provided by host catabolism (Rahav *et al* 1989; Falkowski *et al* 1993). Nitrogen has been studied as the nutrient most likely to be limiting. One source of nitrogen available to the zooxanthellae may be host excretory ammonium, produced through the deamination of glutamate by the enzyme GDH. Rahav *et al* (1989) constructed a nitrogen budget for *Stylophora pistillata* based on the estimation of host excretory nitrogen which is passed to the zooxanthellae. They calculated that 90% of the nitrogen assimilated by the symbionts was generated by the recycling of host excretory nitrogen and the remaining 10% of the nitrogen came from external sources.

Further evidence that the zooxanthellae are capable of assimilating the products of host catabolism and translocating assimilated nitrogenous products back to the host comes from studies with ^{14}C-Carbon and ^{15}N-Nitrogen isotopes (Muscatine & Porter, 1977; Muscatine, 1980; Taylor, 1983). Marian (1979) demonstrated that ^{15}N fed to *Anthopleura elegantissima* as labelled protein was later detected in the zooxanthellae.

Additional sources of nitrogen may include amino acids. It has been shown that corals can take up dissolved free amino acids from nanomolar concentrations in sea water (Drew Ferrier, 1991). Amino acid pools in anemone tissues are high (Shick, 1991), and Carroll & Blanquet (1984a) demonstrated that zooxanthellae, freshly isolated from *Cassiopeia xamachana*, could take up alanine. The extent of amino acid uptake when in symbiosis is not known, but may be confined to periods of darkness.

When zooxanthellae isolated from *Agaricia agaricites* were incubated with ^{14}C-$NaHCO_3$, the major component was glycerol, with traces of glucose and alanine. If zooxanthellae were incubated with ^{14}C glucose *in vitro*, however, the predominant product released from the symbionts was alanine (Muscatine *et al*, 1972). Lewis & Smith (1971) showed, by an inhibition technique, that ^{14}C-labelled alanine from zooxanthellae passed from the host tissue in to alanine-enriched sea water. They found that the amount of alanine released by the symbionts could be increased if the concentration of ammonium in the incubation medium was increased. Marian (1979) demonstrated that $^{15}NO_3$-N was taken up by zooxanthellae in *Pocillopora damicornis* and accumulated in organic form in the animal fraction. In general, estimates of the percentage of fixed ^{14}C-Carbon released as amino acids to different host species vary between 1 and 7% (Trench, 1971b). However Muscatine *et al* (1972) reported the percentage as high as 22% for *Agaricia agaricites*. Quantitative studies estimating the relative contribution of symbiont-derived amino acids to host nitrogen requirements, and therefore its importance as a nitrogen source, are lacking.

The above studies show that the relatively small amount of nitrogenous material translocated from the symbionts is predominantly of alanine, a non-essential amino acid. There is little evidence for the translocation of essential amino acids, despite evidence for the manufacture of essential amino acids by the zooxanthellae. More recently, however, Markell & Trench (1993) found that cultured zooxanthellae released essential amino acids in very small quantities. Rahav *et al* (1989) suggested host predation may be needed as a source of essential amino acids for the host.

Alternative to the idea of nitrogen recycling, Rees (1987) suggested an indirect role of symbionts in the nitrogen economy of the host in the green Hydra symbiosis through nitrogen conservation. This idea is consistent with the suggestion by Rees that the host provides the major ammonium assimilatory pathway (Rees, 1987). Nitrogen conservation would operate by diverting host amino acids away from gluconeogenesis and respiration and therefore increase the supply of amino acids to protein synthesis. The diversion of amino acids from respiration would be dependent on the release of photosynthate by the symbionts (Rees, 1987). He suggested that this mechanism may also apply to the role of zooxanthellae in marine symbioses.

Whether nitrogen recycling or conservation is shown to be acting in cnidarian symbioses, either pathway would be important for species living in oligotrophic waters in conferring on them a competitive advantage over non-symbiotic species, in their ability to exploit and grow rapidly through more efficient utilisation of potentially limiting nitrogen resources.

1.3 Constancy of the symbiont population.

A notable feature of symbiotic associations is the observation of a constant ratio of symbiont:host cell numbers or biomass under a given set of environmental conditions (Taylor, 1969b; Muscatine & Pool, 1979). This has been observed for symbioses involving both *Chlorella* and *Symbiodinium*. A steady state ratio of numbers of *Chlorella* to its host *Hydra viridis* was maintained if illumination and feeding conditions remained unchanged. For example, *Hydra* kept in the light without feeding, maintained a population of 20-25 algae per host cell, but *Hydra* kept in the dark with feeding maintained only 3 to 6 algae per host cell (Muscatine *et al*, 1975). Data from a number of studies on Anthozoa have shown that the density of symbionts range from 0.37 to 2.8 x 10^6 cells.mg protein^{-1} in corals (Smith & Muscatine, 1986; Hoegh-Guldberg & Smith, 1989b) and from 0.5 to 3.52 x 10^6 cells.mg protein^{-1} for anemones, depending on environmental conditions (Clayton & Lasker, 1984; Muller-Parker, 1985; 1987; Cook *et al*, 1988; Berner *et al*, 1993). Densities of zooxanthellae in corals, standardised to coral surface area, range from 0.2 to 4.5 x 10^6 cells.cm^{-2} (McCloskey & Muscatine, 1984; Hoegh-Guldberg & Smith, 1989b; Stambler *et al*, 1991). Data from many studies suggest that symbiont population densities are no less variable within a species than within the range for all anthozoans. However, some anthozoans have been studied more than others and therefore the range of symbiont densities recorded for these anthozoans has tended to be greater.

The observed constant ratio of symbiont:host numbers can be restored in experimentally induced aposymbiotic hosts if they are returned to conditions in which they were previously maintained. Re-infection studies carried out on anemones have shown that symbiont numbers increase until the cell density is similar to those of native associations, thus re-establishing the symbiont:host biomass ratio (Schoenberg & Trench, 1980; Berner *et al*, 1993).

There are potential benefits to both partners of a symbiosis which maintains a constant optimum ratio of symbiont:host biomass. A low symbiont population density would result in a reduced net carbon supply to the host. A high symbiont density would ensure a high carbon supply to the host. However, high algal densities may result in increased competition by the symbionts for nutrients or light, leading to self-shading (Crossland & Barnes, 1977; Jokiel & Morrissey, 1986; Trench, 1987; Hoegh-Guldberg & Smith, 1989b). High symbiont densities have been shown to lead to a lowered photosynthetic productivity per symbiont, which could lead to a proportionately lowered supply of carbon to the host (Hoegh-Guldberg & Smith, 1989b; Falkowski *et al*, 1993). Zooxanthellae also contain pigments and amino acids important in the defence of the host against the damaging effects of ultra-violet radiation. The abundance of these compounds may be affected by the symbiont population density. It would, therefore, be to the host's advantage for the ratio of symbiont:host numbers to be maintained at a level which is optimum with regard to all the above factors.

1.4 Regulation of the symbiont population.

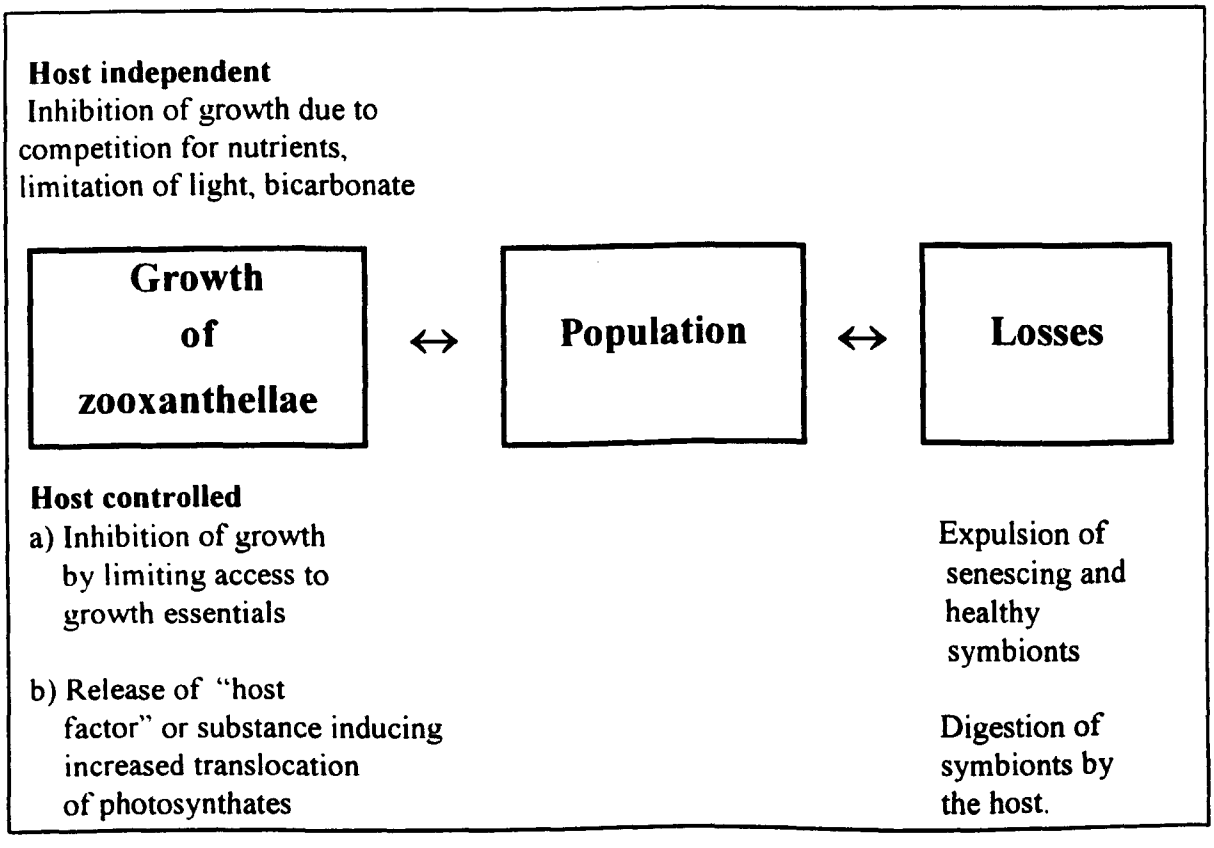
The symbiont:host biomass ratio is generally maintained as the host continues to grow, illustrating that the balance is maintained in a dynamic state, with both host and symbionts growing continuously, but neither 'outgrowing' the other. These observations led some authors to suggest that some form of regulation of the symbionts' growth is occurring within the host (Muscatine & Pool, 1979).

Mechanisms which could potentially control the symbiont population density can be divided into 2 groups:

1) those factors which may act by suppressing or controlling growth rate, and
2) those which may act on the population to remove excess symbionts.

Figure 1.1 illustrates the idea that the steady state symbiont population would be determined by the inputs and losses to the population, and by the maximum capacity (= magnitude of algal density) of the system, which could be determined by a variety of factors such as ambient light, host nutrition and host biomass (Muscatine *et al*, 1986). It is probable that several factors could operate at once to control the population.

Figure 1.1 Potential mechanisms controlling the symbiont population level within anthozoans.



Indirect evidence suggesting that the growth rate of symbionts *in situ* is suppressed comes from a comparison of growth rates of symbionts *in situ* and *in vitro* (Jolley & Smith, 1978). *Chlorella*, isolated from *Hydra* grew 30 times faster than symbionts *in situ* (Jolley & Smith, 1978). Similarly, *Symbiodinium* spp. isolated from tropical corals and anemones grown in culture had specific growth rates between 0.22 and 0.43 cells.cell^{-1}.d^{-1} (Chang *et al*, 1983; Fitt & Trench, 1983a). These are similar to growth rates of free-living phytoplankton species in the North Pacific (see Chapter 4 Table 4.10), but between 3 and 30 times greater than specific growth rates of *Symbiodinium, in situ*, which range from 0.01 to 0.08 cells.cell^{-1}.d^{-1} (Weiler & Chisholm, 1976; Wilkerson *et al*, 1983; Muller Parker, 1985, 1987; Cook *et al*, 1988). These differences in symbiont growth rates might suggest that algal growth in the host is suppressed.

Muscatine *et al* (1985) calculated specific growth rates of symbiont from *Stylophora pistillata* to be between 3 and 9 times the specific growth rates of host tissue. They concluded from these values that there must be mechanisms which remove excess algae to maintain a constant population. So called "post-mitotic" control mechanisms could include expulsion and/or digestion of algae.

Mechanisms implicating the active involvement of the host in restricting the supply of essential nutrients or inducing the leaching of large amounts of photosynthate from the symbionts have been suggested by several authors. Whether regulation can be initiated by the host or is a result of a balance between natural potentially limiting factors, or a combination of both, is not completely understood.

1.4.1 Factors suppressing or controlling growth rate.

Nutrient limitation.

Coral reefs commonly exist in oligotrophic waters where levels of dissolved inorganic nutrients are very low. Typically, concentrations of ammonium and phosphate at reef sites are often at the limits of detection, ranging from 0.2 to 1.4 μmoles.litre^{-1} and 0.15 to 0.6 μmoles.litre^{-1} respectively (Kinsey & Davies, 1979; Cook *et al* 1988; Muscatine *et al*, 1985; 1989). The availability of nutrients may, therefore, be a factor determining the upper limit of the symbiont population density. This led to the suggestion that symbiont growth is nutrient limited (Cook & D'Elia, 1987). Nitrogen and phosphorus are being investigated as potential limiting nutrients in symbioses involving cnidarians and molluscs. Sulphate has been investigated as a potential limiting nutrient involved in the *Hydra-Chlorella* symbiosis (Pool, 1976).

When micro-algae are exposed to situations in which they experience a deficiency in one or more nutrients, they display characteristic physiological changes. These include a relatively

high carbon:nutrient (nitrogen or phosphorus) ratio, decreased cellular photosynthetic and respiration rate, decreased chlorophyll and protein content, increased carbohydrate/lipid content relative to protein content, (Rees, 1991). In studies involving symbiotic associations, changes in these characteristics, brought about by the addition of potentially limiting nutrients, have been taken as evidence for nutrient limitation. An increase in symbiont population density and growth rate following incubation with an increased supply of nutrients have been used as evidence of nutrient limitation by Cook *et al* (1988), however some studies have produced conflicting results on the effects of ammonium enrichment. The effect of nutrient enrichment on some of the above characteristics are mentioned below.

The effect of ammonium enrichment on the population density.

Muscatine *et al* (1989) measured the zooxanthella population density in *Stylophora pistillata* from the Red Sea. They found that when the external concentration of ammonium was increased from undetectable levels to 20 μM, after 14 days the population density was almost double that of the control group - 1.96 x 10^6cells.cm^{-2} compared with 1.11 x 10^6cells.cm^{-2} for the controls. A similar observation was made by Hoegh-Guldberg & Smith (1989b) in *S. pistillata*. After 21 days of ammonium enrichment, the density of zooxanthellae from *S. pistillata* had increased from 0.55 to 1.49 x 10^6cells. mg protein^{-1}. However, the population density of zooxanthellae in *Seriatopora hystrix* maintained with ammonium enrichment in a similar experiment by Hoegh-Guldberg & Smith (1989b) had not increased significantly. Stambler *et al* (1991) reported > 200% increases in the population density of zooxanthellae in *Pocillopora damicornis* kept for 13 days in 15μM ammonium enriched sea water. However, in another paper, Stambler *et al* (1994) report that ammonium enrichment did not affect the symbiont population density of *P. damicornis*.

The effect of ammonium enrichment on symbiont division rate.

When the tropical anemone, *Aiptasia pallida*, was incubated in ammonium enriched sea water, the growth rate of the symbionts, calculated from the mitotic index, increased to 3 times its pre-treatment values within one week of enrichment before decreasing gradually (Cook *et al*, 1988). Muscatine *et al* (1989), however found that ammonium enrichment had not affected the mitotic index of zooxanthellae within *Stylophora pistillata* after 14 days of treatment.

The effect of ammonium enrichment on photosynthetic rates.

Summons *et al* (1986) observed a short-term rise in photosynthetic rate normalised to chlorophyll in corals and tissue slices of symbiotic clams supplemented with ammonium. Similarly, Taylor (1978) found an increased photosynthetic rate of zooxanthellae *in situ* when specimens of *Acropora cervicornis* were enriched with ammonium.

The effect of ammonium enrichment on nitrogen uptake kinetics.

Characteristics of nutrient assimilation of symbiotic associations change under different states of nutrient sufficiency or limitation. An increase in nutrient uptake rate when supplied with the limiting nutrient, is indicative of nutrient limitation (Rees, 1991). Values for K_S for ammonium uptake of zooxanthellae from several studies are less than 1 μmole, which is similar to oligotrophic plankton, indicating a high affinity for ammonium (Muscatine & D'Elia, 1978). The measurement of uptake rates by using radioactive methylamine suggests that the intracellular concentration of ammonium in corals is well below the saturation level for growth of zooxanthellae (Cook & D'Elia, 1987). However, Gunnersen *et al* (1988) found that freshly isolated symbionts from corals had lower ammonium uptake rates than nitrogen starved cultured zooxanthellae which implied that zooxanthellae were not nitrogen limited.

The effect of ammonium enrichment on zooxanthella C:N ratios.

In an environment where the supply of a particular nutrient is limiting to algal division, carbon reserves would accumulate within the cell. Thus, a high carbon reserve and therefore a high C:N ratio is indicative of nutrient deficiency. Nutrient enrichment studies have consistently showed that C:N ratios of zooxanthellae from nutrient supplemented animals are lower relative to field or control samples. Zooxanthellae from unsupplemented *Stylophora pistillata* had a mean C:N ratio of 7.7 compared to a mean ratio of 4.4 for zooxanthellae from ammonium enriched corals (Muscatine *et al*, 1989). Similarly, the mean C:N ratio of zooxanthellae from *Pocillopora damicornis* decreased from 9 to 5 after ammonium enrichment (Snidvongs & Kinzie, 1994).

Rees (1991), however, suggested that in nutrient deficient situations, symbiotic algae may not show C:N ratios characteristic of nutrient deficiency since they release a large proportion of their photosynthate to the host, in contrast to free-living algae which release a very small proportion of fixed carbon (Trench, 1971c; Masuda *et al*, 1994). Therefore zooxanthellae are unlikely to accumulate large photosynthate stores which would give rise to an increased ratio.

As indicated above, there is a large body of evidence suggesting that zooxanthellae are naturally nitrogen limited, although some of the data were inconclusive. It is interesting, therefore, that zooxanthellae should translocate nitrogenous compounds to the host at all.

Suppression of symbiont growth due to host initiated restriction of certain essential nutrients.

Host involvement in the maintenance of the algal population may occur through the regulation of inorganic or assimilatory nitrogen or phosphorus to the symbionts (Cook & D'Elia, 1987; Rees, 1987; 1989). This was suggested as a possible mechanism for the regulation of *Chlorella* symbionts within *Hydra viridis* (Pool, 1976; Muscatine & Pool, 1979). Rees (1987) suggested that *Hydra* may regulate the size of its symbiont population by restricting the

ammonium or amino acid supply to the *Chlorella* symbionts. His evidence came from data indicating that the host assimilated ammonium, together with observations that *Chlorella* was nitrogen limited. *Chlorella* possess low growth rates and a high carbohydrate content which are characteristic of *Chlorella* in nitrogen-deficient cultures, in contrast with nitrogen-sufficient cultures where the major component of the biomass is protein (Myers, 1980; Syrett, 1981). McAuley suggested that division of *Chlorella* is regulated through the restriction of the supply of the amino acid arginine by a change in the perialgal vacuolar pH, since arginine uptake by *Chlorella* was inhibited at low pH (McAuley, 1986b).

Few studies exist to suggest that these mechanisms could operate in marine symbioses. However, two studies provide some evidence of a possible regulatory mechanism involving alanine. Alanine seems to be the major amino acid found to be translocated from alga to host so one might envisage a potential control mechanism based on the supply of alanine. The uptake of ^{14}C-alanine by isolated symbionts from *Cassiopeia xamachana*, *Aiptasia pallida* and *Condylactis gigantea* was suppressed by host tissue homogenates (Carroll & Blanquet, 1984b; Blanquet *et al*, 1988). There is, however, no evidence to suggest that algae *in hospite* take up alanine from the host.

Further evidence that anthozoan hosts may potentially be able to control the flux of substances to the symbionts exists. The host vacuolar and algal membranes surrounding zooxanthellae have ATP-ase activity, suggesting both membrane systems are capable of selective transport processes (Rands *et al*, 1993). Both animal and alga could potentially control the flux of nutrients across the symbiotic interface. Rands *et al* (1993) suggested that the pH of the interface between host and alga may influence whether ammonia exists in mostly ionised or unionised form, and may therefore control its flux across the host:alga interface.

Suppression of symbiont growth by host factors increasing photosynthate leaching.

Symbiotic *Chlorella* excreted maltose to the host. In contrast, cultured *Chlorella* (Smith, 1987) did not. This maltose excreting property was restored after re-infection into *Hydra* (Jolley & Smith, 1978) suggesting that the host induces maltose excretion. Douglas & Smith (1984) found that maltose release of symbiotic *Chlorella* was pH dependent, such that at pH4, when maltose release was at a maximum, growth of *Chlorella* was prevented. These authors proposed that regulation of cell division of *Chlorella* occurred through small reversible changes in the pH of the perialgal vacuole.

Studies on symbionts of *Acropora cervicornis*, freshly isolated or *in hospite*, showed that the percentage of fixed excreted carbon increased with increasing external ammonium concentration (Taylor, 1978). Taylor postulated that intracellular ammonium levels may

function as part of a mechanism controlling the translocation of photosynthates in symbiosis by altering the pH and affecting membrane permeability. Smith (1987), however, reported that the release of photosynthates in anthozoans was not influenced by pH.

Zooxanthellae, isolated from the coral, *Agaricia agaricites*, and incubated with a homogenate of host tissue, released twice as much photosynthate as control isolates in sea water (Muscatine *et al*, 1972). Similarly, algae isolated from *Anthopleura elegantissima* incubated with host homogenate released 59% of the total ^{14}C fixed, while algae incubated in sea water released 31% of the total ^{14}C fixed (Trench, 1971c). These studies have investigated the effects of host homogenate on aspects of symbiont translocation but not on aspects of symbiont productivity or growth rate. Only recently, however, has the substance responsible been identified as a group of free amino acids (Gates *et al*, 1995).

Whether increased translocation rates may act as the mechanism regulating the symbiont population could be tested, perhaps, by looking at the effect of increased translocation, induced by host homogenate, on growth rates of isolated symbionts. Smith (1986) did not find a significant correlation between translocation rate and symbiont growth rate of *in situ* symbionts of *Aulactinia stelloides*, however many factors may influence both translocation rate and symbiont growth rate *in situ*.

Density dependent division of symbionts.

Hoegh-Guldberg *et al* (1986) and Hoegh-Guldberg & Smith (1989b) observed an inverse relationship between symbiont growth rates and population density. They suggested that there may be some feature of the host cell environment which inhibits zooxanthella division at high population densities.

McAuley found a good correlation between total algal volume and animal host protein in the *Hydra-Chlorella* symbiosis and suggested that symbiont division could be limited by the amount of space (i.e. host cell volume) available for colonisation (McAuley, 1981b). Douglas & Smith (1984), however, reported that only 50% of the variation in algal cell volume could be accounted for by host cell volume.

Alternatively, it has been suggested that density dependent division may be consistent with the dependence of algal division on the supply of a factor required for algal division, which at high symbiont densities, would be limiting due to increased competition. Cook & D'Elia (1987) suggested that low densities of zooxanthellae, as in partially aposymbiotic animals would have faster growth rates than symbionts in hosts with high symbiont densities because there would be less competition for available nutrients. It is possible that high population densities may also induce self shading (Hoegh-Guldberg & Smith, 1989b), or carbon dioxide limitation (Weis, 1991), both of which may limit symbiont growth rates. If algal growth were

nutrient limited only at high population densities, it would be expected that the symbionts might display physiological signs of limitation at high densities but not at low densities.

Synchronisation of host and symbiont division.

Work carried out on the *Hydra-Chlorella* symbiosis prompted McAuley (1981a) to suggest another regulatory mechanism. He observed that *Chlorella* do not divide unless host cell division has been initiated, either during regeneration or by feeding. He suggested that symbiont division was closely related to or controlled by host cell division, and that control may involve a substance preventing symbiont division. He reported that during regeneration, symbionts completed their division 24-36 hours before the host cell, whereas during feeding, host and algal division occurred simultaneously. This suggests that a chemical stimulus might be involved, rather than the initiation of symbiont division due to an increase in host cell volume.

Factors limiting Photosynthesis.

Light is required for ammonium and phosphate uptake by free-living algae (Syrett, 1981) and, similarly, by symbionts *in situ* (D'Elia, 1977). Exposure to prolonged darkness following a period in the light leads to the eventual excretion of ammonium, after an initial period of uptake (Wilkerson & Muscatine, 1984). The duration of the initial ammonium uptake in darkness by cnidarians was found to depend on the length of the preceding light period (Muscatine & D'Elia, 1978; Davies, 1988). Photosynthesis is thought to provide a source of fixed carbon which is required for nitrogen fixation (Syrett, 1981). It has been suggested that the duration of ammonium uptake in darkness is proportional to the supply of carbon skeletons, which would depend on the duration of photosynthesis (Wilkerson & Muscatine, 1984; Wilkerson & Trench, 1986). Photosynthetic rates of *Anemonia viridis* increased with light intensity up to an asymptote corresponding to light saturation (Tytler, 1982; Tytler & Davies, 1984). It was, therefore, suggested that ammonium uptake might be proportional to light intensity. This was confirmed by Davies (1988), who showed that rates of ammonium uptake by zooxanthellae within *A. viridis* were proportional to light intensity within the range 50-300 $\mu E.m^{-2}s^{-1}$. Below 50 $\mu E.m^{-2}s^{-1}$, *A. viridis* showed a net efflux of ammonium, and above 300 $\mu E.m^{-2}s^{-1}$, the relationship became curvilinear. Since symbiont division is dependent on nitrogen, light intensity may potentially be a limiting factor of symbiont growth rates through the limitation of dissolved inorganic nitrogen uptake rates.

The rate of photosynthesis may also be limited by bicarbonate availability. Slow rates of water flow may set up diffusion boundary layers which locally deplete bicarbonate concentrations (Dennison & Barnes, 1987).

1.4.2 Regulatory mechanisms acting on the algal population to remove excess symbionts.

Muscatine *et al* (1985) found that specific growth rates of zooxanthellae in the Red Sea coral *Stylophora pistillata* were 3 to 9 fold greater than host specific growth rates and suggested that there must exist a mechanism to remove zooxanthellae from the population. Two mechanisms which may maintain the population level through the removal of excess symbionts have been suggested. These are:

1) the expulsion of excess symbionts
2) the digestion of excess symbionts.

Expulsion of symbionts.

The continual expulsion of symbionts in small numbers in various stages of the life cycle has been observed in many anthozoans. These include *Anemonia viridis* (Taylor, 1969b); *Aiptasia pulchella* (Steen & Muscatine, 1987); *A. tagetes* (Steele, 1975; 1976; 1977); *Zoanthus* spp. (Reimer, 1971; Trench, 1974) *Palythoa* sp. (Trench, 1974); *Stylophora pistillata* (Hoegh-Guldberg *et al*, 1987); and *Pocillopora damicornis* (Stimson & Kinzie, 1991). The expulsion of naturally occurring *Chlorella* symbionts from *Hydra* has not been observed under normal conditions (Smith, 1987). Douglas & Smith (1984), however, reported the regular expulsion of *Chlorella* from *Hydra* which had been experimentally infected with *Chlorella* originally symbiotic with *Paramecium*. During the periodic expulsion of symbionts mentioned above, the numbers of zooxanthellae released is low relative to the expulsion of zooxanthellae from corals brought about by unusually high temperatures or low salinities which cause bleaching of whole colonies on a large scale (Goreau, 1964; Glynn 1984).

The symbionts continually expelled from anemones are often seen as loosely clumped pellets (Taylor, 1969b; Steele, 1976), mucus boli or in strings of mucus (Taylor, 1973; Steele, 1975). It was suggested that this process was a means by which the host could regulate its symbiont numbers (Taylor, 1969b). However, Hoegh-Guldberg *et al* (1987) measured rates of expulsion of zooxanthellae in several species of coral and concluded that expulsion of symbionts represented an insignificant loss relative to growth rates and did not appear to be important in regulating the size of the population in normal conditions.

Whether expulsion is a means by which the host can regulate the number of symbionts is not known. Although periodic expulsion of symbionts takes place, there is no evidence to suggest whether the mechanism for expulsion is entirely random or whether it selects for symbionts of a particular stage in their life cycle or nutritional state.

Alternatively, expulsion may be an important method of transfer and re-infection in bleached corals or in those species which produce aposymbiotic larval forms and rely on expelled symbionts in the surrounding water to recolonise their tissues (Smith, 1987). However this

hypothesis would not account for the periodic release of pellets containing degenerate zooxanthellae.

Host digestion of symbionts.

Since Hoegh-Guldberg *et al* (1987) only found minimal expulsion of zooxanthellae from *Stylophora pistillata* , digestion of zooxanthellae was thought to be a potential mechanism for regulating population density in corals.

Evidence of host digestion of symbionts is limited due to the difficulty in distinguishing degenerate cells produced as a result of natural senescence and autolysis, or as a result of host attack and digestion. Yonge & Nicholls (1930a) found no evidence of digestion of zooxanthellae by coral hosts. Some of their evidence came from observations that the cellulose cell walls of degenerate zooxanthellae were completely intact when stained. There is indirect evidence, however, for the occurrence of a substance produced by the host which can digest its symbionts. Work carried out by Steele & Goreau (1977) on the sea anemone, *Phyllactis flosculifera,* showed that a substance present in the ruff above the column brought about more rapid lysis of isolated symbionts when compared with the lysis time of symbionts not exposed to this substance. It appeared that the extract was specific to algae isolated from the host species. Janssen & Möller (1981) concluded from electron microscopical studies that when *Anemonia viridis* was starved in darkness, the algae were digested. Their conclusions were based on evidence from the presence of acid phosphatases surrounding many symbionts, and the presence of residual bodies with myelin-like figures in micrographs taken from animals which had been starved or kept in the dark. They suggested that the presence of breaks in the thickened algal periplast with subsequent leaching of cell contents were indications of autolysis.

Digestion of symbionts by the host could potentially provide an important source of nutrients and energy for the host, and therefore may make an important contribution to nitrogen and carbon budgets. However, although a mechanism for the digestion of zooxanthellae exists (Steele & Goreau, 1977), there is no conclusive evidence that the host gains significant nutritional benefit from digesting algae.

The general conclusions of the above review appear to indicate that there is no obvious unifying theory of zooxanthella population regulation within symbiotic associations. It seems important therefore to re-examine some of the regulatory processes in one organism. The organism chosen was *Anemonia viridis* which is symbiotic with the dinoflagellate *Symbiodinium* sp. This successful association is of interest because it has the widest geographical range of symbiotic anthozoans and therefore the greatest environmental range, being the most northerly occurring species. It grows to a large size, is abundant and widespread on the west coast of Scotland and is relatively long lived.

The aims of the present work were to answer the following questions:

1. What are the short term and long term effects of changes in inorganic nitrogen supply, light intensity and feeding on the zooxanthella population density of *Anemonia viridis*?

2. Can changes in the symbiont population density, which may be observed following changes in these environmental factors, be explained by changes in the total zooxanthella population in the whole animal or by changes in the host protein content or host weight?

3. If the population size is considered to be the result of gains and losses to the symbiosis, what are the consequences to the symbiont population and expulsion rate of a change in the zooxanthella division rate? Is there any evidence from this that expulsion is a potentially important population regulatory mechanism?

4. What are the effects of changes in symbiont population density on the symbiont growth rate? Can these effects be explained in terms of increased competition for nitrogen or in terms of an increase in intra-cellular space available to symbionts?

5. What happens to the symbiont population density, the total population and mitotic index when the symbionts are unable to photosynthesise?

CHAPTER 2

GENERAL METHODS AND MAINTENANCE OF ANIMALS.

To avoid unnecessary repetition of methodology, the major methods are set out here. Where methods, or statistical techniques are specific to a particular chapter, details are recorded in the methods section of that chapter.

All statistical tests were performed using Minitab Statistical software.

2.1 Collection and maintenance of symbiotic *Anemonia viridis.*

Anemones were collected from two sites towards the head of Loch Sween, Argyll, and one site in Loch Uiskevagh on the east coast of Benbecula in the Western Isles. Large specimens of the brown morph were collected from a submerged pinnacle in Loch Sween in 4 to 9m depth of water, while smaller specimens of the brown morphs (Figure 2.1) were collected from Tayvallich in 1 to 3 m depth. Anemones from the Benbecula site were collected from depths between 4 and 8 m. The animals were transferred to running sea water aquaria in the Department of Zoology at Glasgow University where they were maintained at a temperature of $11 \pm 1^{o}C$ under a 12 hour light:12 hour dark lighting regime with a light intensity of 120 $\mu E.m^{-2}.s^{-1}$ and fed weekly on mussel tissue. Animals were maintained starved for 2 weeks prior to all experiments. Unless otherwise stated, experiments were carried out at standard conditions of $11 \pm 1^{o}C$ under a 12 hour light:12 hour dark lighting regime with a saturating light intensity of 300 $\mu E.m^{-2}.s^{-1}$.

2.2 General Methods.

2.2.1 Separation of zooxanthellae and animal fractions.

Separation of algal and host material was necessary for algal counts, protein and chlorophyll measurements. Isolation of pure suspensions of zooxanthellae is hampered by the large amounts of mucus produced by the host, which cause clumping of the algae and host material when the tissue is homogenised. When the homogenate is centrifuged, the zooxanthellae pellet produced is highly contaminated with host material, and a thick white layer of mucus is visible on top of the pellet. The mucus causes zooxanthellae in a suspension to be unevenly distributed, due to the clumping, and produces highly variable and inaccurate cell counts when using a haemocytometer. Therefore, a method of removing most of the mucus without

removing the zooxanthellae was needed for cell counts and biochemical analyses. The method used in this study consists of repeated centrifugation and washing, as described by Davies (1988), and slightly modified by the addition of 0.05% sodium dodecyl sulphate in artificial sea water to remove more of the mucus and host debris. Sodium dodecyl sulphate has been used to isolate *Chlorella* from its host, *Hydra* with marked improvements (McAuley, 1986c).

Whole animal samples

This method was used to make measurements of zooxanthellae concentrations within host tissue as cells per animal dry weight or cells per unit animal protein and is summarised in Figure 2.2. Animals were buoyant weighed and rinsed thoroughly in filtered sea water to remove mucus and attached algal debris, then rinsed briefly in distilled water. Individuals were cut longitudinally into two approximately equal halves. Each half was gently blotted dry and wet weighed. One half was immediately freeze-dried. The total dry weight could then be calculated using the actual dry weight of the freeze dried fraction and both wet weight measurements. The other half was cut into pieces and homogenised in artificial sea water containing 0.05% sodium dodecyl sulphate using a Janke & Kunkel Ultra-Turrex T25. Artificial sea water, with a salinity of 33‰, was made up by dissolving 31g sodium chloride, 10g magnesium sulphate and 0.02g sodium bicarbonate in 1 litre of distilled water and adjusting to pH 7.4 (Harvey, 1957). Sodium dodecyl sulphate (SDS) was used to reduce anemone tissue contamination in these experiments. The homogenates were left for 20 minutes before re-homogenising and centrifuging. The homogenate was centrifuged and washed repeatedly in artificial sea water as outlined in Figure 2.2. The centrifugal speed was decreased each time as the algal fraction became purer, to allow the lighter host fraction to remain in suspension. The supernatant of the first centrifugation was decanted, shaken and centrifuged in an attempt to pellet any remaining zooxanthellae. Examination of the supernatants from the second and third centrifugations of the algal pellet showed them to be virtually free of algae. After removal of the supernatant, the white layer of mucus was gently pipetted off and the pellet was washed in artificial sea water three times. The combined supernatants, representing the animal fractions, were made up to 100 ml with distilled water in volumetric flasks. This gave a sufficient dilution to be within the range for protein assays. Zooxanthella pellets were re-suspended in 25 ml of artificial sea water, and counts made on subsamples using an improved Neubauer haemocytometer. Observation of the zooxanthellae under the microscope determined whether the suspensions were suitable for counting. Samples with excessive clumping and numerous nematocysts were recentrifuged until acceptable.

Tentacle samples

A modification of this procedure was used when measuring the population density of zooxanthellae in single tentacles. Tentacles were homogenised using a Janke & Kunkel Ultra-Turrex T25 in 1 ml of artificial sea water containing 0.05% SDS in 2 ml Eppendorf tubes for 2 minutes. The samples were centrifuged at 3000 r.p.m for 5 minutes and the resulting supernatants decanted. The pellets were re-suspended in 1 ml artificial sea water and re-centrifuged. The resulting supernatant was then combined with the first supernatant sample and 2 ml of the combined supernatants diluted to 10 ml with distilled water. Protein assays were carried out on this diluted sample (section 2.2.2). The second centrifugation and re-extraction was needed as it was found that just using the first extracted supernatant only yielded approximately 90% of the total protein in the sample. The pellets containing the zooxanthellae were re-suspended in 1 ml of artificial sea water and the numbers of zooxanthellae were counted from subsamples as before.

2.2.2 Protein assays.

Two methods of protein analysis have been investigated in studies involving symbiotic anthozoans (Cook *et al,* 1988). The most commonly used method is the Lowry assay (Lowry *et al*, 1951). Hoegh-Guldberg & Smith (1989b) used Hartree's modification (Hartree, 1972) which differed from the Lowry method in that it contained a more concentrated alkaline tartrate reagent which produced a more linear calibration curve. Cook *et al* (1988) used a different method involving the Bio-Rad procedure (Bradford, 1976) which utilises the principle of protein dye-binding. However, the values which they obtained for *Aiptasia pallida*, using this method, were lower than those determined by the Lowry procedure by a factor of 1.56. This underestimation was probably due to incomplete solubilisation and hydrolysis of anemone protein. Consequently, Lowry's method has been used here with the modification of Markwell (1978) which involves the use of SDS. This method has not been used with cnidarians, but results compared favourably with published data.

Procedure for Protein analysis.

1 ml samples of a 1 in 10 dilution of the separated anemone protein suspensions (section 2.2.1) in distilled water were either analysed immediately or frozen in 15 ml polyethylene centrifuge tubes for up to one week until analysis.

Reagents:

1. 20g sodium carbonate, 4g sodium hydroxide, 1.6g sodium tartrate and 10g SDS dissolved in 1 litre distilled water.

2. 4g hydrated copper sulphate dissolved in 100 ml distilled water.
3. Folin-Ciocalteu phenol reagent (Sigma no. F-9252) diluted 1:1 with distilled water.

1 part of reagent 2 was added to 100 parts of reagent 1 and shaken until the solution became clear. 3 ml of this reagent was added to 1 ml sample and incubated in a water bath at 23°C for 45 minutes. 0.3 ml of the Folin-Ciocalteu reagent was added quickly whilst mixing the sample using a whirlimixer. The samples were incubated in a water bath at 23°C for a further 45 minutes. Absorbance was read at 750 nm in disposable macrocuvettes on a Philips PU8700 series ultra-violet/visible spectrophotometer. Calibration standards were made using bovine serum albumin at concentrations up to 100μg protein.ml^{-1} dissolved in distilled water and allowed to mix using a magnetic stirrer for 1-2 hours.

2.2.3 Staining procedure for Feulgen stain.

Growth rates are usually estimated by calculating the percentage of "doublet cells." A doublet cell consists of two cells where the cytoplasm is still joined and the cell plate is visible. This method gives a measure of the mitotic index, which can be a useful measure of relative growth for comparisons of experimental conditions. A drop in mitotic index is often correlated with poor or stressed growing conditions (Nganro, 1992). Cell plate formation, however, only occurs in the final stage of cell division (late telophase). As a result, not all of the dividing cells in a sample will be visible. By staining the cells using a nuclear stain, the earlier stages of anaphase, when the nuclear material divides but the cell plate has not yet formed, can also be observed. Thus, dividing cells appear as those cells with two nuclei (Figure 2.3). The staining method used in this work, to stain zooxanthellae for mitotic index counts, was modified from a method used by Brown & Zamani (1992).

Preparation of stain

1 g of powdered Feulgen Schiff's (Fuchsin Basic BDH catalogue no. 340324J) was added to 200 ml of boiling distilled water. The solution was then cooled to 50°C and 30 ml of 1M hydrochloric acid and 3g potassium metabisulphite were added. The stain was stored in a dark bottle in a refrigerator overnight before adding 1g activated charcoal. The solution was filtered and the filtrate stored in a dark bottle in a refrigerator.

Tissue staining procedure

1. Tentacle tissue was fixed in 3:1 alcohol:acetic acid for 24 hours.
2. The fixed tissue was stored in 70% alcohol in a refrigerator until required.

3. Tentacles were hydrolysed in 1M Hydrochloric acid at 60°C for exactly 12 minutes.
4. The preparation was washed in tap water twice for 2 minutes and stained in Feulgen stain for 2 to 3 hr.
5. The stained tentacles were then homogenised for a few seconds in the staining solution.
6. Observations were made at 1000x under oil immersion.
7. Mitotic index data were calculated as the numbers of cells dividing per 300 cells.

2.2.4 Determination of Ammonium.

The method used was taken from Liddicoat *et al* (1974) and is a modification of the method used by Solórzano (1969). These modifications included the substitution of potassium ferrocyanide as the catalyst in place of sodium nitroprusside, which resulted in a lower, more reproducible blank. Sodium dichloroisocyanurate was used in place of a commercial solution of hypochlorite since its use was found to reduce variation between samples. Incubation of the samples in natural day light produced differences in colour development due to variation in the amount and quality of natural light from day to day. Therefore incubation under a portable UV light source was used (Liddicoat *et al*, 1974).

Reagents

1. Phenol-alcohol reagent: 10g phenol in 75 ml absolute ethanol and 25 ml acetone.
2. Catalyst: 0.5g potassium ferrocyanide in 100ml distilled water. (stored in an amber bottle).
3. Oxidising solution: 0.2g sodium dichloroisocyanurate in a solution of 1.6g sodium hydroxide in 40 ml distilled water to which was added 20g trisodium citrate dissolved in 40 ml water and made up to 100 ml. This reagent was prepared fresh daily.
4. Calcium-free artificial sea water (Harvey, 1957). 31g sodium chloride, 10g magnesium sulphate, 0.02g sodium carbonate. Dissolved in 1 litre with distilled water. pH 7.4, salinity of 33‰.

All reagents were of analytical grade and de-ionised distilled water was used. The reaction is particular sensitive to contaminants therefore particular care was taken to decontaminate all glassware used. This was done by soaking in Decon-90, rinsing thoroughly and soaking in 15% hydrochloric acid, followed by further rinsing and a final rinse in de-ionised water.

Procedure.

40μl phenol solution was added to 1 ml sea water samples and the vials frozen for up to a week if necessary, until analysis. Once thawed, 40μl catalyst and 100μl oxidising solution

were added to the samples which were then shaken and placed under a portable ultraviolet light (365 nm wavelength) for 2-3 hours for colour development. Absorbance was read at 640nm in disposable semi-microcuvettes against a reagent blank (control) of 1 ml artificial sea water. The analysis was calibrated using a series of ammonium sulphate standards ranging from 2 to 64 μmoles ammonium in artificial sea water.

2.2.5 Chlorophyll extraction and determination.

Chlorophyll *a* determinations were carried out on suspensions of purified zooxanthellae prepared as shown in Figure 2.2. The method of chlorophyll extraction used was modified slightly from the method of Davies (1988) which was based on the procedure of Jeffrey & Humphrey (1975). Two methods were used depending on whether the sample was whole anemone or tentacle homogenate

Procedure for extraction from zooxanthellae isolated from whole anemone homogenates

2.5 ml or 5 ml of the pure zooxanthellae suspension was filtered on to a Whatman cellulose nitrate filter (0.45μm pore size). Two drops of a suspension of 1 g magnesium carbonate in 100 ml distilled water were added to prevent acidic decomposition of the chlorophyll (Jeffrey & Humphrey, 1975). The filter was placed in a glass test tube containing 10 ml of 90% acetone, covered with nescofilm and aluminium foil and allowed to stand for 12 h overnight in a refrigerator. After extraction the tubes were shaken and centrifuged for 5 min at 2000 r.p.m. to clear the supernatant. Absorbance was read in 1 cm glass cuvettes at 750 nm, 663 nm and 630 nm on a Philips PU8700 series UV/visible spectrophotometer. The extinctions at 750nm are subtracted from the 663 nm and 630 nm extinctions to correct for sample turbidity. The formula of Jeffrey & Humphrey (1975) for dinoflagellates containing chlorophylls a and c was used to calculate total chlorophyll a in each sample. These values could be converted to total chlorophyll a in each animal.

Chlorophyll *a* (μg/ml) in sample (C) = $11.43\ E_{663} - 0.64\ E_{630}$

Total chlorophyll *a* in animal (mg) = $C \times V/v \times T \times 1000$

where V = total volume of acetone in ml (V = 10), T = total volume of algae in ml (50), v = volume zooxanthellae filtered in ml (v = 2.5 or 5)

Procedure for chlorophyll extraction from zooxanthellae isolated from tentacle homogenates.

1 ml zooxanthellae suspensions (see 2.2.1) were centrifuged down to a pellet in 2 ml Safe-lock Eppendorf tubes. The supernatant was poured off and immediately replaced with 1 ml of 90% acetone and a few drops of a suspension of 1 g magnesium carbonate in 100 ml distilled water. The tubes were mixed thoroughly and left in a refrigerator for 12 hours. The absorbance of the samples were read in quartz microcuvettes at the same wavelengths as above. The suitability of the plastic Eppendorfs for use with acetone was checked as certain plastics will dissolve in acetone. Eppendorfs were filled with 90% acetone and left for 12 hours. The absorbances at 750 nm, 663 nm and 630 nm were compared with a control of stock 90% acetone kept in a glass bottle. There were no differences in the absorbances so it was assumed that the plastic Eppendorfs were chemically stable and suitable for use with acetone.

Sodium cacodylate buffer

A cacodylate buffer was used after for dissolving pronase and trypsin for cell maceration.

Sodium cacodylate buffer:
10 ml 1M sodium cacodylate
20 ml 1M sodium chloride
0.05 ml calcium chloride
50 ml filtered sea water

The buffer was made up to 100 ml with distilled water. pH 7.8, osmolarity 980 mM

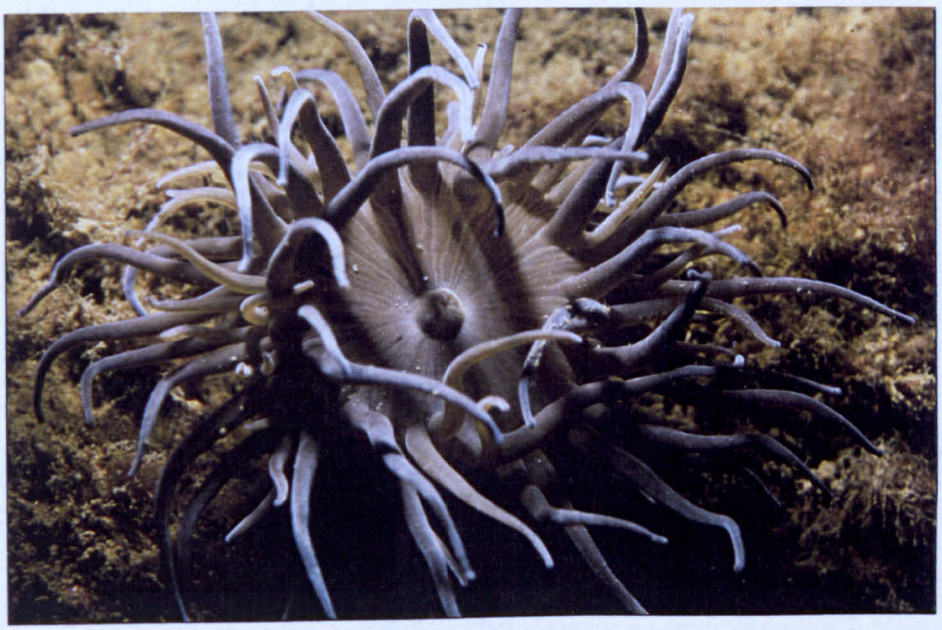
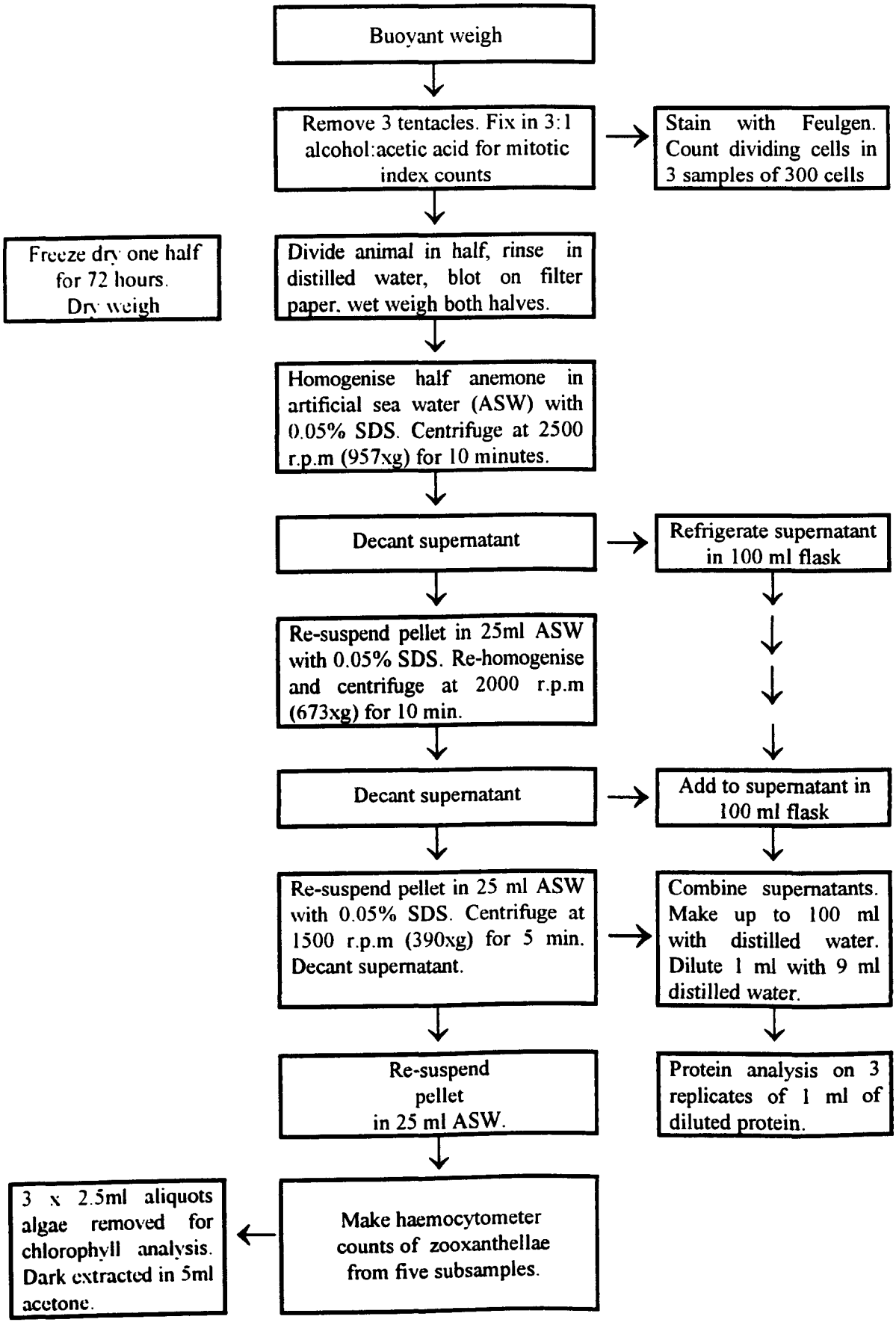


Figure 2.1 *Anemonia viridis* (Forskal).

Figure 2.2 Procedure for separation of total zooxanthellae from anemone protein for total symbiont counts, symbiont density counts, total protein and total dry weight.



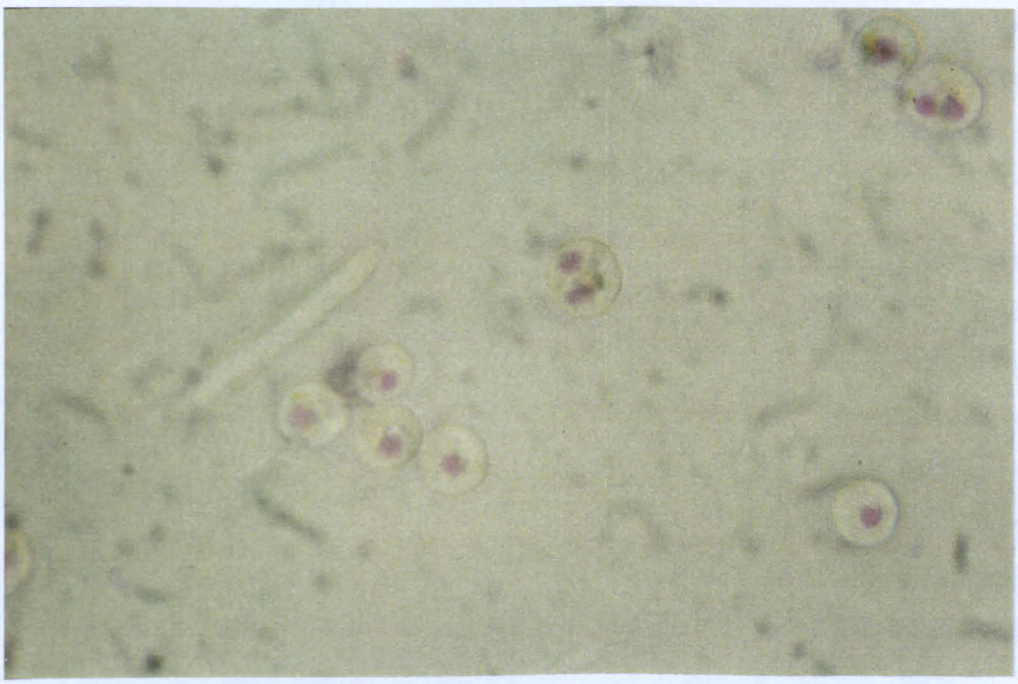


Figure 2.3 Zooxanthellae from tentacles of *A. viridis* stained with Feulgen stain and viewed under bright field under the light microsope.

CHAPTER 3

THE EFFECT OF SOME ENVIRONMENTAL VARIABLES ON THE ZOOXANTHELLA POPULATION OF *ANEMONIA VIRIDIS*.

3.1 Introduction

Zooxanthellae of anthozoans are located in vacuoles within host cells throughout the gastroderm layer (Taylor, 1968; Glider *et al* 1980). The population of zooxanthellae in *Anemonia viridis*, however, is not distributed evenly throughout the animal. The highest densities of zooxanthellae in *A. viridis*, measured as cells per animal protein, were found in the tentacles, and the lowest in the column and foot. The zooxanthellae in the tentacles make up 75% of the total zooxanthellae in the whole animal (Appendix 1).

Under conditions of constant light and feeding, a relatively constant ratio of symbiont:host cell numbers or biomass is maintained (Taylor, 1969b; Muscatine & Pool, 1979; Muscatine *et al*, 1985). When the host is growing, the symbiont population would, therefore, be expected to grow at a similar rate, so maintaining the symbiont density specific to the set of environmental conditions (Muscatine *et al*, 1985, Hoegh-Guldberg & Smith, 1989b). In the natural environment, however, light, temperature, nutritional status of the host and inorganic nutrient levels may vary periodically. Spatial differences relating to water depth, and temporal differences, relating to the seasons, in these environmental variables have been shown to bring about differences in symbiont population density (Drew, 1972; Muller Parker, 1987).

In short term experiments, changes in the symbiont population density have been observed in response to both light and concentration of ammonium ions in the sea water. The effects of these environmental factors, however, appear to be equivocal. Muller Parker (1987) demonstrated that the zooxanthella population density of *Aiptasia pulchella* did not change with increasing irradiance, as did Harland & Davies (1994) in *Anemonia viridis*. However, Steele (1976) found that the zooxanthella population density in *Aiptasia tagetes* increased with increasing irradiance, while Thinh (1991) reported higher densities in low light maintained corals. Experiments involving ammonium enrichment showed that the zooxanthella population density in *Stylophora pistillata* increased dramatically, after 2-3 weeks enrichment, compared to untreated corals (Muscatine *et al*, 1989; Hoegh-Guldberg & Smith, 1989b), however the change in population density, measured as cells.mg protein^{-1}, in another coral, *Seriatopora hystrix*, was not significant (Hoegh-Guldberg & Smith, 1989). The observation that some corals show an increase in the zooxanthella population density under ammonium enrichment

has led to the general suggestion that zooxanthellae *in hospite* are nitrogen limited (Cook & D'Elia, 1987; Falkowski *et al*, 1993). The ammonium enrichment experiments of Muscatine *et al* (1989) and Hoegh-Guldberg & Smith (1989b) took the form of pulsed additions of ammonium added over periods of 2-3 weeks, where concentrations were depleted before the next addition. A system involving continuous enrichment would ensure depletion did not occur. No information is available on the longer term effects of continuous exposure to elevated ammonia concentrations. The previous experiments were essentially short-term, with observations being made after 2-3 weeks. It is not known whether population levels measured after these time periods represent new stable population levels or whether the population would increase further or decrease with longer term exposure.

There are no reports on the effect of ammonium enrichment on zooxanthella population numbers or population density and host growth of symbiotic anemones. *Anemonia viridis* is a temperate water anthozoan, which, in comparison with tropical associations, lives in conditions of lower light intensities, higher nutrient concentrations and lower temperatures. Changes in these factors may affect the symbiont population differently and indicate different limiting factors or different population regulatory mechanisms.

In order to deal with the problems introduced by having to use symbiotic hosts of different sizes in experiments, changes in the population of zooxanthellae are, for convenience, expressed as changes in their population density by normalising to a unit of biomass. In corals, the unit chosen is usually cm^2 of skeletal surface (Falkowski & Dubinsky, 1981; McCloskey & Muscatine, 1984; Muscatine *et al*, 1985; Muscatine *et al*, 1989; Hoegh-Guldberg & Smith 1989 a, b; Stambler *et al*, 1991), whilst in anemones, the normalising units most frequently used are dry weight and weight of animal protein (Smith & Muscatine, 1986; Cook *et al*, 1988). Many previous workers have not recognised that experimentally induced changes in population density could result from changes in the normalising unit rather than, or in addition to, changes in the population of zooxanthellae. An increase in the population density could result from any of the following:

1. Increase in zooxanthella population; host biomass unchanged,
2. Increase in zooxanthella population; smaller increase in host biomass,
3. No change in zooxanthella population; decrease in host biomass,
4. Decrease in zooxanthella population; larger decrease in host biomass.

For instance, Cook *et al* (1988) found that the protein content of specimens of *Aiptasia pallida* dropped from a mean of 0.9 mg to 0.5 mg per anemone over 20 days of starvation. In order

to interpret the cause of any changes in the density of the algal population over time, it is necessary, therefore, to measure concomitant changes in the host.

If the symbiont:host biomass remains constant under conditions of feeding, growth of the host would be accompanied by a *pro rata* growth of the zooxanthellae, so that the population would remain unchanged. Similarly under starvation, if the host and the symbiont population both decreased at the same rate, the population density would remain constant. However under starvation, both an increase and a decrease in the zooxanthella population density have been observed in corals and anemones (Szmant-Froelich & Pilson, 1980; 1984; Muller Parker 1987; Cook *et al*, 1988). Muscatine *et al* (1989) showed that feeding of the host did not result in an increase in zooxanthella population density, measured as cells.cm^{-2} surface area, suggesting that the nitrogen of the amino acids assimilated following digestion was used for growth of the host tissues and was not available to the symbionts.

Changes in population density could result from changes in the number of host gastroderm cells containing zooxanthellae, or from a change in the mean number of zooxanthellae per gastroderm cell. Published data on the mean number of zooxanthellae per gastroderm cell shows that there is variation between associations (Muscatine & Pool, 1979; Glider *et al*, 1980; Gates & Muscatine, 1992), but there is no information on variation in relation to experimentally induced changes in population density. Variation in the number of symbionts within gastroderm cells may reflect variation in the relative rates of symbiont and host cell division so that host cells with many symbionts may indicate a high rate of zooxanthella division and a relatively low rate of host cell division, and *vice versa*.

This chapter is concerned with the effect of environmental variables on the population of zooxanthellae in *Anemonia viridis*. In particular, the following questions were asked:

1. What are the short term effects of increased light and ammonium on the zooxanthella population density?
2. Does the mean number of zooxanthellae per gastroderm cell change in response to elevated levels of ammonium?
3. What are the long term effects of exposure to increased ammonia, to starvation and to feeding on the population density?
4. Can these changes in population density be explained by changes to the total population of zooxanthellae or by changes in the biomass of the host?

3.2 Materials and Methods.

Animals were maintained at illumination levels of 100-120 $\mu E.m^{-2}.s^{-1}$ in running, aerated sea water at 11±1°C for at least 2 weeks. Animals were fed weekly on mussel tissue until two weeks before experiments began.

3.2.1 Short-term effects of ammonium and light intensity on the zooxanthella population density.

Two questions were addressed:

1. What is the effect of ammonium enrichment under a) low light intensity and b) high light intensity on the zooxanthella population density?
2. What is the effect of light on the zooxanthella population density under control and ammonium enriched conditions?

Two groups of 20 animals were photoadapted to either 300 $\mu E.m^{-2}.s^{-1}$ or 100 $\mu E.m^{-2}.s^{-1}$ in running, aerated sea water at 11±1°C for 14 days. After 14 days (day 0 of the experiment), 2 tentacles from each animal were removed and their combined protein content and zooxanthellae density measured, as described in section 2.2.1. Ten of the animals from each light treatment were then placed into sea water enriched with ammonium sulphate to a concentration of 20μM ammonium. Sea water flowed from a constant head tank to a reservoir containing a magnetic stirrer bar. Ammonium sulphate solution was injected into the reservoir at a constant rate by a peristaltic pump. The enriched sea water was continuously pumped down a series of perforated tubes running the length of the tank, so that the animals were constantly bathed in ammonium supplemented sea water. Determination of ammonium (section 2.2.4) was carried out to check for fluctuations in the levels of ammonium in the reservoir and in the tank of both control and enriched systems. The other 10 animals from each light treatment remained in unenriched sea water. The ammonium concentration in the control sea water ranged between <0.5 to 1.3μM. The animals were maintained unfed in these treatments for a total of 21 days. After 14 days, 2 tentacles were removed from each animal for protein content assays and zooxanthella population density counts. A second series of samples was taken after a further 7 days.

3.2.2 The effect of ammonium enrichment on the mean number of zooxanthellae within gastroderm cells.

Isolated gastroderm cells were obtained by a maceration technique based upon the modification of Gates *et al* (1992) of the method first described by Glider *et al* (1980) to

investigate changes in the distribution of zooxanthellae in gastroderm cells under ammonium enrichment. Five animals were maintained at 300 $\mu E.m^{-2}.s^{-1}$ in 20μM ammonium enriched sea water and a control group of 5 animals were maintained without ammonium enrichment for 3 weeks. At the end of this period, the animals were relaxed for 30 minutes in 7% magnesium chloride in calcium free artificial sea water (see section 2.2.1). Three tentacles were removed from each animal and cut transversely into three smaller portions of 2 mm length. The pieces were washed repeatedly in calcium-free sea water to remove mucus. Each piece was cut longitudinally using two scalpels and opened out to form two flat sheets. The sections were transferred to deep cavity slides containing 0.1% pronase made up in cacodylate buffered saline (see section 2.2), covered and left for 30 minutes. After this time, the gastroderm cells (facing uppermost on the slide) were separated from the lower ectoderm layer by gently brushing the surface with a very fine paintbrush. The resulting clumps of gastroderm cells from all pieces of the three tentacles were transferred to one watch glass containing 2 ml of 0.1% trypsin in sodium cacodylate buffered sea water and left for 30 minutes at room temperature to separate the clumps of cells. The individual cells settled to the bottom of the watch glass. 10μl of the fresh suspension was pipetted gently on to a haemocytometer slide and viewed at x 400 under bright field illumination. 1000 gastroderm cells per animal were examined, and the number of zooxanthellae per gastroderm cell were recorded.

3.2.3 Effects of long term exposure to ammonium enrichment, starvation and feeding on the population density and total population of zooxanthellae within *A. viridis*.

The population density of zooxanthellae and biomass of *A. viridis* were measured at intervals over 80 days in animals maintained in flowing sea water at 11±1°C and 300 $\mu E.m^{-2}.s^{-1}$ under 2 experimental regimes: 20μM ammonium enrichment (see section 3.2.1) or weekly feeding with 4 freeze-dried mysid shrimps. The control group was not fed and received no ammonium enrichment. Twenty five animals per treatment were assigned to each regime.

On day zero all animals were buoyant weighed (Tytler, 1982) and the weights converted to dry tissue weights using a calibration graph. These initial weighings and all subsequent ones were made at 11°C. The water was changed after weighing 3 animals to avoid inaccuracies introduced by changes in specific gravity of the sea water due to temperature change or from mucus release by the anemones. Five animals from each regime were then sacrificed and their zooxanthella density, dry weight and animal protein content used as a reference for comparison with those sacrificed at subsequent stages in the experiment.

Each animal was divided in two by a vertical cut. The two halves were quickly wet-weighed to determine the relative weight of each half. One half was freeze dried for 48 hours and then re-weighed. The other half was homogenised and aliquots taken for zooxanthella counts and protein content (section 2.2.1). This enabled the determination for each anemone of:

1. Anemone dry weight
2. Anemone total protein
3. Total zooxanthella number
4. Population density as zooxanthellae per μg dry weight and as zooxanthellae per μg protein.

In order to compensate for differences in the body sizes of anemones, all values for anemone dry weight, total protein and total zooxanthella populations were standardised to an initial body weight on day zero of 500 mg dry weight.

Five anemones from each regime were sacrificed on days 17, 35, 57 and 80 and treated as for the initial group above. Patterns of growth or weight loss for a standard animal of 500 mg dry weight were calculated from differences in the dry weights at time of sacrifice from the predicted dry weights of the animals on day zero. These weight changes together with measured zooxanthella population densities for each treatment were used to calculated changes in the total zooxanthella population and animal protein content of a normalised animal of 500 mg dry weight.

3.3 Results.

Data on symbiont population densities, total symbiont numbers and protein content were tested using the Anderson-Darling's test for normality and Bartlett's test for homogeneity of variances. All data sets, unless otherwise mentioned, were found to be normal and homoscedastic.

3.3.1. Short-term effects of ammonium and light intensity on the zooxanthella population density.

The results of the experiments are presented in Figure 3.1 and Table 3.1. Two way ANOVA analyses on the data for population density in tentacles are given in Table 3.2.

Table 3.1 Zooxanthella population density (cells.mg protein^{-1}) in tentacles ± s.e of means (n=10) of *A. viridis* kept at 100 and 300μE.m^{-2}.s^{-1} with & without 20μM ammonium enrichment for 21 days.

	Control		Ammonium supplemented	
Group	A	B	C	D
Day	100 μE.m^{-2}.s^{-1}	300 μE.m^{-2}.s^{-1}	100 μE.m^{-2}.s^{-1}	300 μE.m^{-2}.s^{-1}
0	3072 (249)	3519 (248)	3153 (319)	3501 (265)
14	4200 (289)	3503 (130)	4040 (371)	4648 (392)
21	3455 (464)	3300 (236)	4095 (552)	4281 (321)

Table 3.2 Table of two-way ANOVA tests carried out on the data presented in Table 3.1. Statistics only presented for treatment effect. Only values followed by * are statistically significant at 5% level.

Treatments	Groups compared	Treatment effect
Effect of ammonium at 100μE.m^{-2}.s^{-1}	A C	F=0.39 p= 0.5
Effect of ammonium at 300μE.m^{-2}.s^{-1}	B D	F=6.88 p= 0.01 *
Effect of light with no ammonium enrichment	A B	F=0.22 p= 0.6
Effect of light under ammonium enrichment	C D	F=1.69 p= 0.2

a) Effect of ammonium.

Under 100 μE.m^{-2}.s^{-1}, there was no significant difference in the zooxanthella population density in tentacles over the 3 week experimental period between the controls and those kept in 20μM ammonium (F=0.39, p=0.5). However at high light intensity (300 μE.m^{-2}.s^{-1}) there was a significant difference in the mean population density (as cells.μg protein^{-1}) between ammonium treated and control animals (two way ANOVA treatment effect: F=6.88 p=0.014). At 300μE.m^{-2}.s^{-1}, the mean density of zooxanthellae in tentacles of ammonium

supplemented animals had increased from 3501 to 4281 cells.μg protein^{-1} by day 21 (Table 3.1). This increase was significant (Tukey test for pairwise comparisons $p<0.05$). Zooxanthella population densities in the control group at 300μE.m^{-2}.s^{-1} had not changed significantly after 21 days (Tukey test for pairwise comparisons $p>0.05$).

b) Effect of light intensity.

In the control anemones there was no significant difference in the zooxanthella population density in tentacles between those kept at 300 μE.m^{-2}.s^{-1} and those kept at 100 μE.m^{-2}.s^{-1} ($F=0.22$ $p=0.6$). The same was true with anemones kept in sea water enriched to 20μM ammonium: there was no significance between those kept at 100 μE.m^{-2}.s^{-1} and those kept at 300 μE.m^{-2}.s^{-1} for 3 weeks ($F=1.69$ $p=0.2$).

It is clear from these experiments, therefore, that over the short term (3 weeks), exposure to high levels of ammonium causes a significant increase in the zooxanthella population density of the tentacles at high light intensities, but light intensity *per se* does not have an effect.

3.3.2. The effect of ammonium enrichment on the mean number of zooxanthellae within gastroderm cells.

Gastroderm cells of the tentacles of *A. viridis* maintained under ammonium treatment and control were found to contain between 1 and 7 symbiotic algae. Host cells with one symbiont per cell made up the highest proportion of gastroderm cells from control animals (42% of the total gastroderm cells counted) while in the ammonium enriched anemones, host cells with two symbionts made up the highest proportion of gastroderm cells (45% of the total gastroderm cells counted) (Table 3.3 and Figure 3.2).

Table 3.3 Number of symbionts per gastroderm cell in tentacles of *A. viridis* at 300 μE.m^{-2}.s^{-1} with or without 20μM ammonium enrichment for 21 days. s.e. of the means given in brackets (n=5).

	control animals			ammonium-enriched animals		
Symbionts per gastroderm cell	Mean no. host cells counted	%	total symbionts	Mean no. host cells counted	%	total symbionts
1	422 (20)	42.2	422	314 (28)	31.4	314
2	382 (19)	38.2	764	448 (52)	44.8	896
3	141 (10)	14.1	423	157 (11)	15.7	471
4	39.5 (3)	3.95	158	56.7 (12)	5.67	227
5	10 (2)	1.0	50	14.3 (3)	1.43	72
6	5 (1)	0.5	30	7 (1.9)	0.7	42
7	0.5 (0.3)	0.05	4	2.9 (1.1)	0.29	21
Total	1000		1851	1000		2043

A Mann-Whitney test was carried out as the data sets were not normal. The mean number of zooxanthellae per gastroderm cell was significantly greater in animals supplemented with ammonium ($p < 0.05$). A Mann-Whitney test was also carried out on the difference in the number of gastroderm cells with 1 and 2 cells between control and ammonium supplemented samples. Ammonium treated animals had proportionately fewer cells with 1 zooxanthella than control animals, at the 5% significance level, but there was no difference in the proportion of cells with 2 symbionts between treatment and control animals.

3.3.3 Effects of long term exposure to ammonium enrichment, starvation and feeding on the zooxanthella population density, total zooxanthella population, and animal biomass.

The objective of this experiment was to examine the effects of long term enrichment with ammonium (which provides nitrogen to the zooxanthellae directly), and feeding (which provides nitrogen to the host) on the population of zooxanthellae in *A. viridis*. Unfed animals were used as a control. Initially, results are presented as changes in the population density, as in previous studies. Since changes in the density could result from independent changes in the biomass units in which density is expressed (see Introduction 3.1), the effects of the treatments on biomass is presented using a 500 mg dry weight anemone as a basis. Finally, the predicted changes to the number of zooxanthellae in a standard 500 mg anemone over the 80 day experiment is calculated for the control and experimental treatments.

3.3.3.1 Effects on the zooxanthella population density.

Results are presented in Tables 3.4, 3.5 and 3.6, and in Figure 3.3.

Table 3.4 Zooxanthella population density as cells.μg dry weight^{-1} $\pm$ s.e. of mean (n=5) of *A. viridis* at 300 μE.m^{-2}.s^{-1} with or without 20μM ammonium supplementation, or with weekly feeding.

	Control starved	Ammonium supplemented	Fed weekly
Day 0	405.12 (17.5)	398.55 (21.7)	384.96 (17.2)
Day 17	392.51 (27.9)	400.63 (24.9)	352.0 (21.6)
Day 35	386.27 (14.6)	425.3 (35.1)	
Day 57	380.58 (36.2)	395.26 (9.6)	307.45 (25.3)
Day 80	366.50 (25.2)	371.2 (33.4)	284.6 (50.2)
ANOVA	F=0.45 p=0.77 n.s.	F=0.4 p=0.8 n.s.	F=3.22 p=0.05 significant

The zooxanthella population density measured as cells.μg dry weight^{-1} did not change significantly during the experiment in the control starved animals or those under ammonium enrichment (F=0.45 p=0.77; F=0.4 p=0.8 respectively). There was a significant fall,

however, in the population density of the fed animals, from 385 to 285 cells.μg dry weight^{-1} (Tukey test for pairwise comparisons $p<0.05$). When zooxanthella density was measured as cells.μg protein^{-1}, there was no significant change in the population density in animals from all treatments at the 5% significance level (Table 3.5; Figure 3.3b).

Table 3.5 Zooxanthella population density per μg animal protein of *A. viridis* kept at 300 μE.m^{-2}.s^{-1} with and without 20μM ammonium supplementation, and with weekly feeding for 80 days. s.e. of the mean (n=5) given in brackets.

	Control	Ammonium supplemented	Fed weekly
Day 0	1267.2 (104)	1159 (55.7)	1255 (58)
Day 17	1060.9 (103)	1058.9 (90)	1050.3 (98.7)
Day 35	997.1 (103)	1149.6 (100)	
Day 57	1155 (52)	1050.4 (70.3)	979.8 (92)
Day 80	1049.9 (42)	1104.8 (65.1)	805.3 (58)
ANOVA	F=0.6 p=0.67 n.s.	F=0.32 p=0.86 n.s.	F=2.63 p=0.098 n.s.

Table 3.6 Table of two way ANOVA tests carried out on the difference in zooxanthella population density between treatments.

Treatments compared	zooxanthellae per μg dry wt		zooxanthellae per μg protein	
	F	p	F	p
control v ammonium supplemented	0.32	0.577 n.s.	1.28	0.267 n.s.
control v fed weekly	4.55	0.044 significant	2.0	0.171 n.s.
ammonium supplemented v fed weekly	7.82	0.009 significant	0.88	0.357 n.s.

There was no significant difference in zooxanthella population density, measured as cells per μg dry weight, between control and ammonium supplemented animals. However, there was a significant difference in population density between control and fed anemones, and between fed and ammonium supplemented anemones (Table 3.6). Differences in population density between treatments, when measured as zooxanthellae per μg protein, were not significant for all pairwise comparisons.

3.3.3.2 Effect on animal biomass.

On day 0 all animals were buoyant weighed. In order to predict their dry weights, a dry weight:buoyant weight relationship was established. This was achieved by buoyant weighing all animals at time of sacrifice before the animals were dry weighed. Plots of the buoyant

weight and corresponding dry weight data for all animals in each regime are given in Figure 3.4. The regression equations for the relationship between buoyant and dry weight were:

fed weekly: $y = -50.9 + 7.17\ x$ $(r^2 = 99.3\%)$;
+ ammonium: $y = -31.1 + 6.94\ x$ $(r^2 = 96.2\%)$;
control: $y = 79.4 + 6.33\ x$ $(r^2 = 96.0\%)$.

There were no statistical differences between either the slopes or the intercepts for any of the treatments and so data were pooled and a common regression equation calculated. Dry weights on day 0 were predicted from the common regression equation:

mg dry weight = -1.4 + (6.74 * mg buoyant weight) $(r^2 = 97.6\ \%)$

The mean standard dry weights are given in Table 3.7 and plotted in Figure 3.5. Results of regression analyses and analysis of covariance carried out on the differences in dry weights between regimes are given in Table 3.8.

Table 3.7 Mean changes in dry weight of a standard animal of 500 mg initial dry weight calculated from weight changes in animals maintained at 300 $\mu E.m^{-2}.s^{-1}$ for 80 days ± s.e. of mean (n=5).

	Control starved		Fed weekly		20μM ammonium	
Day	Dry weight	% change	Dry weight	% change	Dry weight	% change
0	500	-	500	-	500	-
17	479.96 (2.1)	-4.0	581.31 (21.5)	16.3	505.17	1.0
35	459.04 (7.0)	-4.4	625.49 (12.0)	7.6	532.27	5.4
57	421.9 (11.0)	-8.1	666.13 (5.48)	6.5	551.03	3.5
80	360.6 (7.0)	-14.5	727.27 (13.6)	9.2	557.20	1.1
Total	-139.4	-27.9	227.27	45.5	57.2	11.4

Table 3.8 Results of regression analyses carried out on changes in total dry weight of animals from the three treatments over time. Regression coefficients with different superscripts were statistically different ($p<0.05$) from each other when pairwise comparisons were carried out using analysis of covariance.

Regression analysis	Slope	significance of slopes
Control	-1.72^{a}	$p<0.001$ significant
Ammonium supplemented	$+0.8^{b}$	$p<0.001$ significant
Fed weekly	$+2.73^{c}$	$p<0.001$ significant

When changes in dry weight of standard animals from each of the three regimes were compared, significant differences were found when all pairwise comparisons were carried out (Table 3.8). Starved animals lost weight while ammonium supplemented and fed animals gained weight. Fed animals gained more weight than ammonium supplemented animals.

The measured values of animal protein per mg dry weight were used to calculate the expected protein content of standard 500 mg dry weight animals. Changes in protein content are shown in Table 3.9 and Figure 3.6b. Regression and covariance analyses were carried out on the changes in protein content between regimes and are given in Table 3.10. Animal protein content as a percentage of the total dry weight remained close to 34% in the fed animals but fell significantly from 37 to 31% in the control group. Protein content of ammonium supplemented animals had increased significantly. When the changes in total protein content of animals from the three regimes were compared, significant differences were found in all pairwise comparisons (Table 3.10). Protein content of a standard 500 mg animal increased by 76.2 mg in a fed animal (33.5% of total weight gain), decreased by 65 mg in starved animals (47% of total weight loss), and increased by 32.8 mg in ammonium supplemented animals (57.3% of total weight gain).

Table 3.9 Changes in protein content (as mg/mg dry weight) or total protein content (mg) in *A. viridis* maintained for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ for a standard 500 mg dry weight animal calculated from actual weight changes and measured zooxanthella population densities.

Day	Control		20μM Ammonium enriched		Fed	
	Protein per dry wt	Total protein (mg)	Protein per dry wt	Total protein (mg)	Protein per dry wt	Total protein (mg)
0	0.3529	176.44 (16.4)	0.3539	176.95 (7.0)	0.3401	170.05 (5.5)
17	0.3764	180.58 (21.1)	0.3756	188.51 (7.8)	0.3422	197.11 (21.9)
57	0.3279	138.35 (4.9)	0.3806	209.53 (8.7)	0.3436	229.17 (20.3)
80	0.3087	111.32 (7.0)	0.3764	209.70 (12.9)	0.3313	246.28 (5.4)
		-65.12		32.75		76.23

Table 3.10 Results of regression analyses carried out on changes in protein content of animals from the three treatments over time. Regression coefficients with different superscripts were statistically different ($p < 0.05$) from each other when pairwise comparisons were carried out using analysis of covariance.

Treatments	Slope	significance of slope
Control	- 0.717 ^{a}	p = 0.002 significant
Ammonium supplemented	+ 0.866 ^{b}	p = 0.006 significant
Fed weekly	+ 0.328 ^{b}	p = 0.039 significant

3.3.3.3 Changes in the total zooxanthella population.

Measured zooxanthella population densities and predicted weights for a standard animal were used to predict the total number of zooxanthellae for a standard animal with an initial dry

weight of 500 mg at each sampling time under each regime. Values are shown in Tables 3.11, 3.12 and 3.13 and in Figure 3.6a.

Table 3.11 Changes in dry weight and total zooxanthellae s.e of mean (n=5) of *A. viridis* starved for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ for a standard 500 mg dry weight animal calculated from actual weight changes and measured zooxanthella population densities.

Day	Dry weight (mg)	Weight change (mg)	Zooxanthella density/μg dry wt	Total zooxanthella numbers
0	500	-	405.1	2.026×10^8 (0.12)
17	479.96	-20.05	392.51	1.884×10^8 (0.14)
35	459.04	-20.91	386.27	1.772×10^8 (0.06)
57	421.91	-37.14	380.58	1.597×10^8 (0.14)
80	360.6	-61.31	366.5	1.322×10^8 (0.12)
Change		-139.4		-7.04×10^7

Table 3.12 Changes in dry weight and total zooxanthellae ± s.e of mean (n=5) of *A. viridis* maintained with weekly feeding for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ for a standard 500 mg dry weight animal.

Day	Dry weight (mg)	Weight change (mg)	Zooxanthella density/μg dry wt.	Total zooxanthella numbers
0	500	-	384.96	1.925×10^8 (0.12)
17	581.31	81.31	352.0	2.034×10^8 (0.08)
35	625.45	44.14		
57	666.13	40.68	307.45	2.043×10^8 (0.24)
80	727.27	61.14	284.62	2.056×10^8 (0.29)
Change		227.27		1.306×10^7

Table 3.13 Changes in dry weight and total zooxanthellae ± s.e of mean (n=5) in *A. viridis* maintained with 20μM ammonium supplementation for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ for a standard 500 mg dry weight animal from actual weight changes and measured zooxanthella population densities.

Day	Dry weight (mg)	Weight change (mg)	Zooxanthella density/μg dry wt	Total zooxanthella numbers
0	500	-	398.55	1.993×10^8 (0.11)
17	505.17	5.17	400.63	2.022×10^8 (0.13)
35	532.27	27.10	425.31	2.266×10^8 (0.21)
57	551.03	18.76	395.26	2.180×10^8 (0.07)
80	557.20	6.17	371.16	2.068×10^8 (0.24)
Change		57.2		7.5×10^6

Results of regression and covariance analyses carried out on the changes in total zooxanthella numbers in animals under each treatment total are given in Table 3.14.

Table 3.14 Results of regression analysis carried out on changes in total zooxanthellae in animals from the 3 treatments over time. Regression coefficients with different superscripts were statistically different ($p<0.05$) from each other when pairwise comparisons were carried out using analysis of covariance.

Treatments	Slope	significance
Control	-744512 [a]	p = 0.008 significant
Ammonium supplemented	+147873 [b]	p = 0.56 n.s.
Fed weekly	+146616 [b]	p = 0.626 n.s.

When the changes in total zooxanthellae in standard animals from the three regimes were compared, the changes in zooxanthella population of ammonium supplemented animals and fed animals were not significant while the total zooxanthellae in fed animals decreased significantly (Table 3.14).

Anemones maintained for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ without feeding lost 28% of body weight and 35% of the initial zooxanthella population. Anemones maintained at the same light intensity with weekly feeding gained 45.5% in weight, and the zooxanthella population increased by 6.8%, resulting in a significant fall in the zooxanthella population density. Ammonium supplementation led to an increase in weight of 11.4% and an overall increase of 4% in zooxanthella numbers. In the first 35 days the total zooxanthella population of *A. viridis* increased by 14% but fell over the next 45 days.

3.4 Discussion

3.4.1 Effect of ammonium enrichment and light intensity on the symbiont population density within tentacles.

Increased nitrogen levels have been shown to increase symbiont population densities. This has lead to the idea that zooxanthellae are nitrogen limited (Cook & D'Elia, 1987; Falkowski *et al*, 1993). Muscatine *et al* (1989) found that the population density of zooxanthellae in *Stylophora pistillata* from the Red Sea, maintained in sea water enriched to 20μM ammonium sulphate at 1000 $\mu E.m^{-2}.s^{-1}$ for 14 days, was almost double that of the control group - 1.96 x 10^6cells.cm^{-2} compared with 1.11 x 10^6 cells.cm^{-2} for the controls. Similarly, Hoegh-Guldberg & Smith (1989b) reported that after 21 days of ammonium enrichment under 450 $\mu E.m^{-2}.s^{-1}$ of light, the population density of zooxanthellae in *S. pistillata* from the Great Barrier Reef had increased from 0.55 to 1.49 x10^6 cells.mg $protein^{-1}$. Stambler *et al* (1991) reported > 200% increases in the density of zooxanthellae in *Pocillopora damicornis* from Hawaii kept for 13 days in 15μM ammonium enriched sea water at full solar radiation.

However, a more recent study showed little or no effect of ammonium enrichment on symbiont population densities of *P. damicornis* and *Montipora verrucosa*. Stambler *et al* (1994) found that symbiont population densities of coral nubbins maintained in sea water enriched with 20 or 50μM ammonium for 21 days were not significantly different to controls. Studies on *Tridacna gigas* showed that the zooxanthella population density (as symbionts per animal tissue weight) of animals maintained in sea water enriched with ammonium to 10μM for 3 months was not significantly different to those of unenriched controls (Belda *et al* 1993).

In *Anemonia viridis* an increase in ammonium input for 21 days at 100 $\mu E.m^{-2}s^{-1}$ did not affect the zooxanthella population density in tentacles when compared to control samples. Both control and ammonium supplemented animals showed increases in zooxanthella numbers and densities in tentacles. These increases in both groups may have been due to increased water flow rates in experimental and control tanks compared to the larger stock tanks, which is thought to increase nutrient and carbon-dioxide uptake (Dennison & Barnes, 1988). However, water flow rates were not measured accurately so the difference in flow rates between control, ammonium enriched and stock tanks could not be quantified. At a higher light intensity of 300 $\mu E.m^{-2}.s^{-1}$, however, ammonium enrichment for 21 days increased the symbiont density by approximately 23%. This increase was very small when compared with the effect of ammonium supplementation on tropical corals and anemones. *A. viridis* lives in waters where the concentration of ammonium is relatively high compared to tropical coastal waters. On the south coast of Britain, *A. viridis* occurs in rock pools where the ammonium concentration reaches 3.0μM. In Scotland, however, it always occurs subtidally where ammonium concentrations of 0.9μM (open coast), and from <0.5 to 1.3μM (Loch Sween) have been recorded (Davies, 1988). The concentration of ammonium in the recirculating sea water aquaria used during the present experiments varied between <0.5 and 1.5μM which is similar to concentration found at Loch Sween. In tropical coastal waters, ammonium concentrations are almost always below detectable levels of $<0.5\mu$M (Cook 1980; Cook *et al* 1988; Muscatine *et al*, 1989). The difference in the effect of ammonium enrichment on the zooxanthella population density of *A. viridis* compared to the effect on corals may indicate that the symbiont population was already close to maximum levels, that some other factor had become limiting to the zooxanthella population or its growth or that temperate symbiotic anemones respond differently to tropical corals.

A significant increase in zooxanthella population density was only found in the animals maintained at 300 $\mu E.m^{-2}.s^{-1}$ and not at the lower light intensity. It is known that rates of ammonium uptake by zooxanthellae within *A. viridis* is proportional to light intensity within

the range 50 to 300μE.m^{-2}.s^{-1} (Davies, 1988). The difference in zooxanthella densities under ammonium enrichment may be due in part to the difference in the rates of ammonium uptake at high and low light intensities. However, a comparison of the treatments does not show any differences in zooxanthella densities between light intensities either with or without ammonium enrichment. Harland & Davies (1994) also found no difference in zooxanthella density of high and low light adapted *A. viridis* in unenriched sea water. Photo-adaptive responses are not thought to involve changes in symbiont density. Other laboratory studies of zooxanthella densities in response to decreasing light intensity in marine anthozoans have found different trends. Higher densities of symbionts have been reported in low light adapted corals (Thinh, 1991), while Steele (1976) found that zooxanthella density in *Aiptasia tagetes* decreased with decreasing irradiance. The zooxanthella density within *Aiptasia diaphana*; (Svoboda & Pormann, 1980) and *A. pulchella* (Muller Parker, 1985) did not change with decreasing irradiance. Field data on the effects of reduced light intensity due to shading are also variable. Steele (1976) reported that specimens of *A. tagetes* found in areas exposed to higher illumination had higher densities than specimens in shaded areas. Muller Parker (1987) found that populations of *A. pallida* growing in a shaded site had higher symbiont densities than populations of anemones growing at sunny sites at the same depth.

3.4.2 Effect of ammonium enrichment on the distribution of symbionts in gastroderm cells.

Gastroderm cells of the tentacles of *Anemonia viridis* at 300 μE.m^{-2}s^{-1} were found to contain between 1 and 7 symbiotic algae. Muscatine & Pool (1979) reported that gastrodermal cells of anthozoans rarely contained more than one or two zooxanthellae but no data were presented to substantiate this. Fitt & Trench observed that the endoderm cells of *Condylactis gigantea* also contained only one or two symbionts per host cell (unpublished, Trench, 1980). However, work by Glider *et al* (1980), using electron microscopy, showed that the gastroderm cells of *Aiptasia pallida* contained between 2 and 5 symbiotic algae. More recent work by Gates & Muscatine (1992) found that freshly isolated gastroderm cells of *A. pulchella* contained between 1 and 5 algal cells.

Variation in the number of symbionts within gastroderm cells may reflect variation in the relative rates of symbiont and host cell division such that host cells with many symbionts may indicate a relatively high rate of zooxanthella division compared to that of the host cells. Gastroderm cells in tentacles of animals under ammonium supplementation had significantly fewer cells containing just one symbiont than gastroderm cells from control animals, which may indicate an increase in zooxanthella division relative to host division. The increase in the

proportion of gastroderm cells containing 2 or more symbionts with ammonium supplementation found in *A. viridis* would give rise to a 12% increase in total zooxanthella numbers per tentacle provided the number of gastroderm cells in the tentacles did not change.

3.4.3 Effects of long term exposure to ammonium enrichment, starvation and feeding on the zooxanthella population density, total zooxanthella population, and animal biomass.

3.4.3.1 Effects of feeding and starvation on the zooxanthella population density.

Zooxanthella population densities of whole anemone tissue in animals starved for 2 weeks (day 0 of experiment) averaged 1270 cells.μg protein^{-1} while in animals starved for 80 days, densities averaged 1050 cells.μg protein^{-1}. Animals fed weekly had population densities of 805 cells.μg protein^{-1} after 80 days. These values are slightly higher than zooxanthella densities of starved *Aiptasia pallida* found by Clayton & Lasker (1984) (Table 3.15), but between one half and one third of the zooxanthella density of *A. pulchella and A. pallida* (Muller Parker, 1985; 1987; Cook *et al*, 1988).

Table 3.15 Some values for zooxanthella densities for different anthozoans measured as algal cells per μg protein for polyps or whole animal. * denotes s.d. † denotes s.e.

Species	Zooxanthella density (cells.μg protein^{-1})		Reference
Montastrea annularis	671 (270) *	freshly collected	Smith & Muscatine, 1986
Stylophora pistillata	368 (130)*	freshly collected	Smith & Muscatine, 1986
	550 (124)†	freshly collected	Hoegh-Guldberg
(Red Sea)	830	starved 2 weeks	& Smith, 1989b
	960	fed 2 weeks	Muscatine *et al*, 1989
Seriatopora hystrix	2110 (1030) †		Hoegh-Guldberg & Smith, 1989b
Aiptasia pulchella	1950-3520	fresh	Muller-Parker, 1987
	1750-2750	starved	Muller-Parker, 1985
	1240-1560	fed	Muller-Parker, 1985
	1200 (58) †	fed every 14 days	this study
Anemonia viridis	1050 (42) †	starved 80 days	this study
	1105 (65) †	+20μM NH_4^+	this study
	2570 (930) †	fed	Cook *et al*, 1988
Aiptasia pallida	3400	fresh	Cook *et al*, 1988
	750 (90) †	starved 4 weeks	Clayton & Lasker, 1984
	1240 (100) †	fed weekly	Clayton & Lasker, 1984

The specimens of *Anemonia viridis* used in these experiments had oral discs of between 10 and 20 mm in diameter and were much larger than the specimens of *Aiptasia* spp. used by Cook *et al* (1988), which had orals discs of 4-7 mm in diameter. Larger animals contain

proportionately more tissue making up the base and column, where there are generally fewer zooxanthellae (Appendix 1). This is confirmed by preliminary observations which show a negative relationship between the zooxanthella population density and the log of the dry weight of animal (Appendix 2: data from section 3.2.3). This size difference may account for the differences in whole animal densities between *Anemonia viridis* and *Aiptasia* spp.

Feeding led to a significant decrease in zooxanthella population density over 80 days at 300 $\mu E.m^{-2}.s^{-1}$, while the zooxanthella density of starved *A. viridis* had not changed significantly after 80 days. Muller-Parker (1985), similarly found that algal densities measured as cells.mg $protein^{-1}$ in *Aiptasia pulchella*, starved for 5 weeks, were greater than densities in fed animals. Tytler (1982) also found that the algal population density in *Anemonia viridis* starved for 80 days was greater than in animals fed weekly for 80 days. However, Cook *et al* (1988) found a decrease in the symbiont population density to half the original density of 2600 cells.μg $protein^{-1}$ in *Aiptasia pulchella* maintained at 90 $\mu E.m^{-2}.s^{-1}$, after 12 weeks of food deprivation. Specimens of *A. pallida* kept at 65 $\mu E.m^{-2}.s^{-1}$ and fed 3 times weekly on *Artemia* had a mean zooxanthella density of 1560 cells.μg $protein^{-1}$, which was twice the mean density of anemones starved for 10 weeks (Clayton & Lasker, 1984). Colonies of the temperate coral, *Astrangia danae* starved for 2 weeks had a significantly lower mean symbiont density measured as $cells.cm^{-2}$ than colonies fed on *Artemia* (Szmant-Froelich & Pilson, 1980; 1984).

Table 3.16 The effect of feeding on the zooxanthella population density (as cells.μg $protein^{-1}$) of some anthozoans. * indicates density measured as $cells.cm^{-2}$ skeleton surface area.

Animal	zooxanthella density	host growth	host protein content	Reference
Aiptasia pulchella	decreased	difference	increased	Muller Parker, 1985
Aiptasia pulchella	increased			Cook *et al*, 1988
Astrangia danae	increased *			Szmant-Froelich & Pilson, 1984
Anemonia viridis	decreased	difference	increased	This study
Anemonia viridis	decreased			Tytler, 1982
Plesiastrea urvillei	no difference *	no difference		Kevin & Hudson, 1979
Aiptasia pallida	increased			Clayton & Lasker, 1984

3.4.3.2 Effect of ammonium enrichment on the zooxanthella population density

Animals kept in sea water enriched to 20μM ammonium for 80 days had zooxanthella population densities of 1100 cells.μg $protein^{-1}$. As already mentioned in section 3.4.1, not all published studies on the effects of ammonium enrichment show an increase in symbiont

population densities (Belda *et al* 1993; Stambler *et al* 1994). Ammonium enrichment of *Anemonia viridis* only produced a transient increase up to day 35 from 399 to 425 cells.μg dry weight^{-1} (6.5%). After 80 days, however, there was no significant effect. This contrasts with the effect of ammonium supplementation on zooxanthella population densities solely in tentacles of animals which increased significantly by 23% (Figure 3.1).

3.4.3.3 Changes to the total zooxanthella population and animal biomass.

The present results show that the zooxanthella population density in anemones is influenced by the nutritional status of the animal. A constant density could result from no change in the total zooxanthella population and in the host dry weight, or by a decrease in both the biomass of the animal and in zooxanthella numbers, or similarly an increase in both host biomass and zooxanthella numbers. Maintenance of *Anemonia viridis* without feeding for 80 days at 300 $\mu E.m^{-2}.s^{-1}$ with a 12 hour daytime period resulted in a loss in weight. Tytler (1982) also reported weight loss of *A. viridis* maintained starved at 140 and 70 $\mu E.m^{-2}.s^{-1}$ for 80 days. Tytler & Davies (1984; 1986) showed that in *A. viridis* energy fixed in photosynthesis at 140 $\mu E.m^{-2}.s^{-1}$ over a 12 hour daytime period potentially exceeded the 24 hour energy demand for respiration. Therefore weight loss in the light suggests that *A. viridis* needs a source of inorganic nutrients to maintain its body weight and bring about growth. It is likely that nitrogen is required to replace structural and metabolic proteins constantly being metabolised (nitrogen excreted as ammonium exceeds nitrogen uptake). Yonge & Nicholls (1930b) found that starved corals lost weight, while Franzisket (1970) reported that there was no difference in growth rate between fed and starved colonies. Similarly, Kevin & Hudson (1979) reported no difference in growth rate in starved and fed colonies of *Plesiastrea urvillei* after 150 days at 12 $\mu E.m^{-2}.s^{-1}$. This light level is thought to be much lower than would be encountered in the natural environment. Both tropical and temperate anemones have been reported to show a loss in weight with starvation suggesting that anemones cannot exist solely on the products of their symbionts and must rely on heterotrophic input to maintain body weight. The benefits of feeding to coral growth are equivocal and vary between species, and may suggest reliance on predation, bacterial feeding or DOM uptake to different extents. Alternatively, variability may be due to a greater difficulty in measuring coral tissue growth.

The proportion of protein in animal tissue decreased in starved animals to 0.309 mg per mg dry weight over 80 days. Thus, in animals deprived of food, 35% of the total weight lost was protein. The calculated zooxanthella population of *Anemonia viridis* in a standard animal of 500mg initial dry weight had decreased by 7×10^7. Thus, the zooxanthella population density,

measured as cells.mg dry weight^{-1} or as cells per mg protein^{-1} had not decreased significantly after 80 days of starvation, since there was a significant loss in biomass and animal protein as well as a decrease in the zooxanthella population.

The benefits zooxanthellae derive from host feeding are equivocal. Muscatine *et al* (1989) implied that the zooxanthellae derived no benefit from host feeding. However, feeding by anthozoans is thought by some authors to provide symbionts with nitrogen derived from host products of digestion and metabolism which stimulates algal growth (D'Elia & Webb, 1977; Steen, 1986; Cook *et al*, 1988) and that consequently zooxanthella growth and population density may be restricted by the rate of host excretory nitrogen production (Falkowski *et al*, 1993). In the present experiment, the normalised zooxanthella population in fed animals increased by 7% after 80 days, which was an increase of 1.3 x 10^7 zooxanthellae. The proportion of protein in animal tissue did not change under weekly feeding but fed animals increased in weight by 45%. This led to a fall in zooxanthella population density in fed animals such that it would appear that in fed animals, the host seems to "outgrow" its zooxanthella population. Fed animals were consistently larger than starved and ammonium supplemented animals throughout the experiment (ANOVA $F=5.5$ $p=0.008$; Tukey pairwise comparisons $p<0.05$). Animal size appears to influence the zooxanthella population density, since large animals have lower symbiont densities than small animals (Appendix 2). This may be because anemone growth is allometric, such that proportionately more mesoglea (non-zooxanthellate tissue) is added than gastrodermal (zooxanthellae containing tissue) as the anemone grows (Shick, 1991).

Muller-Parker (1985) found that algal population densities measured as cells.mg protein^{-1} in *Aiptasia pulchella*, starved for 5 weeks, were greater than population densities in fed animals. Fed animals doubled their protein content while starved animals lost protein during the experiment giving rise to the densities observed. This implied that the zooxanthella population growth and density had not been affected by host feeding, while the host had increased in biomass due to an increase in protein biomass. Colonies of the temperate coral, *Astrangia danae* which had been starved for 2 weeks had a lower mean symbiont density measured as cells.cm^{-2} than colonies fed once or 3 times a week on *Artemia* (Szmant-Froelich & Pilson, 1980; 1984). Freshly collected colonies had a mean symbiont density of 3.11 x 10^6 cells.cm^{-2} which was similar to fed colonies, implying that starved colonies had lost symbionts while fed colonies had not gained symbionts. The authors suggested that the density within starved corals had reduced because the decreased level of nutrient excretion by the starving corals was less than the symbiont population needed to sustain its growth and maintenance. Reduction in

algal numbers could be brought about by algal digestion or excretion (Szmant-Froelich & Pilson, 1980). Yonge & Nicholls (1930b) reported the loss of zooxanthellae when corals were starved for 52 days in the light. They suggested that the host's lowered metabolic activity led to the expulsion of zooxanthellae. Taylor (1969a) also concluded that the continual culling and removal of zooxanthellae was accelerated under conditions of stress, such that, during starvation, for example, the host was not be able to maintain a large algal population.

Under 20μM ammonium supplementation for 80 days anemones increased in weight by 11.4%. This weight increase was not as great as the weight increase shown by animals fed weekly. The zooxanthella population of a normalised animal of 500 mg initial dry weight under ammonium supplementation for 80 days showed an initial increase of 2.74×10^7 zooxanthellae, however in the latter half of the experiment there was a substantial loss of zooxanthellae leading to an overall increase of only 7.5×10^6 which amounts to an increase of 4%. Thus, under ammonium enrichment, the symbiont population density, which takes into account both the total population and host biomass, did not change significantly. Belda *et al* (1993) found that ammonium enrichment of *Tridacna gigas* over 3 months did not change the zooxanthella population density, but that both the host tissue weight and zooxanthella population had doubled in size.

Under prolonged exposure to ammonium supplementation, as in the present study, some other nutrient (which would normally be provided during feeding) may become limiting to the symbiont population after several weeks under enrichment. It has been suggested that phosphorus is a potentially limiting factor to the zooxanthella population and growth rate (Miller & Yellowlees, 1989; Belda *et al*, 1993).

Under ammonium enrichment the amount of protein in host tissue increased from 0.35 mg to 0.38 mg per mg dry weight, amounting to an increase in host protein of 19% in a normalised animal so that 58% of the total increase in dry weight was due to an increase in host protein. Muscatine *et al* (1989) found that host protein per cm^{-2} surface area of coral increased in *Stylophora pistillata* after 14 days with ammonium enrichment. Muller Parker *et al* (1994) found a 2-fold increase in animal protein per cm^{-2} surface area of coral in *Pocillopora damicornis* kept in sea water enriched with 20μM ammonium for 8 weeks, however, Achituv *et al* (1994) working on the same animal under identical conditions did not find a significant increase in host protein per surface area.

The increase in host protein in ammonium supplemented animals and increase in host dry weight is interesting as it suggests that ammonium is either assimilated by the host for use in

maintenance, growth and storage, or, alternatively, ammonium is assimilated by zooxanthellae and translocated back to host as amino acids which can then be transaminated by host enzymes. Ammonium assimilation enzyme studies suggest that only the zooxanthellae are capable of synthesising amino acids from ammonium (Muscatine *et al*, 1989; Rahav *et al*, 1989). In addition, Ferrier (1992) showed that intracellular free amino acid pools in the host tissue of symbiotic and aposymbiotic *Aiptasia pallida* maintained with ammonium enrichment were no different, which suggests that ammonium is not directly incorporated into host tissue. Under conditions of starvation with ammonium enrichment where a supply of photosynthate is available, less protein may be metabolised or that which is metabolised can be replaced.

Summary

1. Ammonium enrichment increased the zooxanthella population density in tentacles of *A. viridis* at 300 $\mu E.m^{-2}.s^{-1}$ by 23%; no increase occurred in the control animals. No such increase occurred at 100 $\mu E.m^{-2}.s^{-1}$ after 3 weeks of ammonium enrichment.

2. There was no difference in zooxanthella population density between tentacles of animals maintained under high (300 $\mu E.m^{-2}.s^{-1}$) and low (100 $\mu E.m^{-2}.s^{-1}$) light intensity for 3 weeks, indicating that light *per se* does not influence population density.

3. The number of zooxanthellae per gastroderm cell was significantly higher in animals kept in ammonium enriched sea water than in non-enriched sea water.

4. After 80 days, the zooxanthella population density decreased in fed animals but did not change significantly in either ammonium supplemented or starved animals.

5. The decrease in population density in fed animals was due to a substantial increase (46%) in host weight but no significant change in the total zooxanthella population. Starved animals showed a significant loss of host tissue (28%) and a substantial loss of zooxanthellae (35%). Ammonium supplemented animals increased in weight by 11% but the increase in the zooxanthella population was not significant. Animal protein significantly increased in fed and ammonium enriched animals (45% and 19% respectively) but decreased by 37% in starved animals.

6. These results emphasise the problems which are introduced by expressing the zooxanthella population size, as all previous researchers have, in terms of population density. The results also show, for the first time, that under conditions of high light and with high external ammonium concentrations, the flux of nitrogen compounds from zooxanthellae to host is sufficiently high to permit growth of host tissue.

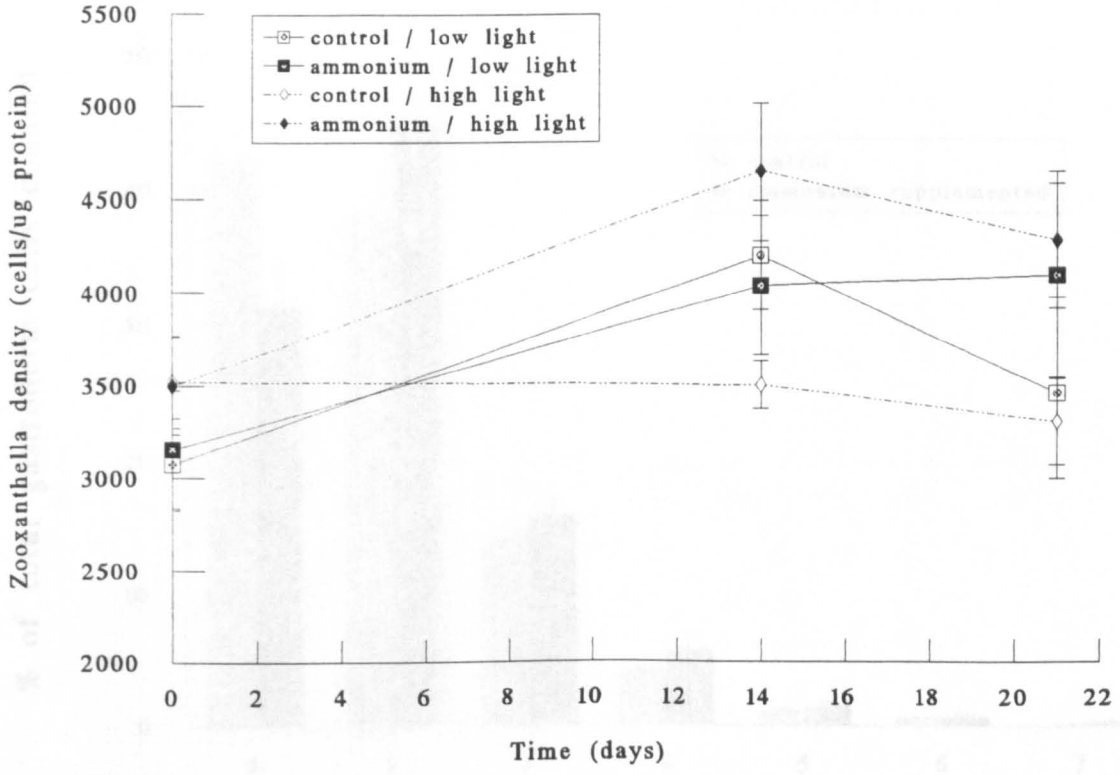


Figure 3.1 Zooxanthella population density in tentacles of *A. viridis* maintained at low light (100 $\mu E.m^{-2}.s^{-1}$) and high light (300 $\mu E.m^{-2}.s^{-1}$) for 21 days without or with 20μM ammonium supplementation. Errors are s.e. of mean (n=10).

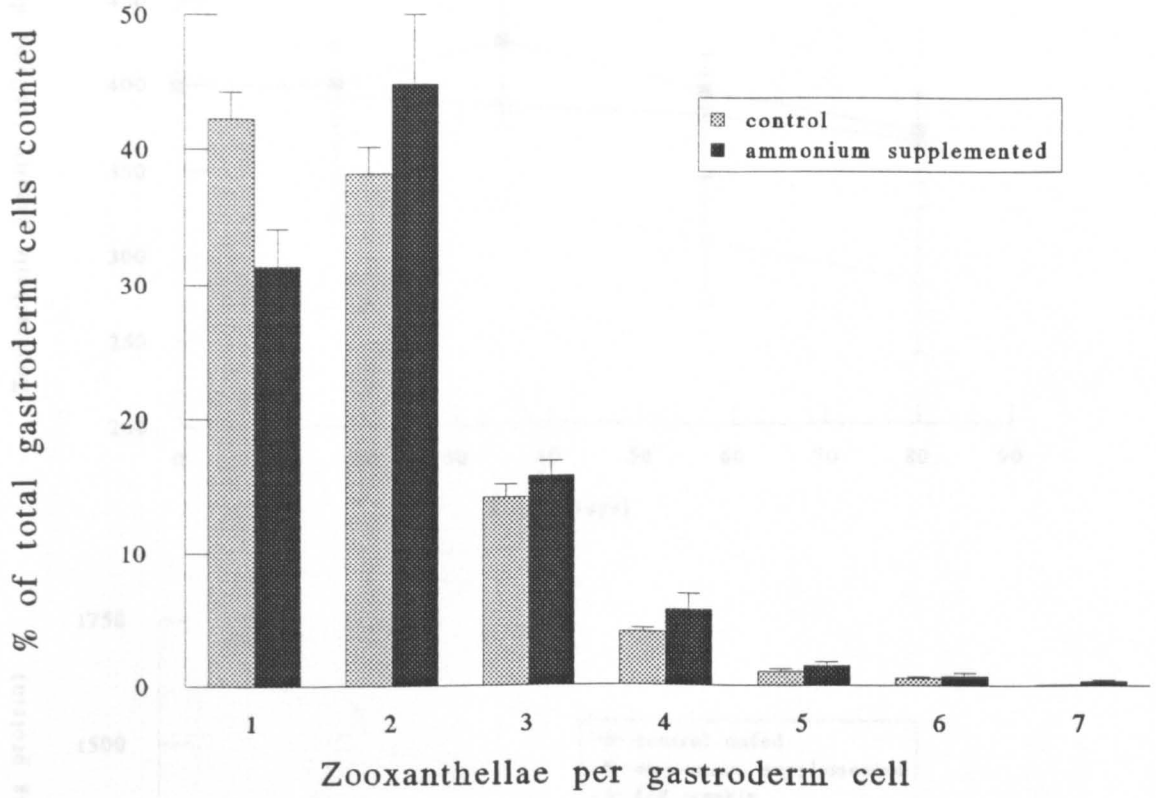
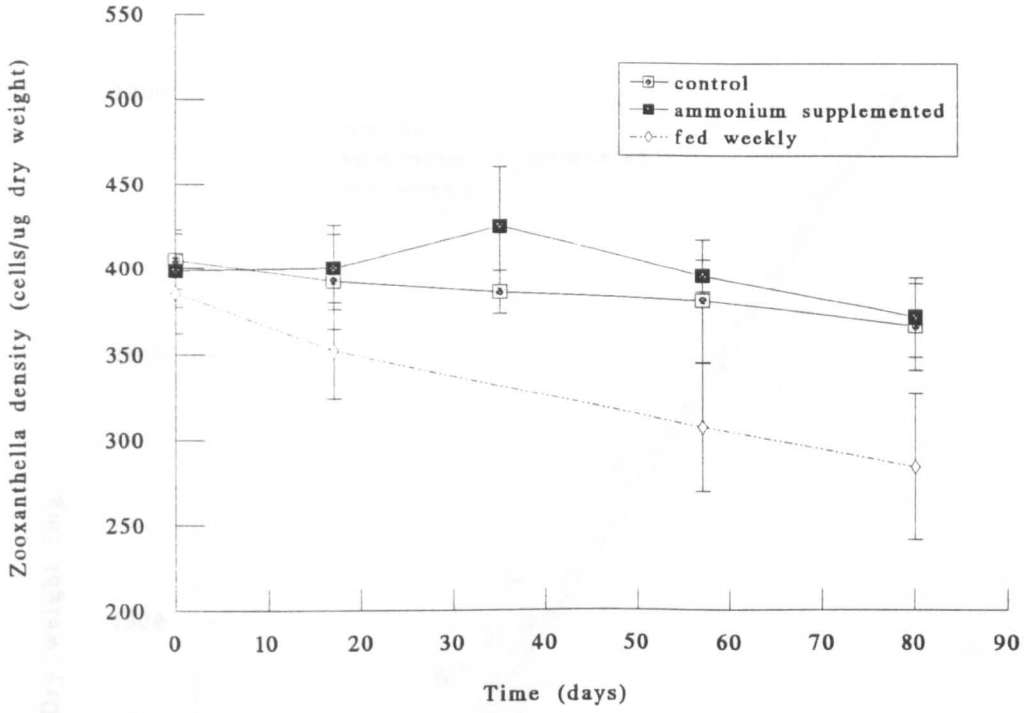


Figure 3.2 Variation in the number of zooxanthellae per gastroderm cell in tentacles of *A. viridis* maintained at 300 $\mu E.m^{-2}.s^{-1}$ in control and 20μM ammonium enriched sea water. Bars are standard errors of the mean of 5 animals.

a)



b)

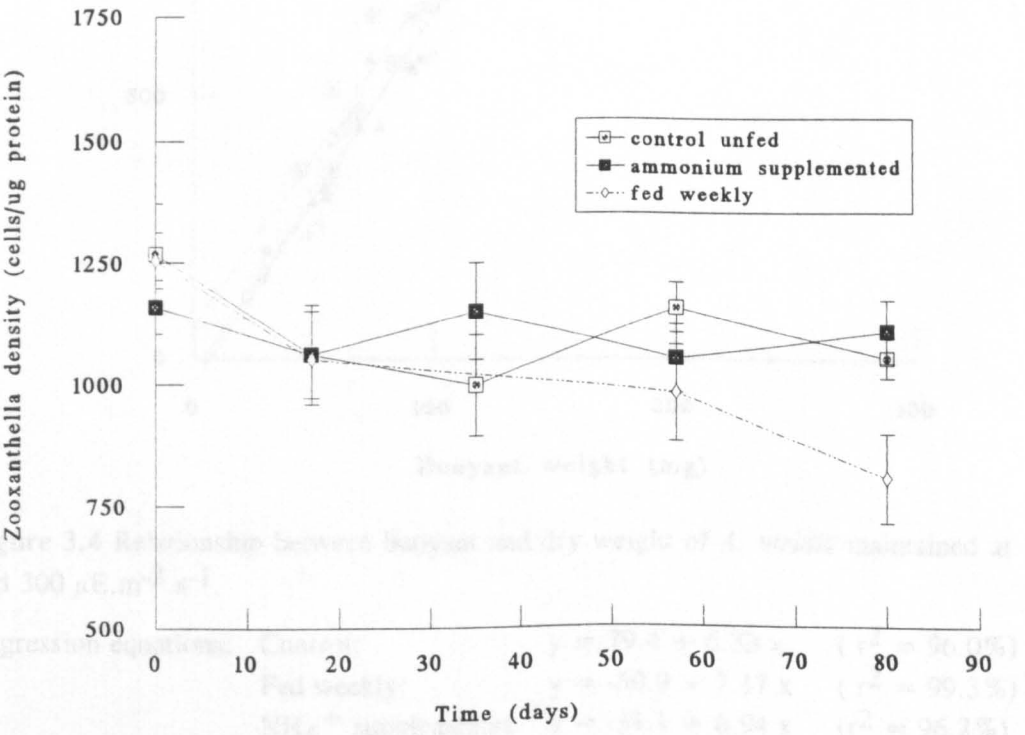


Figure 3.3 Zooxanthella population density as a) cells.μg dry weight^{-1} and b) cells.μg animal protein^{-1} of *A. viridis* maintained at 300 μE.m^{-2}.s^{-1} with or without 20μM ammonium enrichment or with weekly feeding for 80 days. Bars are s.e. of the mean (n=5).

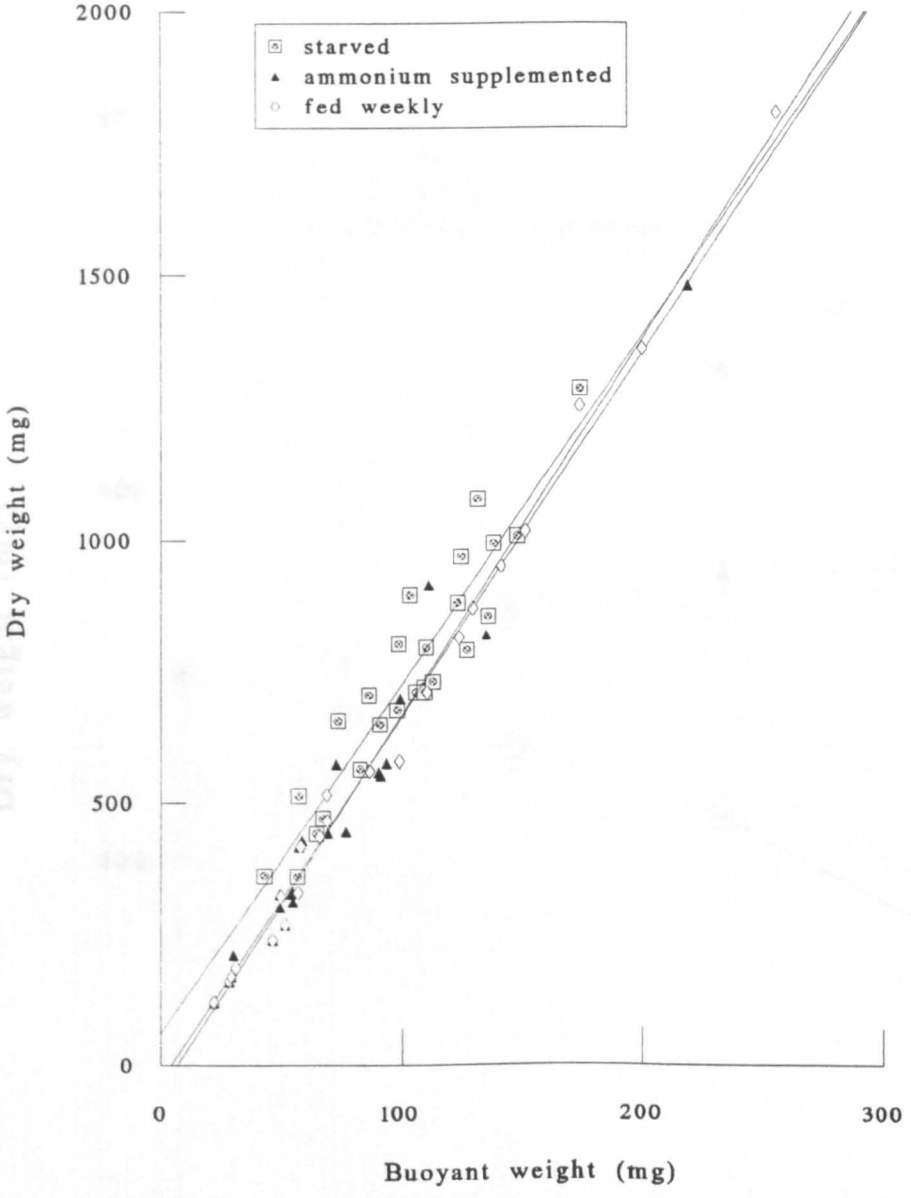


Figure 3.4 Relationship between buoyant and dry weight of *A. viridis* maintained at 11±1°C and 300 μE.m^{-2}.s^{-1}.

Regression equations:	Control:	y = 79.4 + 6.33 x	(r^2 = 96.0%)	n=25
	Fed weekly:	y = -50.9 + 7.17 x	(r^2 = 99.3%)	n=22
	NH_4^+ supplemented	y = -31.1 + 6.94 x	(r^2 = 96.2%)	n=23

(where x = buoyant weight in mg, y = freeze dried weight in mg).

Covariance analysis showed no significant difference between the 3 regression coefficients (F = 1.4102; $F_{0.05(2)2,30}$ =4.18; p=0.26 not significant) and no significant difference between elevations (F = 1.441, $F_{0.05(2)2,32}$ =4.15, p=0.5, not significant). Therefore data were pooled.

combined regression line: y = -1.4 + 6.74 x (r^2 = 97%)

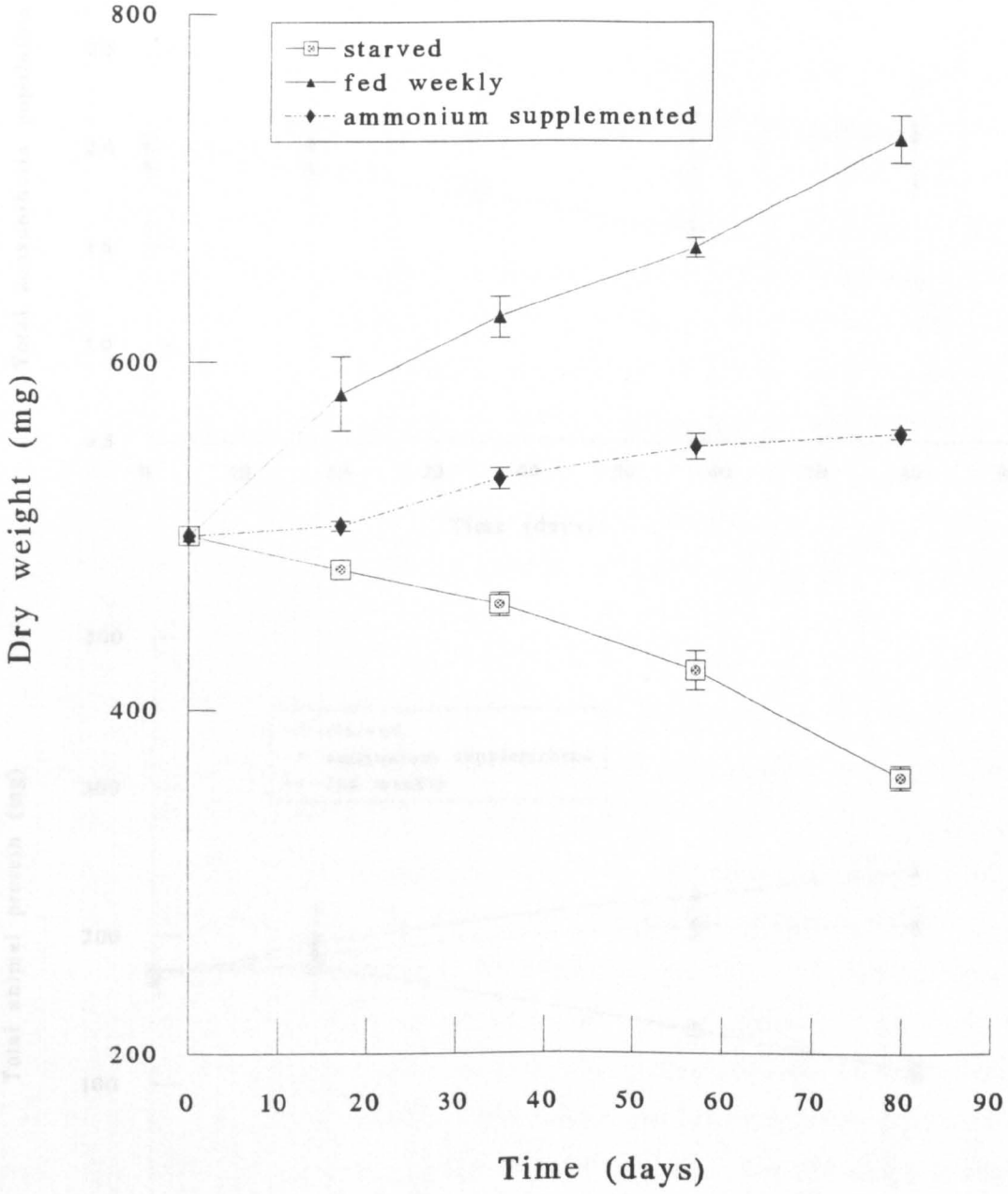
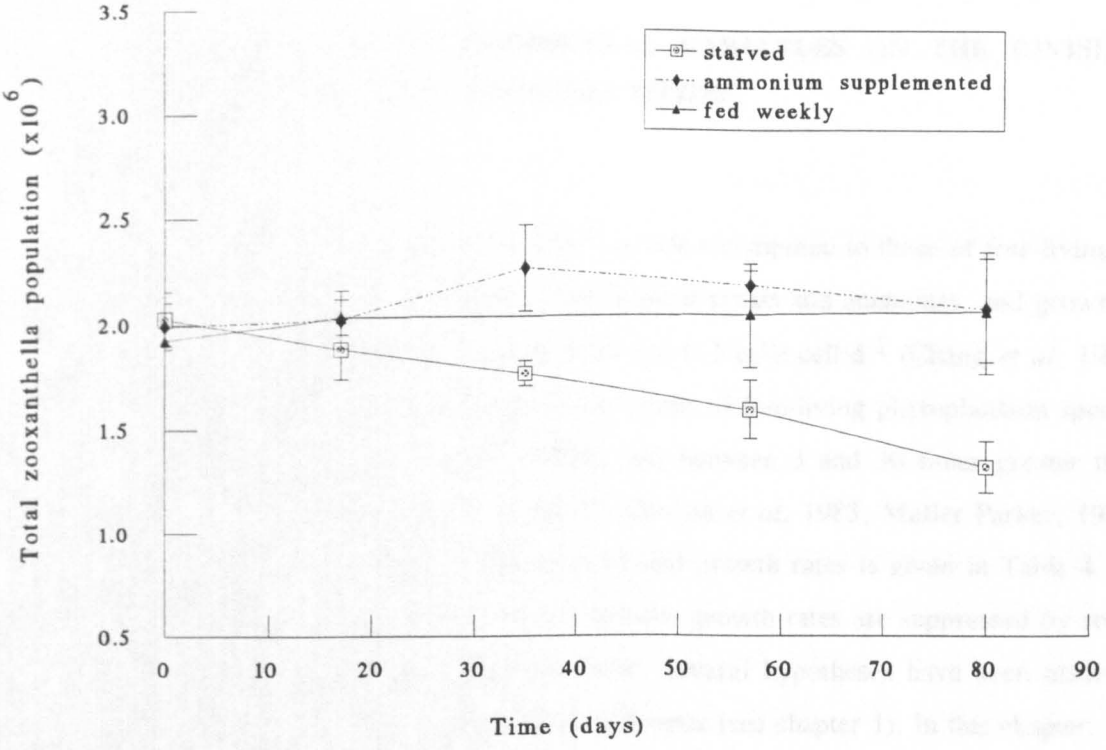


Figure 3.5 Dry weight of *A. viridis* for a standard animal of 500 mg initial dry weight calculated from changes in buoyant weight of animals maintained at 300 $\mu E.m^{-2}.s^{-1}$ under ammonium supplementation, feeding or starvation for 80 days. Bars are s.e. of mean (n=5).

a)



b)

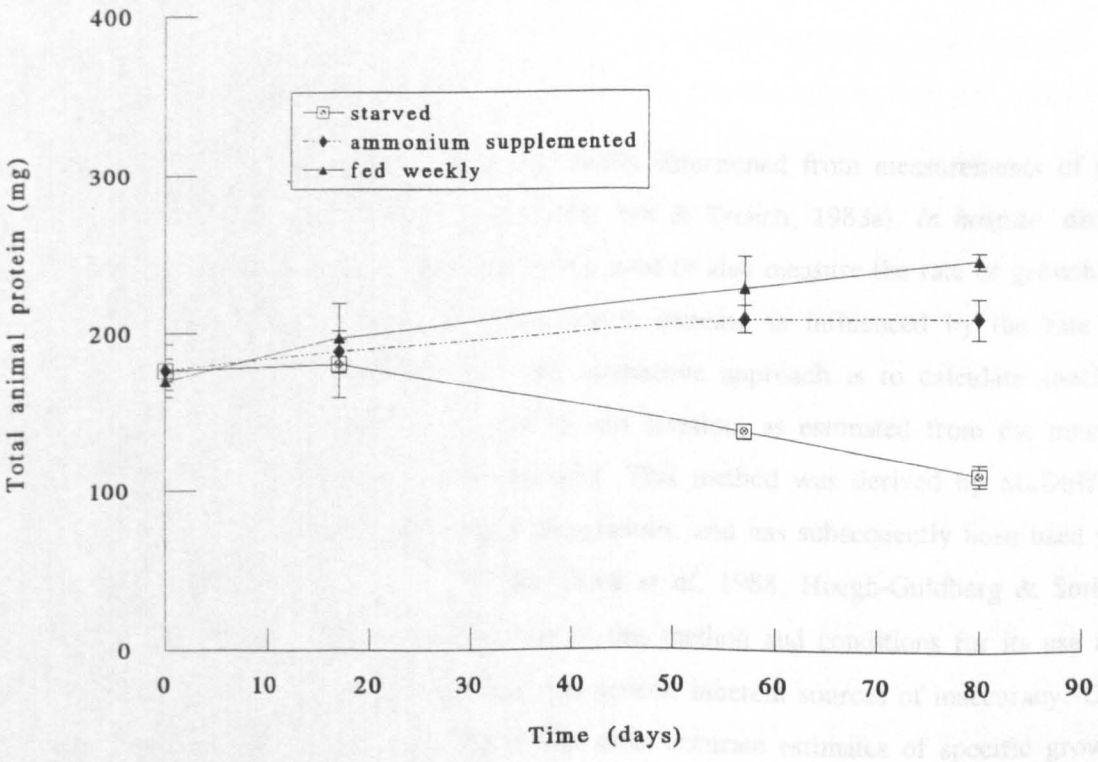


Figure 3.6 a) Total zooxanthella population and b) total protein content of a standard animal of 500mg dry weight under ammonium enrichment, weekly feeding and starvation for 80 days.

CHAPTER 4

THE EFFECTS OF SOME ENVIRONMENTAL VARIABLES ON THE DIVISION RATE OF ZOOXANTHELLAE IN *ANEMONIA VIRIDIS*.

4.1 Introduction

Growth rates of algae in symbiosis are relatively low when compared to those of free-living or cultured algae. *Symbiodinium* spp., isolated from tropical corals and anemones, and grown in culture, had specific growth rates of between 0.22 and 0.3 cells.cell.d^{-1} (Chang *et al*, 1983; Fitt & Trench, 1983a), which are similar to growth rates of free-living phytoplankton species in the North Pacific (Weiler & Chisholm, 1976), but between 3 and 30 times greater than specific growth rates of *Symbiodinium, in situ* (Wilkerson *et al*, 1983, Muller Parker, 1985, 1987; Cook *et al*, 1988). A summary table of published growth rates is given in Table 4.10. These differences led to the suggestion that zooxanthella growth rates are suppressed by some factor/s associated with the symbiont's environment. Several hypotheses have been made to explain the reduced growth rate of zooxanthellae *in hospite* (see chapter 1). In this chapter, the effects of nitrogen, light intensity and host biomass on the division rate of zooxanthellae in *Anemonia viridis* are investigated.

4.1.1 Measurement of growth rates.

Growth rates of zooxanthellae in culture are readily determined from measurements of the population density over time (Chang *et al*, 1983; Fitt & Trench, 1983a). *In hospite*, direct measurements of growth rate are hampered by the need to also measure the rate of growth of the host cells. Furthermore, the rate of population increase is influenced by the rate of expulsion of zooxanthellae from the host. An alternative approach is to calculate specific growth rates from measurements of the rate of cell division, as estimated from the mitotic index of a sample of cells isolated from the host. This method was derived by McDuff & Chisholm (1982) for use with phytoplankton populations, and has subsequently been used for symbiont populations (Wilkerson *et al*, 1983; Cook *et al*, 1988; Hoegh-Guldberg & Smith, 1989b; Muscatine *et al*, 1989). The derivation of this method and conditions for its use are given in Appendix 3. This method, however, has several inherent sources of inaccuracy. One of these is the diel pattern of cell division. The most accurate estimates of specific growth rates can be made if division is phased or synchronous during the 24 hour light:dark cycle. When division is asynchronous, growth rate estimates are subject to greater error (Fitt & Trench, 1983a). Free-living dinoflagellates typically show phased division (Weiler &

Chisholm, 1976; Weiler, 1980; Chisholm, 1981). Cultures of symbiotic dinoflagellates isolated from *Aiptasia tagetes* and *Cassiopeia xamachana* also exhibited phased diel patterns of mitotic index (Fitt & Trench, 1983a). In contrast, many anthozoan zooxanthellae *in hospite* exhibit phased diel patterns of growth rate, while in others growth rate is asynchronous.

The problem of calculating specific growth rates of zooxanthellae *in hospite* in *Anemonia viridis* will be discussed further in Chapter 7. For the present, an index of growth rate, the mitotic index, will used as a measure of the rate of cell division.

4.1.2 Factors influencing zooxanthella division rates.

Dissolved inorganic nitrogen availability.

It is well established that cnidarian symbiotic associations will take up ammonia from the sea water (Wilkerson & Muscatine, 1984; Davies, 1988). Typically, however, the concentration of ammonia in coastal waters is very low and on coral reefs the levels are often at the limits of detection, ranging from 0.2 to 1.4 μmoles.l^{-1} (Kinsey & Davies, 1979; Cook *et al*, 1988; Muscatine *et al*, 1985; 1989). On this basis, Cook & D'Elia (1987) proposed that symbiont growth rates *in hospite* may be limited by the availability of dissolved inorganic nitrogen. This hypothesis could be tested by establishing experimentally a correlation between the division rate and concentration of ammonia in the surrounding water. Nutrient enrichment studies of this sort have yielded equivocal results. Muscatine *et al* (1989) found no difference in the mitotic index of symbionts from *Stylophora pistillata* when colonies incubated in 20μM ammonium were compared with controls. In a similar experiment, Hoegh-Guldberg & Smith (1989b) reported no difference in the mitotic index of symbionts of *Seriatopora hystrix* and *Stylophora pistillata* at the end of 19 days exposure to pulsed addition of ammonium, when compared with controls. By contrast, in an experiment lasting only 4 days, symbionts of the hydroid, *Myrionema amboinense*, had a diel peak mitotic index of 12% when incubated in ammonium or nitrate enriched sea water, compared with 4% in animals freshly collected from nutrient-poor water (Cook & Fitt, 1989). The experiments of Muscatine *et al* (1989) and Hoegh-Guldberg & Smith (1989b) were carried out over 2-3 weeks, with growth rate measurements taken only at the end of the experimental period. Furthermore, ammonium was introduced in pulsed additions rather than as a continuous supply. At the time of the current investigation, no long-term nutrient enrichment studies had been carried out. It is clear that further experiments are required to determine the duration of any enhanced rate of division of zooxanthellae when the host is exposed to ammonium enriched sea water.

Host derived nutrients.

A second source of nitrogen for assimilation by the zooxanthellae are amino acids from the host cytoplasm or ammonia produced by the host's excretory catabolism. Zooxanthellae have the capacity to take up amino acids (Schlichter, 1978; Blanquet, *et al*, 1988) and will assimilate host-derived ammonia during the day and night (Davies, 1988). In *Aiptasia pallida*, the symbiont mitotic index fell from 8% to 3% after 4 weeks starvation at a light intensity of $90\mu E.m^{-2}.s^{-1}$, but increased again after 5 days of feeding (Cook *et al*, 1988). Conversely, Muscatine *et al* (1989) found no difference in the mitotic index of zooxanthellae from *Stylophora pistillata* which had been fed on *Artemia* for 14 days when compared with a control group which were starved, suggesting that amino acids resulting from digestion of food were used for growth of the host and were not available to the symbionts. Again, the role of host-derived nitrogen in determining the division rate of the symbionts remains unresolved.

Light Intensity.

Light is required for ammonium and phosphate uptake by free-living algae (Syrett, 1981), and by symbionts *in situ* (D'Elia, 1977). Photosynthesis is thought to provide a source of fixed carbon which is required for nitrogen fixation (Syrett, 1981). Davies (1988) showed that rates of ammonium uptake by zooxanthellae in *Anemonia viridis* were proportional to light intensity within the range 50 to 300 $\mu E.m^{-2}.s^{-1}$. Below 50 $\mu E.m^{-2}.s^{-1}$, *A. viridis* showed a net efflux of ammonium. Since symbiont division is dependent on a supply of nitrogen, light intensity may potentially be a limiting factor of symbiont growth rates through the limitation of DIN uptake rates. Several studies have shown the effect of changes in light intensity on population density, but very few have investigated the effect on zooxanthella growth rate within the host. Those which are available give conflicting results (Fitt & Trench, 1983a; Muller Parker, 1985, 1987).

Space available for growth.

Division rate may in some way be related to the intracellular space available to the symbionts. Thus, increased division rate of the host cells brought about by feeding or by regeneration of excised tissues would be expected to increase the space available for the algae to occupy and hence their rate of division (McAuley, 1981a). The observation that the mitotic index of zooxanthellae in *Seriatopora hystrix* is inversely related to their population density (Hoegh-Guldberg *et al*, 1986, Hoegh-Guldberg & Smith, 1989b), may lend support to this hypothesis.

However, Cook & D'Elia (1987) argued that, at high densities, competition for certain nutrients would be increased and likely to become limiting with increasing symbiont density. Alternatively, a high symbiont density may lead to self shading and increased competition for carbon dioxide (Weis, 1991), which might lead to lowered photosynthesis and may affect division rates. Thus, an increase in mitotic index with feeding could be due to an increase in nutrient supply by increased host metabolism or an increase in space created by an increase in host cell division.

Further indirect evidence for the space limitation hypothesis comes from the observation of Smith (1986) that the division rate of zooxanthellae in juveniles of the anemone *Aulactinia stelloides* is greater than that of zooxanthellae in adults. A similar relationship was found in the jellyfish *Mastigias* by Muscatine *et al* (1986). Smith (1986) attributed this difference to a higher growth rate of juvenile anemones, although no data on growth rate of the host were presented.

In this chapter, experiments to investigate some of the factors which may influence the division rate of zooxanthellae of *Anemonia viridis* will be described. In particular the following questions will be addressed.

1. Is the rate of division of the zooxanthellae measured by mitotic index synchronous or asynchronous over 24 hours?
2. Is the rate of division affected by the level of dissolved inorganic nitrogen in the form of ammonia in the sea water and if so what is the time-course of the response?
3. Is the division rate affected by levels of irradiance?
4. Is division rate influenced by host-derived nutrients resulting from feeding or starvation?
5. Is there support for the space-limitation hypothesis from correlation of division rate with the size of anemone?

4.2 Materials and Methods.

4.2.1 Diel variation in the mitotic index of zooxanthellae symbiotic with *A. viridis*.

Animals were maintained in running aerated sea water at $11 \pm 1^{o}C$ in 3 regimes of high light (300 $\mu E.m^{-2}.s^{-1}$), low light (20 $\mu E.m^{-2}.s^{-1}$), or high light with 20μM ammonium enrichment (section 3.2.1), for 1 week. After this time, three animals from each of the above treatments were sampled every 2 hours over a 24 hour period. Two tentacles were removed from each animal and fixed in 3:1 alcohol:acetic acid for 24 hours. The samples were stained with Feulgen and counts of the number of dividing cells made using the procedure in section 2.2.3.

Before calculating the mitotic index (as the percentage of cells undergoing division), the raw data comprising the number of dividing cells in a sample of 300 cells, were tested for normality, using Anderson-Darling tests, and for homogeneity of variance using Bartlett's test to determine their suitability for statistical analysis by ANOVA and *t*-tests.

4.2.2 The effect of ammonium supplementation and light intensity on symbiont mitotic index.

4.2.2.1 The relationship between symbiont division rate and ammonium concentration.

Anemones of similar sizes (400-700 mg dry weight) were maintained at 300$\mu E.m^{-2}.s^{-1}$ on a 12 hour light 12 hour dark cycle for one week at a temperature of $11 \pm 1^{o}C$, before being randomly assigned to 1 of 4 regimes. The regimes consisted of a continuous supply of sea water enriched to either 10, 20 or 50μM of ammonium and a control group maintained in unenriched sea water.

The experimental tank was divided into 4 compartments, and 12 animals were placed in each compartment. Illumination was provided by a series of high output fluorescent tubes set on a 12 hour light: 12 hour dark cycle at an intensity of 300 $\mu E.m^{-2}.s^{-1}$. The animals were left in unenriched sea water for a week to acclimatise to the light levels, after which time the ammonium treatments were started. The ammonium concentrations of the recirculating system and in each enriched treatment were monitored regularly. After one week, 3 tentacles from each of 6 animals chosen randomly were removed and fixed for 24 hours in a solution of 3:1 alcohol:acetic acid. Samples to be stained for mitotic index counts were treated following the procedure in section 2.2.3. Sampling was carried out at 14 00h.

4.2.2.2 The relationship between symbiont mitotic index and light intensity with and without ammonium supplementation.

Anemones, of approximately the same size (400-700 mg dry weight), were randomly assigned to 6 treatments. 30 animals were divided equally between 3 compartments of a partitioned tank. A further 30 animals were placed into a similarly partitioned tank. All animals were maintained for one week at a light intensity of 110 $\mu E.m^{-2}.s^{-1}$ provided by a series of fluorescent tubes on a 12 hour light: 12 hour dark cycle at a temperature of $11 \pm 1^{o}C$. At the start of the experiment the sea water to the first tank was enriched with ammonium to a concentration of $20\mu M$ as described in section 3.2.1. The sea water to the second tank was not enriched. At the same time the light levels to the 3 compartments of each tank were adjusted to 300, 60 and 20 $\mu E.m^{-2}.s^{-1}$ and the animals were left for 28 days. After 21 days, 6 animals were randomly selected from each of the 6 treatments. The ammonium concentration in the reservoir and the compartments was monitored regularly. Three tentacles were removed from each animal, and fixed for mitotic index counting. The excised tentacles were fixed for 24 hours in 3:1 alcohol:acetic acid and stained according to the procedure in section 2.2.3. A further 6 animals from each regime were sampled on day 28. Samples were taken at 1400h of each sampling day.

4.2.2.3 The effect of long-term ammonium enrichment, feeding or starvation on the mitotic index of zooxanthellae.

A group of 90 animals were maintained for 1 week at a light intensity of 300 $\mu E.m^{-2}.s^{-1}$ and at a temperature of $11 \pm 1^{o}C$. 30 animals were then randomly assigned to each of 3 treatments. Group 1 were unfed and received standard aquarium sea water; group 2 were unfed but maintained in sea water continuously enriched with ammonium to a concentration of $20\mu M$ (section 3.2.1); group 3 were maintained in standard aquarium sea water and were fed weekly with freeze-dried mysid shrimps. Animals were maintained for 80 days at $11 \pm 1^{o}C$ and at a light intensity of 300 $\mu E.m^{-2}.s^{-1}$. The ammonium level in each compartment was monitored regularly. Two tentacles from 5 animals from each treatment were removed and fixed for mitotic index counts on days 0, 7, 17, 35, 57 and 80.

4.2.3 Effect of body mass on mitotic index.

If "space" for division is a limiting factor (see Introduction), and if smaller anemones have a higher relative growth rate than large anemones, as suggested by Smith (1986), it might be expected that there would be a negative correlation between mitotic index and body mass. To

test this, the anemones used in experiment 4.2.2.3 were used. After removal of tentacles at each sampling period, the sampled animal were sacrificed and then cut into two. Each half was quickly wet weighed and one half was freeze dried. From these data, the dry weight of the whole anemone was calculated. The other half was homogenised and used to provide a measurement of zooxanthella population density in the whole anemone (see 3.2.3). Data for animals sacrificed on day zero were combined with the data obtained over the 80 days for the control unfed group. The sample size for the control group was, therefore, 35. Because of sample losses during the staining procedure, the sample size for the ammonium supplemented and weekly fed group was 19 and 12 respectively. The mitotic index values (obtained in 4.2.2.3) were then plotted against the log of the anemone dry weight and regression lines were drawn.

4.3 Results.

4.3.1 Diel variation in the mitotic index of zooxanthellae in *A. viridis*.

All data was homoscedastic and conformed to a normal distribution. Mean mitotic index values from samples at 2 hour intervals over a 24 hour period are shown in Figure 4.1. Sample indices, for the three animals maintained under low light, did not vary significantly over the 24 hour period (ANOVA: between sample difference $F=1.19$, $p=0.3$). The mean diel mitotic indices were 0.93%, 1.42% and 1.43%.

The mean mitotic indices of zooxanthellae of animals from the high light and high light with ammonium treatments varied between 2 and 4% (Figure 4.1). There was significant variation in the mitotic index over the 24 hours in the high light and ammonium supplemented treatments (ANOVA, high light treatment $F=3.82$, $p<0.001$; ANOVA, ammonium supplemented $F=9.49$, $p<0.001$), however the maximum mitotic index values for high light and ammonium supplemented animals did not coincide. The mitotic index of symbionts from high light maintained animals reached a maximum at 22 00h, after which the index decreased to a minimum at 02 00h, then steadily increased. In the ammonium supplemented animals, the mitotic index of zooxanthellae peaked at 10 00h during the light cycle, then steadily decreased until 04 00h.

4.3.2 The effect of ammonium supplementation and light intensity on symbiont mitotic index.

4.3.2.1 The relationship between symbiont mitotic index and ammonium concentration.

The ammonium concentration in the re-circulating system in the Department of Zoology which flowed through the control chamber varied from 3μM during early morning to $<0.5\mu$M at the end of the light period. All data was homoscedastic ($p=0.358$) and conformed to a normal distribution. Mitotic index data as the percentage of dividing cells is tabulated in Table 4.1. Figure 4.2 shows the mean mitotic index for each treatment plotted against ammonium concentration. ANOVA and Tukey tests were carried out on the differences in numbers of dividing cells between each treatment.

After 1 week, symbionts from animals kept in 50μM ammonium had an average mitotic index of 7.9% (Table 4.1). A Tukey test showed that this was significantly greater than all other treatments ($F=25.6$, $p<0.001$).

Table 4.1 Mitotic Index as percentages of zooxanthellae from *A. viridis* maintained for 1 week under 4 ammonium treatments. Means are given at the bottom of each column. s.e of means given in brackets.

Ammonium concentration			
Control	10μM	20μM	50μM
3.63	5.93	5.93	8.27
4.37	5.13	5.74	7.76
4.27	5.19	6.44	7.67
3.17	3.78	4.89	
3.91		5.67	
		6.0	
3.87	5.01	5.78	7.9
(0.22)	(0.45)	(0.21)	(0.19)

Mitotic indices of zooxanthellae from animals kept in 20μM ammonium were significantly greater than control values, although values for zooxanthellae maintained at 10μM ammonium enrichment were not significantly greater than control values (Table 4.2). The relationship between the mean mitotic index and ammonium concentration closely fitted a logarithmic function (Mitotic index = 1.02 + 3.93*log (concentration); r^2 = 97.9%).

Table 4.2 Table of statistical differences between treatments from experiment 4.3.2.2 calculated using Tukey's test for pairwise comparisons at the 1% level.

	Control	10μM ammonium	20μM ammonium
10μM ammonium	not significant	-	-
20μM ammonium	$p<0.01$	not significant	-
50μM ammonium	$p<0.01$	$p<0.01$	$p<0.01$

4.3.2.2 The relationship between symbiont mitotic index and light intensity with and without ammonium supplementation.

All data was homoscedastic and conformed to a normal distribution. Mitotic index data is given in Table 4.3 and plotted in Figure 4.3. Results of Tukey tests for comparisons between light treatments are given in Table 4.4.

Mitotic indices of zooxanthellae in animals maintained without ammonium enrichment under the three light intensities were significantly different ($F=18.61$ $p<0.001$). Mean mitotic index values at 4 weeks increased with increasing light intensity from 1.7% at 20 $\mu E.m^{-2}.s^{-1}$ to 4.3% at 300 $\mu E.m^{-2}.s^{-1}$. Similarly, mitotic indices of zooxanthellae from ammonium supplemented animals maintained under the 3 light intensities were significantly different

($F=40.27$, $p<0.001$), and also showed an increasing trend with light intensity from 1.9% at 20 $\mu E.m^{-2}.s^{-1}$ to 9.4% at 300 $\mu E.m^{-2}.s^{-1}$ after 4 weeks.

Table 4.3 Mitotic Index, as percentages, of zooxanthellae from *A. viridis* under 3 light intensities with and without 20μM ammonium enrichment. Error bars are standard errors of the mean (no superscript n=6; superscript a n=4; superscript b n=8; superscript c n=5).

	Unsupplemented sea water			Ammonium supplemented		
Sampling time	High Light 300$\mu E.m^{-2}.s^{-1}$	Intermediate 60$\mu E.m^{-2}.s^{-1}$	Low light 20$\mu E.m^{-2}.s^{-1}$	High Light 300$\mu E.m^{-2}.s^{-1}$	Intermediate 60$\mu E.m^{-2}s^{-1}$	Low light 20$\mu E.m^{-2}.s^{-1}$
3 weeks	3.99 (0.21)	2.68 (0.45)	2.21 [c] (0.48)	8.09 (0.76)	4.74 (0.32)	3.60 (0.69)
4 weeks	4.31 (0.53)	2.49 (0.17)	1.73 (0.24)	9.41 [b] (0.58)	6.57 (0.16)	1.92 [a] (0.20)
ANOVA	light effect: $F=18.61$ $p<0.001$			light effect: $F=40.27$ $p<0.001$		

Table 4.4 Results of Tukey tests on the difference in mitotic index between light treatments.

Control	Low Light	Intermediate	**Ammonium enriched**	Low Light	Intermediate
Intermediate	n.s.		Intermediate	$p < 0.05$	
High Light	$p < 0.05$	n.s.	High Light	$p < 0.05$	$p < 0.05$

Table 4.5 Results of ANOVA tests carried out on the data presented in Table 4.3. Treatment effect was the comparison between control and ammonium supplemented data.

	F	p
High Light 300 $\mu E.m^{-2}.s^{-1}$	42.69	<0.001 significant
Intermediate 60 $\mu E.m^{-2}.s^{-1}$	76.63	<0.001 significant
Low light 20 $\mu E.m^{-2}.s^{-1}$	2.4	0.141 n.s

It is clear from this experiment that after 4 weeks there is a small but significant increase in the mitotic index of zooxanthellae living at high levels of irradiation. However, in anemones which had been maintained in ammonium enriched sea water, there was a greater increase in mitotic index at high light levels. As a consequence, at low levels of light, there was no difference in the mitotic index of control and ammonium enriched animals, but at the higher light intensities there was a significant difference (Table 4.5). The increase in mitotic index, as noted in 4.3.2.1, only occurs when anemones are exposed to higher levels of illumination.

4.3.2.3 Effect of long-term ammonium enrichment and feeding on the symbiont mitotic index.

All data was found to be homoscedastic and conformed to a normal distribution. Mean mitotic indices of zooxanthellae over the 80 days are given in Figure 4.4 and Table 4.6. Results of ANOVA tests between treatments and Tukey tests are given in Tables 4.7, 4.8 and 4.9.

Table 4.6 Mean mitotic index values as percentages ± s.e (n=5) of zooxanthellae from *A. viridis* kept for 80 days at 300 $\mu E.m^{-2}.s^{-1}$.

Day	Control	Ammonium supplemented	Fed weekly
0	4.306 (0.52)	3.856 (0.20)	4.197 (0.42)
7	-	6.236 (0.46)	4.586 (0.30)
17	3.752 (0.30)	7.725 (0.77)	5.306 (0.53)
35	4.307 (0.54)	6.702 (0.78)	6.144 (0.26)
57	4.467 (0.41)	5.769 (0.57)	5.806 (0.52)
80	3.750 (0.25)	5.491 (0.77)	5.378 (0.45)
ANOVA	F=0.08 p=0.9	F=2.63 p=0.05	F=7.31 p=0.011

Mitotic indices of zooxanthellae from the control animals averaged 4.1% and did not change significantly throughout the experiment (F=0.08 p=0.9). Mitotic indices of symbionts from the ammonium supplemented group increased significantly to 7.7% by day 17 before gradually decreasing down to 5.5% by day 80 (F=2.6 p=0.05: Tukey's: $p<0.05$). The mitotic index of fed animals increased significantly to a maximum of 6.14% by day 35 (Tukey's: $p<0.05$) but fell over the next 45 days. When the zooxanthella mitotic index of animals from the three regimes were compared, significant differences were found between control and ammonium enriched, and between control and fed, but not between fed and ammonium enriched animals.

Table 4.7 Table of ANOVA tests carried out on the difference in mitotic index between treatments.

Treatments compared	F	p
control v ammonium supplemented	8.53	0.006 significant
control v fed weekly	6.37	0.019 significant
ammonium supplemented v fed weekly	1.65	0.207 n.s.

Table 4.8 Tukey tests between symbiont mitotic index values from animals under 20μM ammonium.

	Day 0	Day 7	Day 17	Day 35
Day 7	$p < 0.05$			
Day 17	$p < 0.05$	n.s.		
Day 35	$p < 0.05$	n.s.	n.s.	
Day 80	n.s.	n.s.	$p < 0.05$	n.s.

Thus, feeding and ammonium enrichment had significant effects on the symbiont mitotic index, although differences between these treatments were not significant. The rise in mitotic index occurred more rapidly in the ammonium enriched group such that a difference was observed after 7 days, whereas in the fed group the difference was observed after 35 days (Table 4.8 and 4.9).

Table 4.9 Results of Tukey tests between zooxanthella mitotic index values from weekly fed animals.

	Day 0	Day 7	Day 17	Day 35
Day 7	n.s.			
Day 17	n.s.	n.s.		
Day 35	$p < 0.05$	$p < 0.05$	n.s.	
Day 80	$p < 0.05$	n.s.	n.s.	n.s.

4.3.3 The effect of body mass on the zooxanthella mitotic index.

Mitotic index values are plotted against the logarithm of the corresponding dry weights for 35 anemones maintained unfed and 19 anemones maintained with 20μM ammonium enrichment at 300 $\mu E.m^{-2}.s^{-1}$ in Figure 4.5. There was an inverse relationship between the logarithm of the dry weight and mitotic index at 300 $\mu E.m^{-2}.s^{-1}$ for both controls and for animals maintained with ammonium. Analysis of covariance showed that the two plots had similar slopes but significantly different elevations (slopes: $F=1.46$ $p=0.23$; elevations: $F=5.5$ $p=0.023$). Zooxanthellae from ammonium supplemented animals were dividing at a faster rate than unsupplemented animals for a given dry weight.

Mitotic index values are plotted against the logarithm of the dry weights for 12 animals maintained with weekly feeding and 35 animals maintained without feeding (controls) in Figure 4.6. There was an inverse relationship between the logarithm of the dry weight and mitotic index. The regression lines were similar in slope but the line corresponding to the fed animals was significantly greater in elevation (slopes: $F=0.48$ $p=0.49$; elevations $F=23.25$ $p<0.001$).

The regression equations had the values:

control:	$y = 28.2 - (8.5*\log x)$	$r^2 = 78.4\%$, $p<0.001$.
ammonium enriched:	$y = 34.9 - (10.7*\log x)$	$r^2 = 62.9\%$, $p<0.001$
fed weekly:	$y = 27.6 - (7.51*\log x)$	$r^2 = 76.2\%$, $p<0.001$

4.4 Discussion.

The majority of studies on the rate of division of zooxanthellae have been based upon determination of the mitotic index from counts of the number of cells showing cell plates (see Table 4.10). Cell plate formation takes place during telophase. However, Brown & Zamani (1992) pointed out that this method underestimates the number of cells undergoing division, since it does not account for those cells where the nucleus has divided but cell wall formation has not yet occurred (ie those cells in anaphase). By the use of the Feulgen nuclear stain, which allows for the differentiation of cells in anaphase as well as those in telophase, Brown & Zamani (1992) showed mitotic index values of 3 to 5 times those determined from counts of zooxanthellae showing cell plates only.

Thus, there arises potential confusion when the two methods, each measuring different stages of the cell division cycle, are both termed the "mitotic index". In future, therefore, the method used to determine the "mitotic index" must be clearly explained. Clearly, mitotic index values obtained using different methods cannot be compared directly.

In the current study and in all data presented in chapter 4, the Feulgen method was adopted and yielded mitotic index values of between 1 and 7.7% (Table 4.11). These values can be compared with those for *Anemonia viridis* of between 10 and 17% obtained using this method by Brown & Zamani (1992). The reasons for this difference have not been addressed fully but may be due to differences in feeding regimes or temperatures or differences in the staining procedures. In particular, the duration of hydrolysis of the material was critical to the whole staining technique. Incomplete hydrolysis resulted in staining of cellular RNA. Brown & Zamani (1992) used a variable hydrolysis period depending on the nature of the tissue (5-15 minutes), whereas, in this study, the hydrolysis period remained constant at precisely 12 minutes regardless of the nature of the tissue or origin of the reagents used.

Table 4.11 Maximum and minimum mitotic index values measured over 24 hour periods of zooxanthellae from *A. viridis* obtained using Feulgen nuclear stain.

Host species	Location	Mitotic Index (%)	Conditions	Reference
A. viridis	Cumbrae, U.K	10.5 - 17	Fed weekly, 15°C	Brown & Zamani (1992)
A. viridis	Loch Sween, U.K	1 - 1.5	$20\mu E.m^{-2}.s^{-1}$ 11°C starved for 2 weeks	This study
A. viridis	Loch Sween, U.K	2 - 4.2	$300\mu E.m^{-2}.s^{-1}$ 11°C starved for 2 weeks	This study
A. viridis	Loch Sween, U.K	5 - 7.7	$300\mu E.m^{-2}.s^{-1}$ 11°C fed 2 days before	This study

Table 4.10 Mitotic indices of zooxanthellae and phytoplankton estimated using the cell plate technique. Specific growth rates included where values were estimated from an assumed duration of cytokinesis (Wilkerson *et al*, 1983). * temperate water species † *in vitro*.

Host Species	Location	Mitotic Index (%)	Specific growth rate cells.cell.$^{-1}$d^{-1}		Reference
Temperate anemones					
Anemonia viridis *	U.K	1.2-2.8		homogenate	Suharsono & Brown, 1992
A. sulcata *	Mediterranean	0.8 / 2.8		1200h / 2200h	Stambler & Dubinsky 1987
Tropical spp.					
Stylophora pistillata	Red Sea	0.61 (±0.3)	0.013	light adapted	Muscatine *et al*, 1984
	Red Sea	0.43 (±0.1)	0.009	shade adapted	
Millipora dichotoma	Red Sea	2.6	0.08		Hoegh-Guldberg *et al* 1987
Aiptasia pulchella	Hawaii, Pacific	0.76-0.78	0.017	shade adapted	Muller Parker, 1987
		0.38	0.008	sun-adapted Sum	
		1.54	0.033	sun-adapted Aut	
Aulactinia stelloides	Caribbean	3.7 (±0.7)	0.094	juveniles	Smith, 1986
		1.9 (±0.5)	0.048	adults	
Aiptasia pallida	Bermuda	1.5-8	0.063	fed for 30 days	Cook *et al*, 1988
		0.25-1		starved 30 days	
Mastigias sp.	Pacific	1-10.8	0.1	min. and max.	Wilkerson *et al*, 1983
Zoanthus sociatus		0.6		unphased	Steen & Muscatine, 1984
Palythoa variabilis		1.4		max./phased	Steen & Muscatine, 1984
Acropora cervicornis	Caribbean	2.1	0.045	1.5m depth	Wilkerson *et al*, 1988
		7.0	0.147	30m depth	
Tridacna gigas	Australia	4.9	0.045		Belda *et al*, 1993
*Pteraeolidia ianthina**			0.069	high cell density	Hoegh-Guldberg *et al*, 1986
			0.28	low cell density	
Symbionts in culture					
Aiptasia pulchella†			0.24	22 μE.m^{-2}.s^{-1}	Chang *et al*, 1983
			0.26	57 μE.m^{-2}.s^{-1}	
			0.30	157 μE.m^{-2}.s^{-1}	
			0.26	248 μE.m^{-2}.s^{-1}	
Tridacna maxima †			0.22	22 μE.m^{-2}.s^{-1}	Chang *et al*, 1983
			0.29	57 μE.m^{-2}.s^{-1}	
			0.33	157 μE.m^{-2}.s^{-1}	
			0.32	248 μE.m^{-2}.s^{-1}	
Montipora verrucosa†			0.3	22 μE.m^{-2}.s^{-1}	Chang *et al*, 1983
			0.38	157 μE.m^{-2}.s^{-1}	
			0.22	248 μE.m^{-2}.s^{-1}	
Cassiopeia xamachama†			0.08	20 μE.m^{-2}.s^{-1}	Fitt & Trench, 1983a
			0.27	180 μE.m^{-2}.s^{-1}	
Aiptasia tagetes †			0.08	20 μE.m^{-2}.s^{-1}	Fitt & Trench, 1983a
			0.16	180 μE.m^{-2}.s^{-1}	
Phytoplankton					
Ceratium spp.	N. Pacific		0.08-0.21		Weiler & Chisholm, 1976
Dinophysis fortii	U.K.		0.5 ±0.04		Weiler & Chisholm, 1976

4.4.1 Diel variation in mitotic index of symbionts from *A. viridis*.

Phytoplankton exhibit strongly phased cell division, with peaks in the early morning. Light/dark photo-period, temperature and nutrient availability have been suggested as possible stimuli for initiating phased division in natural populations of phytoplankton (Fogg & Thake, 1987). Patterns of cell division of *Symbiodinium microadriaticum*, isolated from *Aiptasia tagetes* and *Cassiopeia xamachana* and cultured under a 14 hr light:10 hr dark regime, were clearly synchronous (Fitt & Trench, 1983a) with peaks in cell division at 04 00h, regardless of strain or phase of population growth cycle. They observed that division became asynchronous if the cultures were kept in either continuous light or dark for 9 days. This suggests that the pattern of cytokinesis is circadian and entrained by a light:dark stimulus.

Data from similar studies of symbionts *in situ* have shown that zooxanthellae of some anthozoans exhibit phased diel patterns of growth rate while others are asynchronous. An absence of phasing has been reported for zooxanthellae from *Aiptasia pulchella, Anthopleura elegantissima, Stylophora pistillata* (Wilkerson *et al*, 1983; Gates 1988), *Acropora cervicornis, Montastrea* spp. (Wilkerson *et al*, 1988), *Millepora dichotoma* (Hoegh-Guldberg *et al*, 1987 unpubl. obs.), and *Zoanthus sociatus* (Steen & Muscatine, 1984). Phased division has been reported in *Mastigias* sp. (Wilkerson *et al*, 1983), *Myrionema amboinense* (Fitt & Cook, 1989), *Seriatopora hystrix* (Hoegh-Guldberg & Smith, 1989b), *Stylophora pistillata* (Smith & Hoegh-Guldberg, 1987), *Fungia repanda, Goniastrea edwardsii, Pocillopora damicornis* (Smith & Hoegh-Guldberg, 1987), *Aiptasia pallida* (Cook *et al*, 1988), *Anemonia viridis* (Stambler & Dubinsky, 1987), *Palythoa variabilis* (Steen & Muscatine, 1984) and the bivalve *Tridacna gigas* (Belda *et al*, 1993). In cases of synchronous cell division, the peak occurred during the early morning between 0400h and 0600h and the minimum occurred around 1600h. Exceptions to this occurred in *A. viridis*, where the peak in zooxanthella division rate occurred at 2200h and the minimum at 1200h (Stambler & Dubinsky, 1987). In *Palythoa variabilis* the diel peak in symbiont division corresponded to the timing of nocturnal polyp expansion which coincides with the time of greatest prey capture (Steen & Muscatine, 1984). In contrast, the symbionts of *Zoanthus sociatus* exhibit asynchronous division. This zoanthid relies less on zooplankton capture and has polyps which are continually expanded.

In the present experiments, patterns of division of zooxanthellae in *A. viridis* over 24 hours were considered to be asynchronous as there were no clear peaks in division in animals from all regimes (Figure 4.1). This is in accord with previous observations with *Anemonia viridis* (Gates, 1988; Suharsono, 1990), although Stambler & Dubinsky (1987) reported phased

division of zooxanthellae in the Mediterranean form of this species. The maximum mitotic index of zooxanthellae within ammonium supplemented animals occurred at 1000h, which was different to that of animals not supplemented with ammonium. The observation that the maximum rate occurred during the light period, which is the reverse of most reported observations of symbiont growth rate might be attributed to the increased nitrogen concentration. Experiments involving free-living diatoms showed that ammonium addition altered the timing of the division peak relative to the light:dark cycle (Olson & Chisholm, 1983). This may explain the difference in the division cycle found in zooxanthellae within ammonium supplemented *A. viridis*. Hoegh-Guldberg (1994) found that more symbionts divided out of synchrony in the coral *Pocillopora damicornis* with increasing ammonium concentration, such that without enrichment, division was clearly synchronous, but at 50μM ammonium, division became asynchronous after 8 weeks exposure. Wilkerson *et al* (1983) attributed the phased cell division of symbionts from *Mastigias* sp. to the periodic exposure to elevated ammonium concentrations of up to 15μM during its nocturnal vertical migrations through the water column across an apparent chemocline, where it was still able to take up ammonium (Muscatine & Marian, 1982; Wilkerson *et al*, 1983).

4.4.2 The effect of ammonium supplementation on symbiont mitotic index.

4.4.2.1 The relationship between symbiont division rate and ammonium concentration.

The mitotic index of zooxanthellae from *A. viridis in situ* was proportional to external ammonium concentration up to 50μM at 300 $\mu E.m^{-2}.s^{-1}$. These results suggest that nitrogen concentration is a major factor in determining the growth rate of zooxanthellae *in situ*, and suggests that, at normal sea water levels, nitrogen is limiting to zooxanthella growth rates. Ammonium uptake over 1 hour periods by zooxanthellae in *A. viridis* increased with external ammonium concentration up to 50μM at 190 $\mu E.m^{-2}.s^{-1}$ (Davies, 1988). The relationship of uptake to concentration closely fitted a rectilinear hyperbola. Saturation of uptake rates occurred at concentrations above 50μM. The relationship of mitotic index to ammonium concentration found in the present study gave a similar plot (Figure 4.2).

4.4.2.2 The relationship between symbiont growth rate and light intensity.

The mitotic index of zooxanthellae in *Anemonia viridis* increased with increasing light intensity (Figure 4.3). However, the increase was much larger in the experimental group which received ammonium enrichment for the 4 week period. The latter results were similar to the observations of Chang *et al* (1983) on cultured *Symbiodinium* sp., which were cultured

in medium where nitrogen was not limiting. In their experiments the division rate increased with increasing light intensity between 22 and 157 $\mu E.m^{-2}.s^{-1}$ (Table 4.8). However growth rates were lower in both strains at 248 $\mu E.m^{-2}.s^{-1}$, as were photosynthetic rates, suggesting that photo-inhibition occurred at higher irradiances. Similarly, cultured, photo-adapted *Symbiodinium microadriaticum* from *Aiptasia tagetes* and *Cassiopeia xamachana* showed higher maximum division rates at high light intensity (180 $\mu E.m^{-2}.s^{-1}$) than at low light intensity (15$\mu E.m^{-2}.s^{-1}$) (Fitt & Trench, 1983a). However, Muller-Parker (1985) found no difference between the mitotic index of symbionts from *Aiptasia pulchella* maintained at 45, 115, and 320 $\mu E.m^{-2}.s^{-1}$ for 5 weeks, and concluded that irradiance did not affect the mitotic index of photo-adapted symbionts *in situ*. Values obtained were low in comparison to other published values (see Table 4.7), ranging between 0.23% and 0.35% for fed animals and between 0.2% and 0.29% for starved animals, and suggests the possibility that some other factor, in particular nitrogen availability, was limiting at all irradiances. Her observations are closer to those of the control animals in the current study, where the increase in mitotic index with an increase in light intensity was very much lower than in the ammonium enriched group. The observations from the present study indicate that the rate of cell division may be limited by low light intensity even under ammonium enrichment. Davies (1988) showed that in *Anemonia viridis*, the rate of ammonia uptake was determined by the level of illumination and the concentration of ammonia in the water. It may be hypothesised, therefore, that since mitotic index also increases with both ammonia concentration and light level, the rate of cell division is determined by the rate of inorganic nitrogen uptake by the zooxanthellae.

4.4.2.3 The effect of long-term ammonium enrichment and the effect of feeding on the mitotic index of zooxanthellae.

The study of long term enrichment with ammonia was undertaken because of the conflicting results from previous studies in which mitotic index was measured at one point only in the experiment. Thus, Cook & Fitt (1989), showed that in *Myrionema ambionense*, after 3 days ammonia enrichment, the mitotic index rose to 12% compared with 3% in freshly collected controls. However, Muscatine *et al* (1989) found no increase in ammonium enriched *Stylophora pistillata* at the end of 14 days, and Hoegh-Guldberg & Smith (1989b) found similar results for *S. pistillata* and *Seriatopora hystrix* after 19 days. Similarly in the bivalve, *Tridacna gigas* there was no difference in the mitotic index between controls and those in 5μM and 10μM ammonium enriched sea water at the end of 3 months (Belda *et al* 1993).

In the present study, it was apparent that there was an initial rapid increase in mitotic index, which lasted for about 25 days. This twofold increase probably corresponds to the increase observed by Cook & Fitt (1989) in *Myrionema amboinense*. After 15 days the mitotic index fell back to a new stable level of between 6 and 7% which was nevertheless higher than the control which was about 4%. The stable second phase corresponds to the phase observed by Muscatine *et al* (1989), Hoegh-Guldberg & Smith (1989b) and Belda *et al* (1993) in which mitotic index levels had fallen back to control levels. These later three studies were all conducted on tropical species. In these species the population density of zooxanthellae had increased significantly at the sampling time. Clearly, in these cases (unless there was a dramatic increase in the rate of expulsion), a fall in mitotic index would be required after an initial increase, in order to maintain a new, elevated population level.

Heterotrophic feeding of the host might be expected to increase the availability of amino acids in the host cytoplasm, and since zooxanthellae have the ability to assimilate these (Blanquet et al 1988), the mitotic index would be expected to increase. Results from experiments of this sort are equivocal, possibly because the levels of feeding were different. Cook *et al* (1988) found that when specimens of *Aiptasia pallida* were starved for 30 days at $90\mu E.m^{-2}.s^{-1}$, the symbiont mitotic index fell from 8% to 3%, with the most rapid drop occurring in the first 10 days. On resumption of daily feeding the mitotic index increased to 8% within 5 days. The zooxanthellae from *Myrionema amboinense,* fed twice daily, maintained a maximum daily mitotic index of 10 - 16% while freshly collected specimens starved for 3 days had a mean maximum mitotic index of 1% (Fitt & Cook, 1989). Conversely, Muller-Parker (1985) found no difference between the mitotic index of symbionts from *Aiptasia pulchella* starved for 5 weeks and that of symbionts from animals fed for 5 weeks, and Muscatine *et al* (1989) showed no change in mitotic index of zooxanthellae from colonies of *Stylophora pistillata* which were fed daily with *Artemia* nauplii for 14 days and those which were starved. Since there was virtually no change in the population density of zooxanthellae, these authors concluded that amino acids from digestion were used exclusively for the growth of host cells. In the current experiments, there was a significant increase in the mitotic index of fed *Anemonia viridis* when compared with starved animals and this was maintained throughout the 80 day period (Figure 4.4). Nevertheless, as shown in Chapter 3, the major part of the nitrogen assimilated through feeding is used for host growth, with a smaller proportion being accounted for by an increase in the size of the zooxanthella population.

4.4.3 Space available for growth.

Smith (1986) observed a greater mitotic index in the zooxanthellae from juveniles of the anemone *Aulactinia stelloides* than from adults, and suggested that this may be due to a higher growth rate of juveniles, which would create more space for growth of the symbionts. However, no evidence for differences in growth rates between juveniles and adults was presented. Hoegh-Guldberg *et al* (1986) found a similar inverse relationship between mitotic index and host size in *Pteraeolidia ianthina*. He suggested that smaller hosts supported faster growing symbionts since smaller hosts are faster growing themselves providing more space for symbiont growth, although there is no evidence to support this. Relatively faster growth rates of host cells at coral tips, where zooxanthella growth rates are also relatively higher, have been observed (Wilkerson *et al*, 1988; Hoegh-Guldberg & Smith, 1989b) which lends support to the idea that symbiont division is, to some extent, influenced by host division. Muscatine *et al* (1986) working with *Mastigias* also found a similar relationship. In *Anemonia viridis*, it is clear that there is a similar inverse relationship between mitotic index and body mass. Furthermore, this relationship is not influenced by either exposure to 20μM ammonium enriched sea water or by feeding or starvation under high light conditions. However, these observations should not necessarily be interpreted as conclusively substantiating the hypothesis that mitotic index is determined by space available for growth.

Since there is strong evidence that the rate of division is limited by the rate of inward flux of dissolved inorganic nitrogen, it could be hypothesised that the higher mitotic index of zooxanthellae in smaller anemones could be related to lower competition between zooxanthellae for available nitrogen, since the zooxanthella:host space ratio would be lower at any time. It would follow from this hypothesis that if there is competition between zooxanthellae for nitrogen, that under nitrogen limiting conditions there should be an inverse relationship between mitotic index and population density of the symbionts. Conversely, if nitrogen is not limiting, the competition and hence the inverse relationship would not be present. To test this hypothesis the mitotic index data together with determinations of symbiont population density of experiment 4.2.2.3 were used. These are plotted in Figures 4.7 and 4.8. It is clear that under conditions of starvation (Figure 4.7) the mitotic index is reduced at high population densities, as expected. However, in animals maintained in 20μM ammonium enriched sea water, a regression equation with a slope of zero was expected, indicating that nitrogen availability was no longer limiting the division rate. Instead a significant positive slope showing an enhanced division rate with an increase in population density was observed.

Summary

1. Cell division in zooxanthellae of *Anemonia viridis*, as measured by the mitotic index, does not follow a phased or synchronous pattern over a 24 hour period.

2. When maintained under high light (300 $\mu E.m^{-2}.s^{-1}$) for 1 week, the mitotic index increased in proportion to external ammonia concentrations up to a maximum of around 8% in 50μM ammonium.

3. In unenriched sea water, there was only a small increase in the mitotic index with an increase in light level from 20 to 300 $\mu E.m^{-2}.s^{-1}$. However, there was a large increase from 2% to >8% with an identical increase in light intensity when animals were maintained in 20μM ammonium enriched sea water for 4 weeks. At 20 $\mu E.m^{-2}.s^{-1}$ there was no difference between the mitotic index of zooxanthellae from enriched sea water and that of controls. This is interpreted as a response to the rate of inward flux of ammonia, suggesting that mitotic index is limited by inorganic nitrogen availability.

4. Long term (80 day) experiments indicated that there was an initial large increase in mitotic index to 8% between 10 and 20 days when exposed to 20μM ammonia enriched sea water but this subsided to an elevated but stable value of about 6% thereafter. Compared to starved controls, the mitotic index of zooxanthellae from fed animals was higher (6%) than controls (4%), suggesting that some of the ingested nitrogen is made available to the symbionts.

5. The mitotic index is inversely related to body mass. This may be interpreted in relation to increased host growth leading to the creation of space for the newly divided symbionts to occupy, assuming that small anemones have a higher relative growth rate.

6. The mitotic index is inversely proportional to zooxanthella population density. This observation could result from competition between zooxanthellae for available nitrogen for cell division. Alternatively, this may also be interpreted in relation to availability of space for the symbionts to occupy.

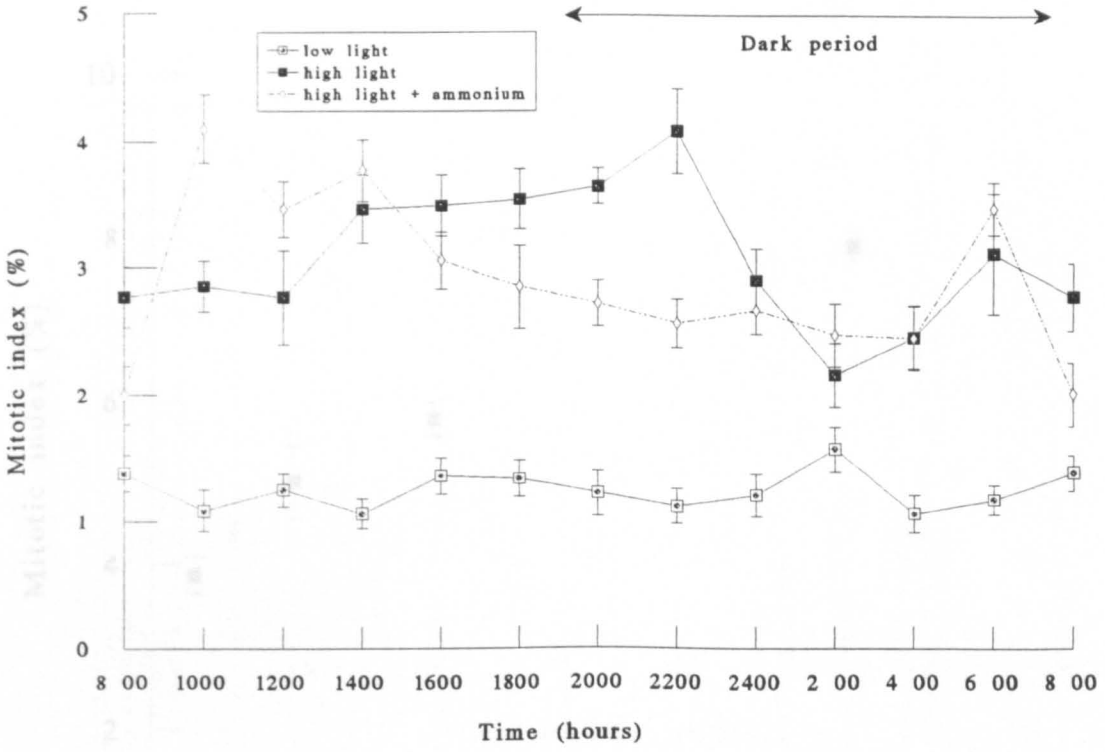


Figure 4.1 Diel changes in mitotic index of zooxanthellae from tentacles of *A. viridis* maintained in a 12 hour light: 12 hour dark regime. Each point is the mean of counts from 2 tentacles of each of 3 animals. Bars are standard errors of the mean. Animals maintained for 1 week in 20 $\mu E.m^{-2}.s^{-1}$ (low light), 300 $\mu E.m^{-2}.s^{-1}$ (high light), or 300 $\mu E.m^{-2}.s^{-1}$ with 20μM ammonium supplementation.

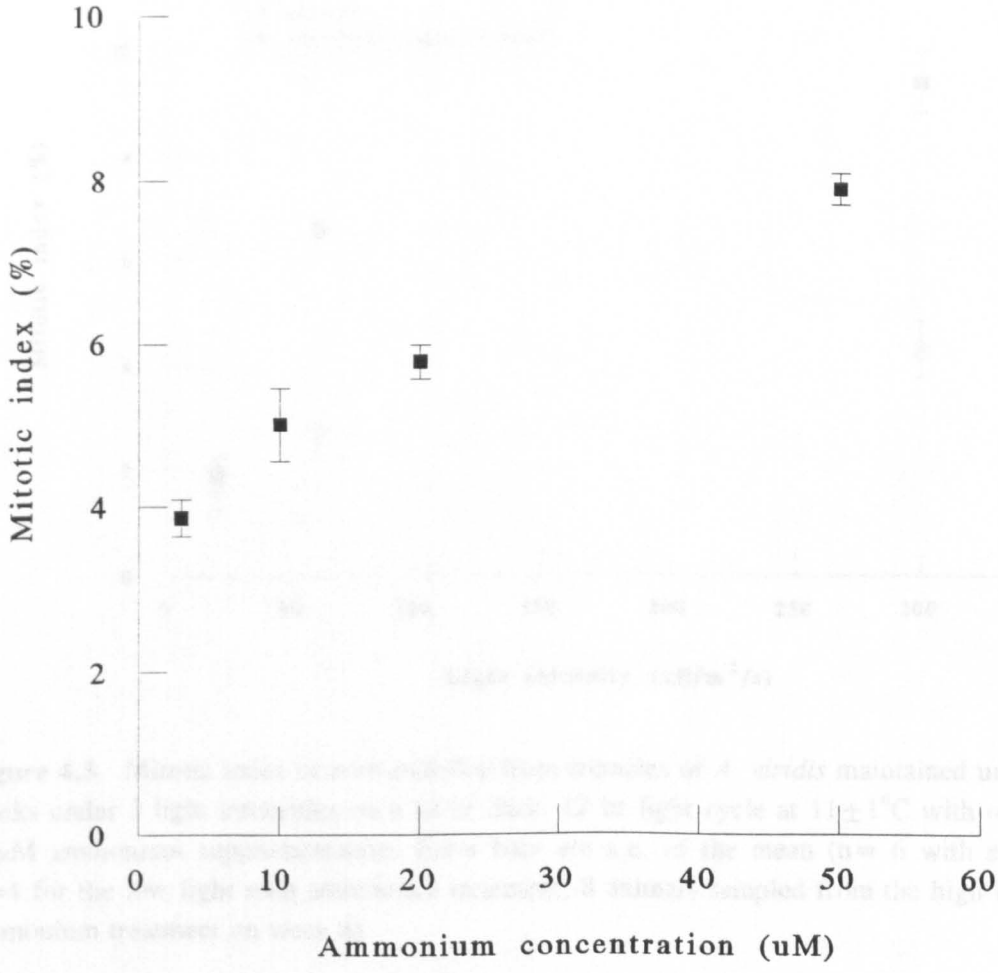


Figure 4.2 Mitotic index of zooxanthellae from tentacles of *A. viridis* maintained unfed at 300μE.m^{-2}.s^{-1} under 3 ammonium supplementation treatments or control sea water (≤3μM ammonium) for 1 week. Error bars are standard errors of the mean of all animals in each treatment (control: n=5, 10μM: n=4, 20μM: n=6, 50μM: n=3).

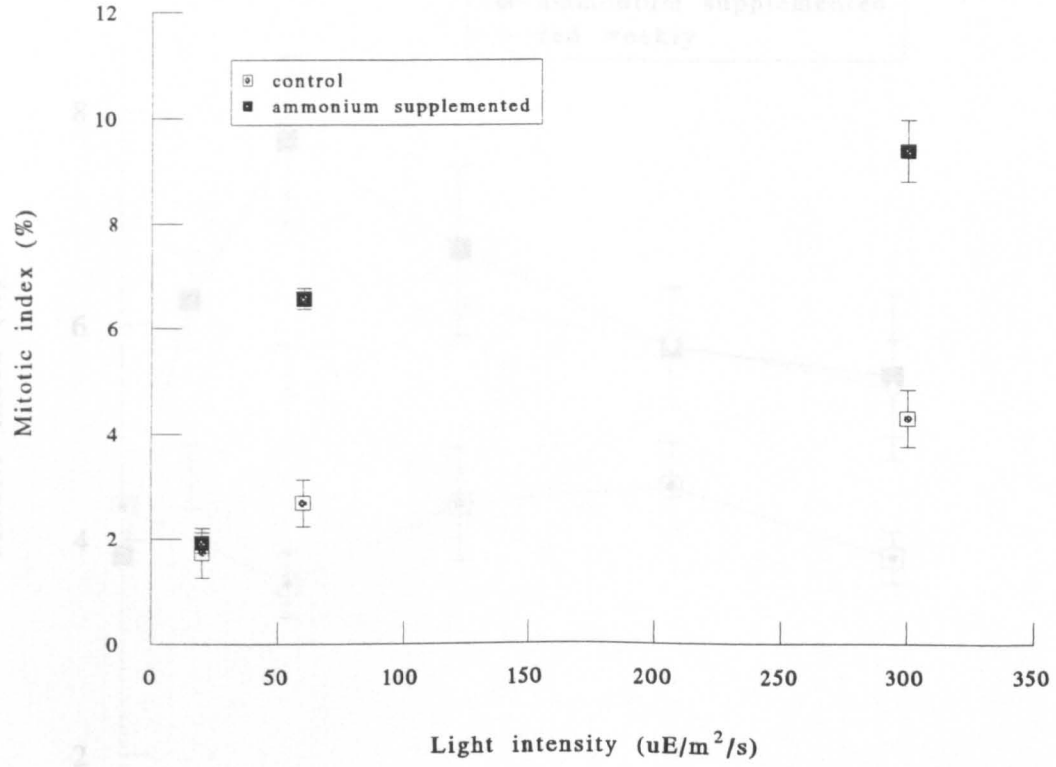


Figure 4.3 Mitotic index of zooxanthellae from tentacles of *A. viridis* maintained unfed for 4 weeks under 3 light intensities on a 12 hr dark: 12 hr light cycle at $11 \pm 1^{\circ}C$ with or without $20\mu M$ ammonium supplementation. Error bars are s.e. of the mean (n= 6 with exceptions n=4 for the low light with ammonium treatment; 8 animals sampled from the high light with ammonium treatment on week 4).

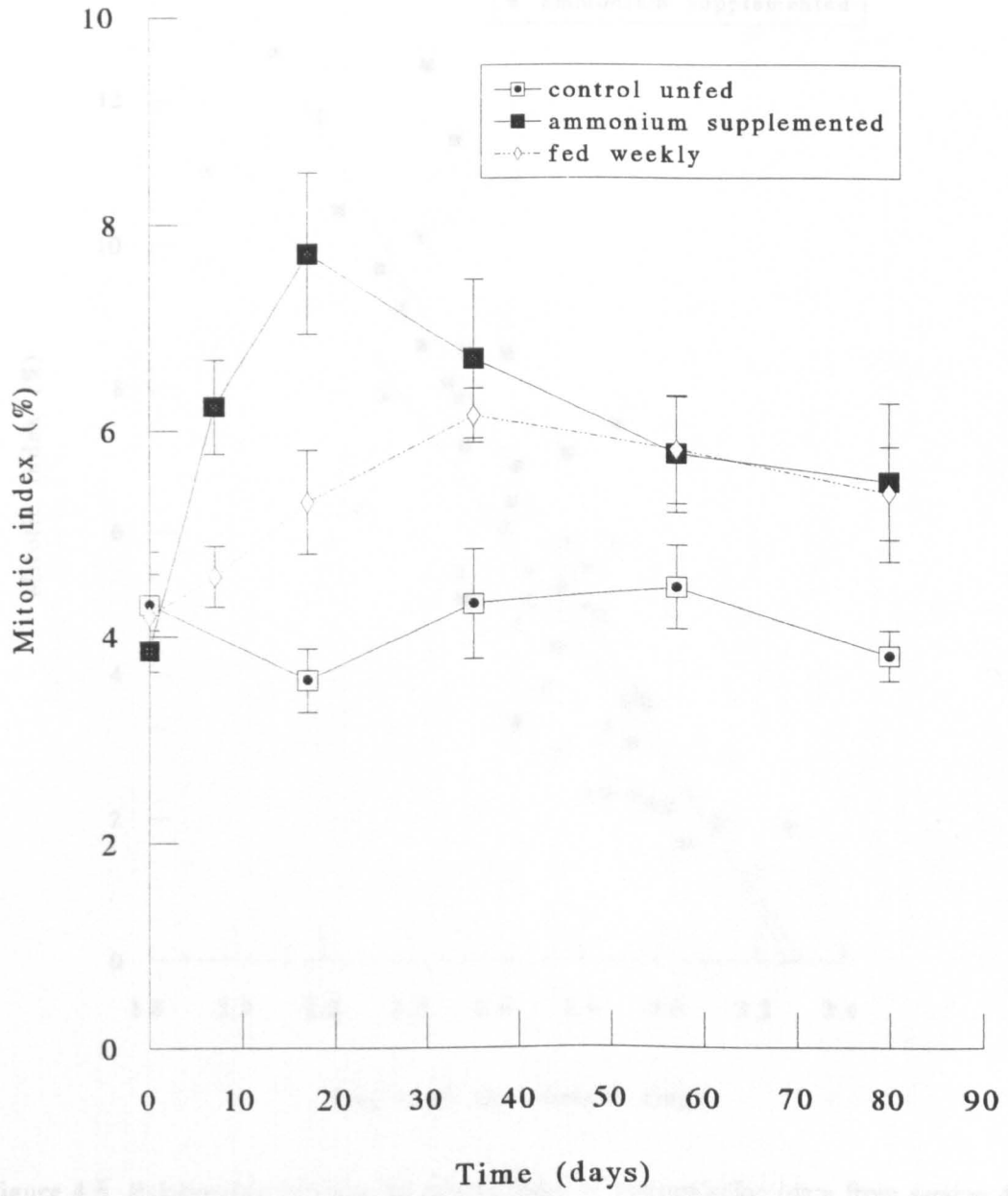


Figure 4.4 Mitotic index of zooxanthellae from tentacles of *A. viridis* kept at 300 $\mu E.m^{-2}.s^{-1}$ for 80 days in either ammonium enriched (20μM) or unenriched sea water or with weekly feeding. Bars are standard errors (n=5). A different sample of animals were used for each measurement.

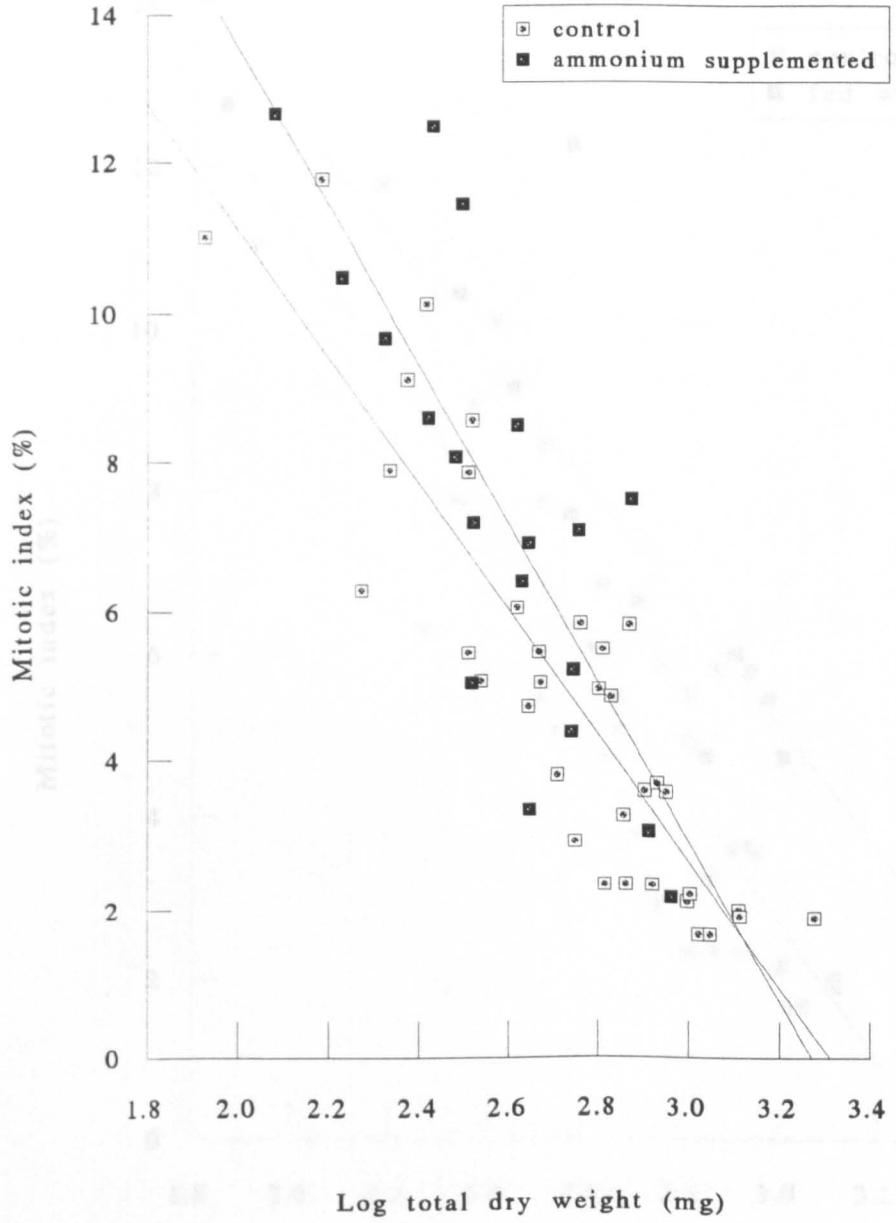


Figure 4.5 Relationship between the mitotic index of zooxanthellae (data from section 4.3.2.3) within tentacles of *A. viridis* and the corresponding total dry weight (data from section 3.3.3) for animals maintained unfed at 300 $\mu E.m^{-2}.s^{-1}$ (control) and animals in 20μM ammonium enriched sea water. The data best fitted a semi-logarithmic relationship.

Regression analysis gave equations

control:	$y = 28.2 - (8.50*\log x)$	$r^2 = 78.4\%$	(n=35)
+ ammonium:	$y = 34.9 - (10.7*\log x)$	$r^2 = 62.9\%$	(n=19)

slopes: $F = 1.46$ $p = 0.232$ not significant

elevation: $F = 5.50$ $p = 0.023$ significant.

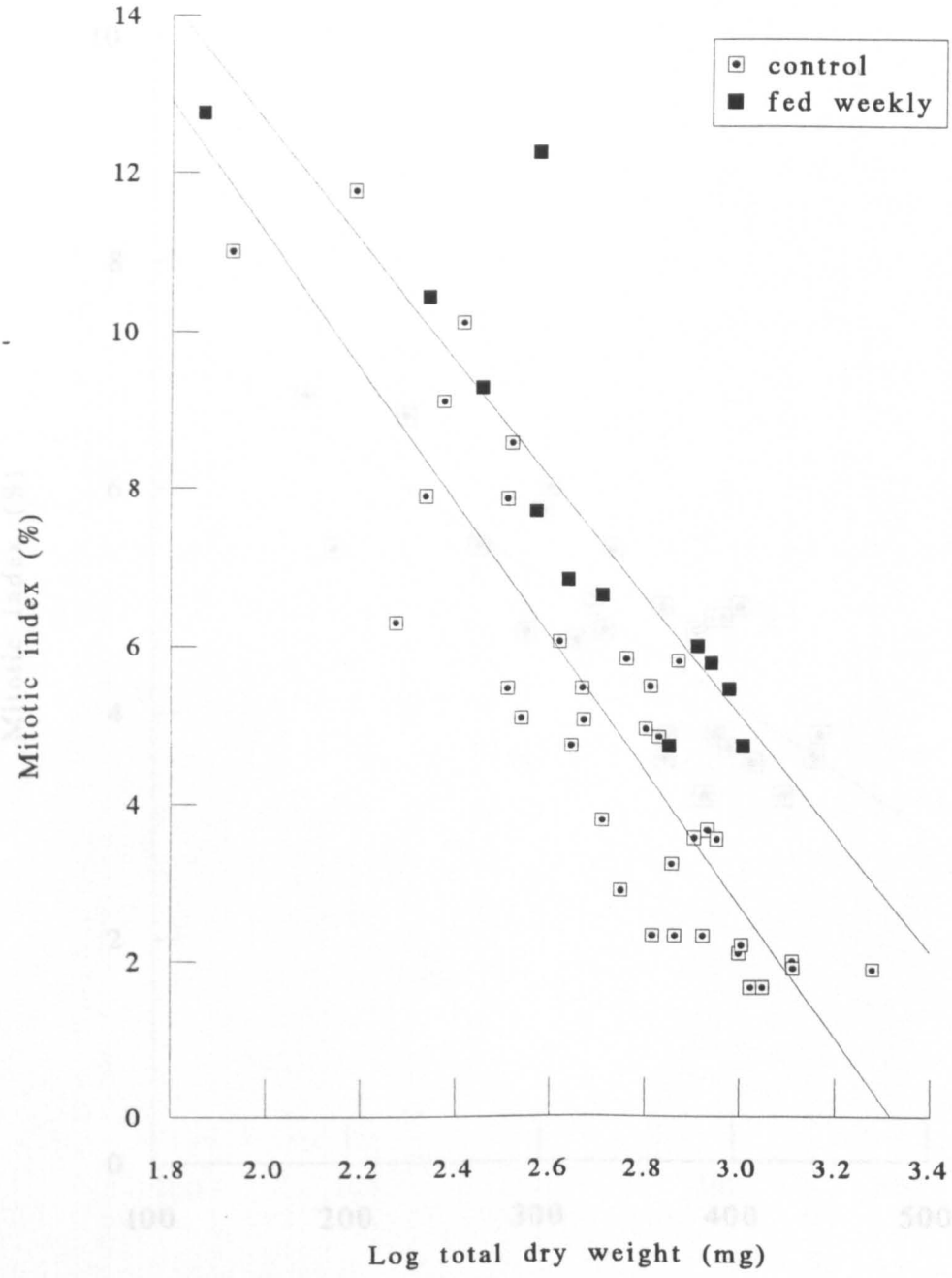


Figure 4.6 Relationship between the mitotic index of zooxanthellae within tentacles of *A. viridis* and the total dry weight (data from section 3.3.3) for animals maintained at 300μE.m^{-2}.s^{-1} either starved or fed weekly. Regression analysis gave equations:

starved : $y = 28.2 - (8.5 * \log x)$ $r^2 = 78.4\%$ (n=35)

fed weekly: $y = 27.6 - (7.51 * \log x)$ $r^2 = 76.2\%$ (n=12)

slopes: $F = 0.48$ $p = 0.49$ not significant

elevation: $F = 23.25$ $p < 0.001$ significant.

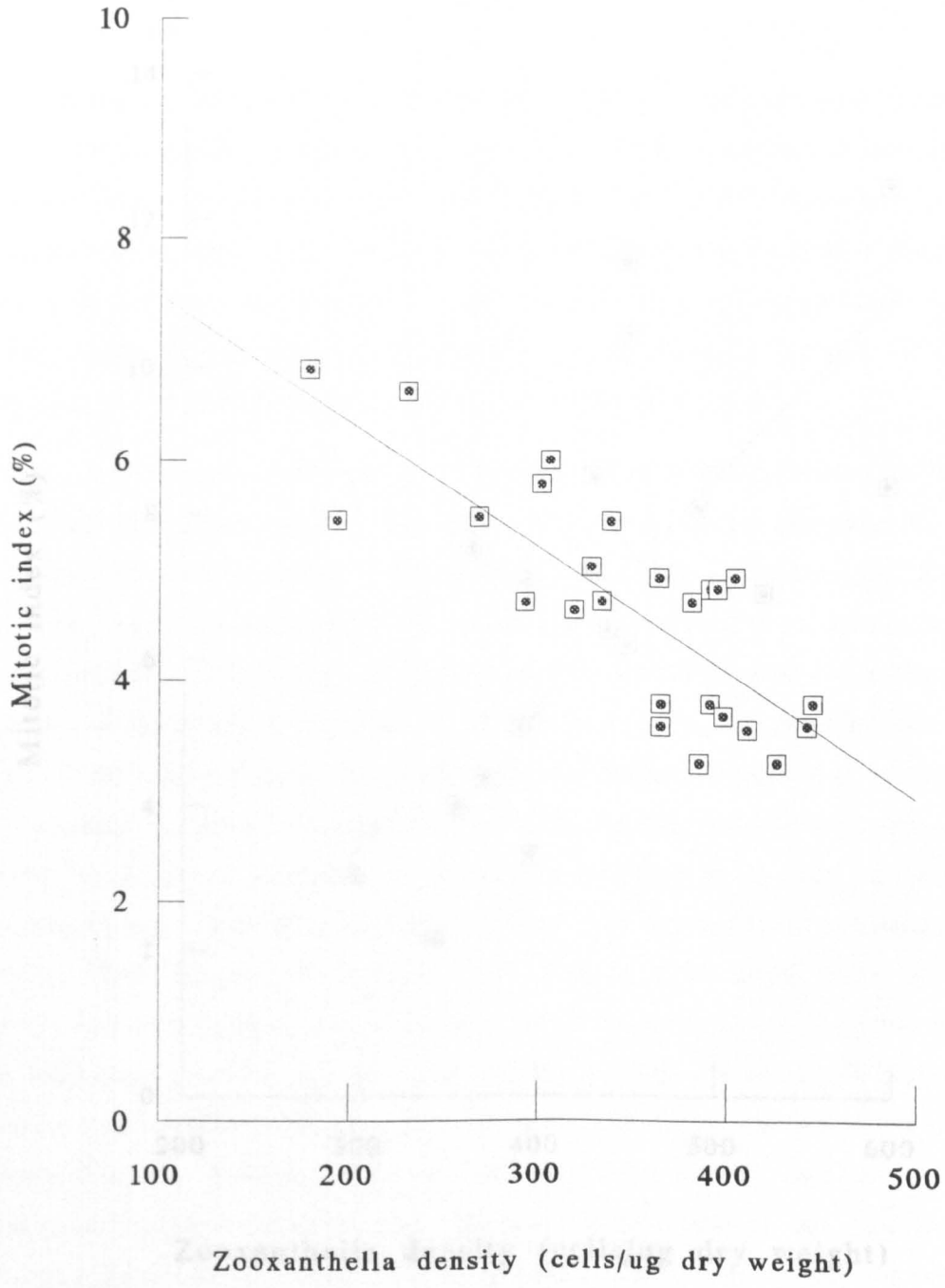


Figure 4.7 Relationship between the mitotic index of zooxanthellae (data from section 4.3.2.3) within tentacles of *A. viridis* and the corresponding population density within the whole animal (data from section 3.3.3) for anemones maintained unfed at 300 $\mu E.m^{-2}.s^{-1}$ at 11±1°C for 80 days.

Regression equation:

$$y = 8.613 - 0.0114\,x \qquad (r^2 = 68.3 \quad n=25)$$

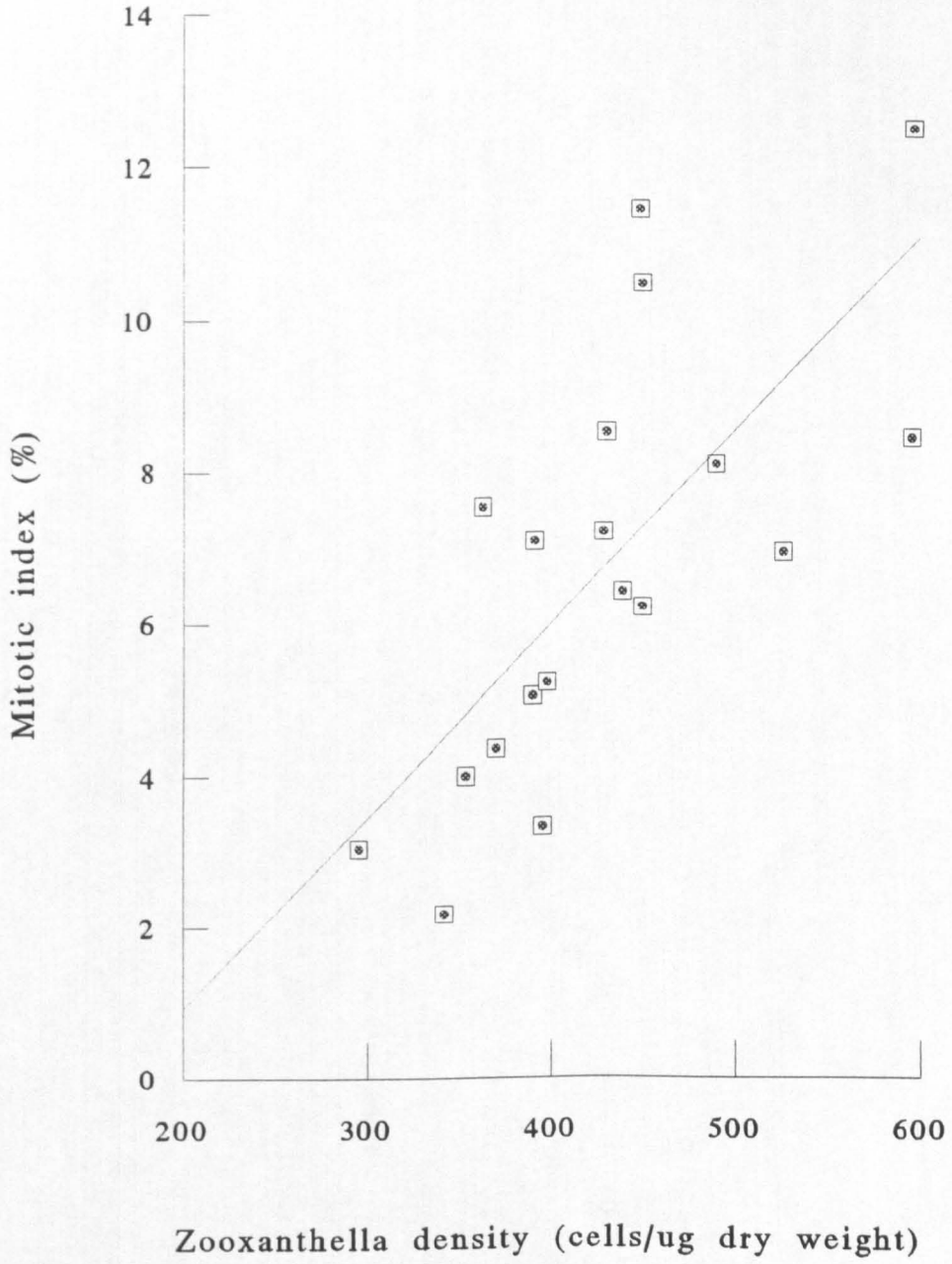


Figure 4.8 Relationship between the mitotic index of zooxanthellae (data from 4.3.2.3) within tentacles of *A. viridis* and the corresponding population density (data from section 3.3.3) within the whole animal of anemones maintained at 300 $\mu E.m^{-2}.s^{-1}$ at $11\pm1^{o}C$ with 20μM ammonium enrichment for up to 80 days.

The regression for the line of best fit was:

$$y = -4.0 + 0.0263\,x \qquad r^2 = 51.4\% \qquad \text{(ANOVA } p = 0.001, n=20)$$

CHAPTER 5

EXPULSION OF ZOOXANTHELLAE.

5.1 Introduction.

The continual expulsion of zooxanthellae has been observed from a variety of corals and both tropical and temperate anemones. The symbionts periodically expelled from anemones are often seen as loosely clumped pellets (Taylor, 1969b; Steele, 1976), mucus boli or in strings of mucus (Taylor, 1973; Steele, 1975). Expulsion of symbionts in the form of discrete pellets from corals has been reported by Fankboner & Reid (1981) and Hoegh-Guldberg & Smith (1989a) during the bleaching of heat-stressed corals, by Yonge & Nicholls (1930a) during starvation, and by Kevin & Hudson (1979) under prolonged darkness.

Whether expulsion occurs through factors beyond host or symbiont control, as for example during expulsion induced by heat stress (Gates *et al*, 1992), or is a mechanism by which the symbiont population is regulated, is unclear. Taylor (1968) commented that degenerating symbionts were always present in material expelled continually from anemones, and that viable symbionts were never seen in expelled material. He later concluded that these forms of expulsion were a means of "weeding" out the dead or senescing zooxanthellae that were of no benefit to the host, and put forward the idea that the continual expulsion of symbionts might be a potential mechanism by which the host could regulate its symbiont numbers (Taylor, 1969b). Subsequently, workers have reported that symbionts in all stages of the life cycle, including mature and dividing cysts and zoospores, are expelled from anthozoan symbioses (Taylor, 1969b; Reimer, 1971; Yonge, 1973; Trench, 1974; Steele, 1975; 1976). Gates (1988) found dividing algae in the sea water in which *Anemonia viridis, Phyllactis flosculifera* and *Montastrea cavernosa* had been kept, and Hoegh-Guldberg *et al* (1987) also reported dividing cells expelled from *Xenia macrospiculata* and *Heteroxenia fuscescens* under non-stressful conditions. Suharsono & Brown (1992) observed a greater zooxanthella mitotic index when using tentacle homogenates than when using tentacle sections, and suggested that this difference was due to the inclusion within the homogenate sample of symbionts expelled into the coelenteron. They concluded that expelled symbionts divide at a faster rate than symbionts *in situ* since they were no longer under host cell control. All of the above observations indicate an alternative underlying mechanism to that proposed by Taylor (1968). Steele (1976) suggested that exposure to thermal or osmotic stress damaged the algae, causing the onset of degenerative changes which lead to their extrusion. However this would not explain the expulsion of large numbers of viable symbionts, often still within host gastroderm cells,

during elevated temperatures, as observed by Muscatine *et al* (1991) and Gates *et al* (1992). Their studies suggest that the host cell, rather than the symbiont, is affected by the stress. Douglas & Smith (1984) reported an interesting phenomenon of particular strains of the *Hydra-Chlorella* association. They found that strains of *Hydra* in which induced maltose release was low and subsequently algal growth rates were higher, expelled their symbionts regularly, whereas in strains with high maltose releasing symbionts and relatively lower growth rates, no expulsion occurred. This suggests that the expulsion of symbionts has a population regulatory function in the *Hydra-Chlorella* association.

Although many observations on the expulsion of zooxanthellae have been made, only a very few studies have measured the rates of expulsion (Hoegh-Guldberg *et al*, 1987; Gates 1988; Hoegh-Guldberg & Smith, 1989a, b; Stimson & Kinzie, 1991). Measuring rates of expulsion can be used to determine whether symbiont expulsion represents a significant loss to the host, either in terms of carbon and nitrogen losses, or in terms of the total symbiont population. Expulsion rates have been determined using chlorophyll fluorescence analysis to measure the number of zooxanthellae in the incubation medium (Hoegh-Guldberg & Smith, 1989a; Muscatine *et al*, 1991), or by microscopical counting of the zooxanthellae in the incubation medium (Steele, 1976; Muscatine *et al*, 1991) and are recorded as either numbers of cells expelled per surface area of colony per day ($cells.cm^{-2}.d^{-1}$), as used for corals, or as a specific expulsion rate ($cells.cell^{-1}.d^{-1}$). Some values are given in Table 5.10.

Rates of expulsion have been observed to increase under certain physiological stresses, although the mechanisms leading to such changes are not known. Starvation (Yonge & Nicholls, 1930b), and changes in light intensity (Steele, 1976), temperature (Muscatine *et al*, 1991) and salinity (Hoegh-Guldberg & Smith, 1989a) have been shown to affect expulsion. Although much work has been carried out on the effects of nitrogen supplementation on zooxanthella population density and division rate, only one study, carried out by Stimson & Kinzie (1991), has investigated its effect on symbiont expulsion rates. Nitrogen enrichment was shown in Chapter 4 to increase symbiont division rates in *Anemonia viridis*, but, unlike most studies involving corals, where enrichment dramatically increased zooxanthella population densities, effects of increased nitrogen on *A. viridis* only produced a relatively small increase in the population density. This suggests that symbionts are, perhaps, being expelled or removed at a faster rate. This hypothesis could be tested by establishing experimentally a correlation between the symbiont expulsion rate and division rate or population density. In this study, both expulsion rates and division rates of zooxanthellae were measured in animals maintained in control and ammonium enriched sea water. Since an

increase in light level was also found to increase the mitotic index (Chapter 4), the expulsion rate and mitotic index were measured under different light intensities.

The questions asked in this chapter were:

1. What is the zooxanthella expulsion rate of *Anemonia viridis* under standard conditions of 300 $\mu E.m^{-2}.s^{-1}$ at $11 \pm 1^{o}C$?
2. What is the effect of increasing light intensity on the zooxanthella expulsion rate?
3. What is the effect of ammonium enrichment on the zooxanthella expulsion rate?
4. Is the mitotic index of expelled symbionts different to that of symbionts within host cells?

5.2 Materials and Methods.

The animals used in the following experiments were acclimatised to experimental conditions for 2 weeks before measurements were made.

5.2.1 Symbiont expulsion rates of *A. viridis* under standard conditions.

Five anemones were maintained without feeding for 2 weeks. They were then placed on small perspex tiles in individual 150 ml glass beakers. Each beaker was maintained at $11 \pm 1^{o}C$ with its own air supply and sea water supply with a flow rate of 10 $ml.min^{-1}$ and with a light level of 300 $\mu E.m^{-2}.s^{-1}$ on a cycle of 12 hr light: 12 hr dark. Ammonium concentration in the sea water ranged between <0.5 and 2 μM depending on the time of day. The sea water from the beakers overflowed on to detachable plankton net filters of 5μm pore size which trapped zooxanthellae expelled that did not collect in the beakers. The animals were allowed to settle for at least 24 hours in running sea water before the filters were attached. The first samples were taken after 2 hours incubation. After these initial samples were taken, expelled zooxanthellae were collected every 2, 3 or 4 days for a total of 31 days. After each incubation, the animals, still attached to the tiles, were transferred to clean beakers. The filters and beakers were rinsed to remove any attached zooxanthellae. The expelled symbionts were counted immediately using the method given below. Both live cells and degenerate cells were counted. Degenerate cells in each sample were distinguished from viable cells by staining the cells suspensions in 0.05% methylene blue in sea water. Degenerate cells could be distinguished as those which readily take up the stain (Steele, 1976). The animals were allowed to settle for a further 24 or 48 hours in running aerated sea water before the filters were replaced for the next sample.

At the end of the experiment, animals were divided into approximately equal halves and wet weighed. One half was freeze dried while the other half was homogenised and the zooxanthellae counted as described in section 2.2.1. The dry weight of the homogenised half was calculated using the dry weight:wet weight ratio of the freeze dried half. Thus, the number of cells expelled per unit dry weight per day could be calculated. Specific expulsion rates were calculated using total zooxanthella population estimates.

Samples of suspended expelled algae, collected from experimental beakers and filters, were centrifuged in 50 ml or 500 ml centrifuge tubes for 5 minutes at 1000 r.p.m. The majority of the water was removed and the pellet resuspended in 1-10 ml sea water. The volume used to resuspend the pellet depended on the approximate number of cells present and was estimated by the colour intensity. The samples were shaken vigorously or homogenised using a Turrex for 10 seconds to break up any clumps of algae. 10 replicate counts were made using an improved Neubauer haemocytometer to estimate total cells expelled.

In a few samples of expelled symbionts, the number of cells was too low to count using the above method. In these samples an alternative method was used. Samples of sea water containing very small numbers of cells were filtered on to 0.45μm pore size 47 mm diameter cellulose nitrate filters. These filters were placed on slides suspended in a few drops of water and viewed under a microscope at x400 magnification. The number of zooxanthellae present in each of 20 fields of view were counted. Since the area of one field of view and the area of the filter were known, the total number of cells on the filter could be estimated as shown below:

$$\text{No. of zooxanthellae} = \text{mean no. cells per field of view} \times (\text{area of filter / area of field of view})$$

where the area of the filter = 1452.2 mm^2 and the area of one field of view = 0.1257 mm^2. The two methods were tested for any significant differences in the estimates of numbers expelled by using both methods on a range of samples. There were no significant differences between the values obtained from both methods when a two sample *t*-test was applied to the data (Appendix 5). It was therefore assumed that both methods gave sufficiently similar estimates of the numbers of symbionts expelled.

5.2.2 The effect of increasing light intensity on the zooxanthella expulsion rate.

Twenty animals were divided equally and randomly assigned to one of 4 light levels of 50, 120, 250 or 300 $\mu E.m^{-2}.s^{-1}$ where they were maintained in running, aerated sea water at $11 \pm 1^{o}C$ for 18 days as in section 5.2.1.

Expelled zooxanthellae were collected over 2 or 3 day intervals as in section 5.2.1. Samples of expelled zooxanthellae were counted and then fixed in 3:1 alcohol:acetic acid for future counts of dividing cells. Two tentacles were removed from each animal for nuclear staining. Before calculating the mitotic index (as the percentage of cells undergoing division), the raw data comprising the number of dividing cells in a sample of 300 cells, were tested for differences between treatments using ANOVA and Tukey tests. At the end of the experiment, animals were treated as in section 5.2.1 to obtain dry weights and total population numbers.

5.2.3 The effect of ammonium supplementation on zooxanthella expulsion rate.

Five animals previously maintained at a light level of $300\mu E.m^{-2}.s^{-1}$ were transferred to $20\mu M$ ammonium enriched sea water (section 3.2.1) where they were maintained with continuous ammonium enrichment as in section 5.2.1 for 21 days. A further five animals acted as controls and were maintained without ammonium enrichment for 21 days. The sampling procedure followed that of section 5.2.1. Both live and senescent cells were counted. Two tentacles were removed from each animal for nuclear staining on each sampling day.

5.3 Results

ANOVA tests were carried out on the data unless stated otherwise. Tukey tests, when used, were carried out at the 5% significance level.

5.3.1 Expulsion of zooxanthellae from *A. viridis* under standard conditions.

Symbionts were expelled individually or in irregularly shaped, loosely clumped pellets 1-2 mm in diameter, occasionally reaching 5 mm in diameter. All expelled samples contained senescent and viable zooxanthellae. Dividing cells were always present and only very rarely were no degenerate symbionts present. The majority of zooxanthellae expelled after 2 hour incubations were viable (cells which did not take up methylene blue stain). Observations made by transmission electron microscopy showed that the majority of expelled zooxanthellae were surrounded by what appeared to be host cell membranes, and some were clearly contained in host gastrodermal cells, as evidenced by the presence of host nuclei and host cytoplasm.

However, no quantitative data were obtained for either viability or presence of host material for samples expelled over 2 hours. These samples also contained a large amount of host material including nematocysts, gastroderm cells without symbionts and amoeboid mesoglea cells, some of which contained degenerate zooxanthellae.

Table 5.1 and Figure 5.1 shows the specific expulsion rates and their means of 5 animals on the 5 sampling occasions. All animals showed different patterns in consecutive measurements of μ_x.

Table 5.1 Specific expulsion rates of zooxanthellae (cells expelled/10^3cells/d) from *A. viridis* maintained unfed for 31 days at 300μE.m^{-2}.s^{-1} and 11 ± 1°C. Sampling periods are given in brackets.

	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Mean for each day
Days 0-3 (3)	3.47	0.63	0.89	0.79	6.36	2.44
Days 4-8 (4)	1.72	0.41	0.14	3.77	0.12	1.23
Days 12-15 (3)	4.96	0.12	1.95	1.56	2.93	2.30
Days 24-26 (2)	1.51	0.28	1.54	1.97	1.25	1.30
Days 28-31 (3)	2.02	0.95	0.22		0.10	0.82
Mean for each animal	2.74	0.48	0.95	2.02	2.16	no significant difference between days
	no	significant	difference	between	animals	

Table 5.2 Expulsion rates of zooxanthellae normalised to dry weight (cells/mg dry weight/d) of animals maintained unfed for 31 days at 300μE.m^{-2}.s^{-1} and 11 ± 1°C. Duration of sampling periods are given in brackets.

	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Mean for each day
Days 0-3 (3)	3567	644.0	429.6	588.3	6387	2323
Days 4-8 (4)	1761	424.3	68.8	2822	117.9	1039
Days 12-15 (3)	5094	120.6	940.9	1166	2940	2053
Days 24-26 (2)	1546	289.1	741.2	1476	1260	1063
Days 28-31 (3)	2071	966.6	107.9		98.3	811
Mean for each animal	2808	488.9	457.7	1513	2161	no significant difference between days
	no	significant	difference	between	animals	

There was a high degree of variation between sampling intervals ($F=0.87$, $p=0.5$) and between animals ($F=0.87$ $p=0.5$), therefore data from animals and samples were pooled to give a mean specific expulsion rate of 0.00167 cells.cell^{-1}.d^{-1} (± 0.0004 cells.cell^{-1}.d^{-1} s.e.). There was a decreasing trend in the mean specific expulsion rate with increasing experimental

duration, from 0.00244 to 0.00082 cells.cell^{-1}.d^{-1}. Table 5.2 shows the expulsion rates (μ_X) normalised to dry weight of 5 animals on the 5 sampling occasions. There was, similarly, a large amount of variation in expulsion rates normalised to animal dry weight between sampling intervals (F=0.79 p=0.54) and between animals (F=2.5 p=0.08). The mean expulsion rate normalised to dry weight decreased throughout the experiment from 2323 to 811 cells.mg dry weight^{-1}.

5.3.2 The effect of increasing light intensity on the zooxanthella expulsion rate.

Expulsion rates did not change significantly over the duration of the experiments at each light intensity (Table 5.3), neither was there any significant differences between animals under all light regimes ($p > 0.05$), and so data were pooled to give means for each of the 4 light levels. Mean expulsion rates at each light intensity are given in Table 5.3 and 5.4 and Figure 5.2.

Table 5.3 Mean specific expulsion rates ± s.e (n=5) from *A. viridis* maintained unfed at 4 light intensities. Values are cells expelled/10^3 cells/d. Symbionts expelled were sampled after 3 days.

	Days 0-3	Days 5-8	Days 10-13	Days 15-18	significance of difference
50 μE.m^{-2}.s^{-1}	0.28 (0.1)	0.18 (0.1)		0.32 (0.1)	F=0.62 p=0.56
120 μE.m^{-2}.s^{-1}	0.302 (0.16)	0.484 (0.21)		0.145 (0.03)	F=1.35 p=0.29 n. s.
250 μE.m^{-2}.s^{-1}	0.842 (0.4)	0.87 (0.4)	0.89 (0.01)	0.548 (0.23)	F=0.31 p=0.82 n.s.
300 μE.m^{-2}.s^{-1}	2.43 (11)	1.93 (0.8)	2.26 (0.8)	1.06 (0.3)	F=0.84 p=0.49 n. s.

Table 5.4 Mean expulsion rates of symbionts normalised to dry weight (cells.mg d wt.$^{-1}$d^{-1}) ± s.e. (n=5) from *A. viridis* maintained unfed at 4 light intensities at 11±1°C. Zooxanthellae expelled were sampled after 3 days.

	Days 0-3	Days 5-8	Days 10-13	Days 15-18	significance of difference
50 μE.m^{-2}.s^{-1}	97.96 (33.1)	96.8 (21)		171.9 (24)	F=2.89 p=0.11 n. s.
120 μE.m^{-2}.s^{-1}	56.09 (21.8)	158.9 (87)		34.6 (8)	F=1.66 p=0.23 n. s.
250 μE.m^{-2}.s^{-1}	173.8 (85)	122.5 (10)	125.7 (62.4)	167.1 (67.6)	F=0.08 p=0.97 n. s.
300 μE.m^{-2}.s^{-1}	2326 (1181)	1023 (534)	2010 (869)	903 (258)	F=1.05 p=0.39 n. s.

The results show a trend towards increasing expulsion rate with increasing light intensity from 50 to 300 $\mu E.m^{-2}.s^{-1}$. Tukey tests showed significant differences between the specific expulsion rate at 300 $\mu E.m^{-2}.s^{-1}$ and all other light intensities. Values at 50, 120 and 250 $\mu E.m^{-2}.s^{-1}$ were not significantly different (Table 5.5).

The mean mitotic index of zooxanthellae from tentacles of animals at 300 $\mu E.m^{-2}s^{-1}$ was significantly higher than values obtained from animals at all other light intensities, but there was no significant difference in mitotic index values when animals maintained at 50, 120 and 250 $\mu E.m^{-2}.s^{-1}$ were compared using Tukey tests (Table 5.5).

Table 5.5 Mean symbiont expulsion rates and mitotic index ± s.e (n=5) of *A. viridis*. Differences between means with similar superscripts across a row were not significant (Tukey test, 5% level).

	50 $\mu E.m^{-2}.s^{-1}$	120 $\mu E.m^{2}.s^{-1}$	250 $\mu E.m^{-2}.s^{-1}$	300 $\mu E.m^{-2}.s^{-1}$
(μ_x) Specific expulsion rate (cells.10^3 cell.d^{-1})	0.26 ^{a} (0.4)	0.31 ^{a} (0.1)	0.79 ^{a} (0.08)	1.92 ^{b} (0.31)
Expulsion rate (cells.mg dry weight^{-1})	122.2 ^{a} (25)	83.2 ^{a} (38)	147.3 ^{a} (13)	1543 ^{b} (355)
Mitotic index of zooxanthellae in tentacles (%)	4.15 ^{a} (1.5)	3.645 ^{a} (0.64)	3.08 ^{a} (0.68)	5.68 ^{b} (0.39)

5.3.3 The effect of ammonium supplementation on zooxanthella expulsion rate

Expulsion rates under ammonium enriched and control regimes at 300 $\mu E.m^{-2}.s^{-1}$ are given in Table 5.6 and Figure 5.3.

Ammonium supplemented animals had significantly greater specific expulsion rates and showed increasing rates with increasing exposure to ammonium enrichment (analysis of covariance $F=9.38$ $p=0.004$). The weight specific expulsion rates of ammonium supplemented animals were similarly significantly greater than controls over the experiment (analysis of covariance $F= 9.6$ $p=0.004$).

Table 5.6 Expulsion rates of *A. viridis* maintained unfed at 300 $\mu E.m^{-2}.s^{-1}$ for 21 days ± s.e (n=5). Zooxanthellae expelled were sampled after 3 days. Regression coefficients with different superscripts were statistically different when tested using analysis of covariance.

	Specific expulsion rate (cells. 10^3 cell.$^{-1}$.d^{-1})		Expulsion per dry wt (cells.mg dry.wt^{-1}.d^{-1})	
	Control	Ammonium	Control	Ammonium
Days 0-3	2.43 (1.1)	4.00 (1.48)	2326 (1181)	2157 (1027)
Days 5-8	1.93 (0.8)	6.19 (1.4)	1023 (534)	2875 (345)
Days 12-15	2.26 (0.8)	6.89 (0.3)	2010 (869)	4142 (381)
Days 18-21	1.06 (0.3)	8.31 (1.2)	903 (258)	5024 (1040)
Regression coefficient	- 0.00007 ^{a}	+ 0.00023 ^{b}	- 56.0 ^{a}	+ 182 ^{b}
significance of slope	p=0.197 n.s.	p=0.02 significant	p=0.29 n.s.	p=0.005 significant
Two way ANOVA	treatment factor: F= 42.5 p<0.001		treatment factor: F= 13.67 p= 0.001	

Figure 5.4 and Table 5.7 show zooxanthella mitotic index values from tentacles of control and ammonium enriched animals maintained at 300 $\mu E.m^{-2}.s^{-1}$. Regression and covariance analysis could not be carried out as the data sets were not linear with time. Mitotic index samples from the ammonium supplemented animals increased significantly throughout the experiment (Tukey test for pairwise comparisons $p<0.05$) but there was no significant change in the mitotic index in tentacles from control animals (one way ANOVA on the difference between days: $F=2.23$, $p=0.12$). The mean mitotic index of symbionts in tentacles from ammonium supplemented animals was significantly higher than the control (Table 5.7).

Table 5.7 Mitotic index of zooxanthellae from tentacles of *A. viridis* maintained unfed at 300$\mu E.m^{-2}.s^{-1}$ for 21 days ± s.e (n=5) with and without 20μM ammonium enrichment. Zooxanthellae expelled were sampled after 3 days.

	Mitotic index (%)	
	Control	Ammonium supplemented
Day 3	6.50 (0.64)	10.55 (1.33)
Day 8	4.81 (0.44)	6.07 (0.55)
Day 15	6.10 (0.60)	8.21 (0.60)
Day 21	5.32 (0.42)	10.16 (1.16)
Mean	5.77 (0.39)	8.75 (0.60)
Two way ANOVA	Treatment factor F=26.2 p<0.001 significant Time factor F = 4.9 p=0.005 significant	

When expulsion rates normalised to animal dry weight were plotted against corresponding mitotic index values, there was a positive correlation between specific expulsion rate and zooxanthella mitotic index when data from both treatment and control groups were pooled (Figure 5.5). No correlation was found between symbiont population density and specific expulsion rate.

The mitotic index of expelled symbionts was significantly higher than the mitotic index of symbionts in tentacles for both ammonium enriched and control animals (Table 5.8).

Table 5.8 Mitotic index of zooxanthellae from tentacles of animals and zooxanthellae in expelled samples maintained unfed at 300 $\mu E.m^{-2}.s^{-1}$ for 21 days ± s.e (n=5). Zooxanthellae expelled were sampled after 3 days.

	Control		Ammonium supplemented	
	M I of zooxanthellae from homogenated tentacles (%)	M I of expelled zooxanthellae (%)	M I of zooxanthellae from homogenated tentacles (%)	M I of expelled zooxanthellae (%)
Day 3	6.5 (0.64)		10.55 (1.33)	8.67 (0.44)
Day 8	4.81 (0.44)	8.1 (0.61)	6.07 (0.55)	13.19 (1.78)
Day 15	6.10 (0.60)	9.89 (2.57)	8.21 (0.60)	9.58 (0.31)
Day 21	5.32 (0.42)	11.38 (1.13)	10.16 (1.16)	10.84 (1.44)
Mean	5.77 (0.39)	9.59 (1.0)	8.75 (0.6)	10.70 (0.7)
ANOVA	treatment factor F=8.24 p=0.012		treatment factor F=5.42 p=0.028	

Between 31 and 79% of the total symbionts expelled in samples were viable and did not take up the methylene blue stain. There was no significant difference in the proportion of senescent cells expelled between ammonium supplemented and control groups (F= 0.16 p=0.69).

Table 5.9 Proportion of expelled cells that were degenerate ± s.e (n=5) from counts of zooxanthellae which took up a methylene blue stain in animals maintained at 300$\mu E.m^{-2}.s^{-1}$.

	Control	Ammonium supplemented
Days 0-3	43.24 (12)	25.7 (8)
Days 5-8	38.69 (10)	35.3 (7)
Days 12-15	42.06 (13)	31.16 (9)
Days 18-21	42.46 (12)	64.76 (10)
Mean	44.99 (4)	39.2 (9)

5.4 Discussion

5.4.1 Expulsion of zooxanthellae from *Anemonia viridis*.

The mean specific expulsion rate of *A. viridis* maintained at 300 $\mu E.m^{-2}.s^{-1}$ decreased over 31 days from 0.00243 to 0.0008 $cells.cell^{-1}.d^{-1}$. There was a large amount of variation between animals on each sampling occasion, and no consistent pattern was observed for all animals.

Steele (1976) found a similarly large variation in consecutive daily measurements of expulsion rates of *Aiptasia tagetes* measured over 14 days. Stimson & Kinzie (1991) found a high degree of variation between expulsion rates of *Pocillopora damicornis* measured over consecutive one hour periods.

Table 5.10 Expulsion rates for some anthozoans. * from cell counts † from chlorophyll fluorescence.

Species	Expulsion rate ($cells.\ cell^{-1}.d^{-1}$)		Source
Pocillopora damicornis	0.011	control	Stimson & Kinzie, 1991 *
	0.0055	nitrogen enriched	
	4300 $cells.cm^{-2}.d^{-1}$	control	
	6000 $cells.cm^{-2}.d^{-1}$	nitrogen enriched	
Heteroxenia fuscescens	0.000803	field experiments	Hoegh-Guldberg *et al*, 1987*
Xenia heterocrypta	0.00158	lab. kept. 150$\mu E.m^{-2}.s^{-1}$	Hoegh-Guldberg *et al*, 1987*
	0.00048	field experiment	
Millepora dichotoma	0.00005	field experiments	Hoegh-Guldberg *et al*, 1987*
Seriatopora hystrix	0.0001 - 0.001	measured over 24 hours in full sunlight / 25% sunlight	Hoegh-Guldberg & Smith 1989a
Stylophora pistillata	0.000167	field experiments	Hoegh-Guldberg *et al*, 1987.*
	0.0001 - 0.002	laboratory experiments 25% or full sunlight	Hoegh-Guldberg & Smith, 1989a
Aiptasia tagetes	0.0017	1000 lux (≡ 19 $\mu E.m^{-2}.s^{-1}$)	Steele, 1976*
	0.0015	1600 lux (≡ 30 $\mu E.m^{-2}.s^{-1}$)	
	0.0029	1800 lux (≡ 35 $\mu E.m^{-2}.s^{-1}$)	
Anemonia viridis	0.00166	300 $\mu E.m^{-2}.s^{-1}$	present study
	0.00027	50 $\mu E.m^{-2}.s^{-1}$	
	0.00635	300 $\mu E.m^{-2}.s^{-1}$ 20$\mu M\ NH_4^+$	

The rates of symbiont expulsion from *Anemonia viridis* at 300 $\mu E.m^{-2}.s^{-1}$ fall within the range of values found by Steele (1976) for *Aiptasia tagetes* (Table 5.10). However, the latter were maintained at much lower light intensities of between 19 and 35 $\mu E.m^{-2}s^{-}$

specific expulsion rate for *A. viridis* was much higher than values for 4 species of Red Sea coral, measured in the field, which ranged from 0.00005 to 0.0008 cells.cell^{-1}.d^{-1} (Hoegh-Guldberg *et al*, 1987). Values for *A. viridis* at 300 μE.m^{-2}.s^{-1} in this study were close to the rates obtained for the soft coral, *Xenia heterocrypta* maintained at 150 μE.m^{-2}.s^{-1} in the laboratory but much greater than expulsion rates of *X. heterocrypta* measured in the field (Hoegh-Guldberg *et al*, 1987).

5.4.2 The effect of increasing light intensity on the zooxanthella expulsion rate.

These results show a trend of increasing specific expulsion rate with increasing light intensity between 50 and 300 μE.m^{-2}s^{-1} in animals maintained for 21 days at the 4 light levels (Figure 5.2). Steele (1976), similarly reported a rise in the rate of symbiont expulsion from *Aiptasia tagetes*, with a relatively low change in light intensity from 20 to 35 μE.m^{-2}.s^{-1} (see Table 5.10). The light intensities used in the latter experiments are very low relative to the range of natural illumination experienced by anemones in the field. Rates of symbiont expulsion from *Anemonia viridis* at 50 μE.m^{-2}.s^{-1} averaged 0.00027 cells.cell^{-1}.d^{-1} which is at least 6 times lower than values for *Aiptasia tagetes* observed by Steele (1976) at similar light intensities. The symbiont mitotic index also increased with increasing light intensity. The same trend was also observed in the data from a separate experiment presented in Chapter 4.

5.4.3 The effect of ammonium supplementation on zooxanthella expulsion rate.

When *Anemonia viridis* was maintained in 20μM ammonium enriched sea water, symbiont specific expulsion rates and mitotic index both increased relative to control values which did not change significantly. The mean specific expulsion rate of animals in ammonium enriched sea water was 0.00635 cells.cell^{-1}.d^{-1}, which is slightly higher than rates reported for colonies of *Pocillopora damicornis* maintained in ammonium enriched sea water by Stimson & Kinzie (1991). These authors reported an increase in the expulsion rate of symbionts measured as cells expelled per unit area from corals in ammonium supplemented sea water, compared with expulsion rates of control colonies. However, in their experiments, symbiont population densities in ammonium supplemented corals were 3 times the densities in controls, and this resulted in specific release rates being lower in ammonium supplemented corals. They attributed the differences between control and experimental groups to the increased symbiont population densities in the ammonium supplemented corals. There was no significant increase in the final total zooxanthella population density in ammonium supplemented *Anemonia viridis* in this experiment. Furthermore, earlier experiments involving ammonium supplementation

did not show a significant increase in the total zooxanthella population density in *A. viridis* (section 3.3.3).

The mitotic index of symbionts within ammonium supplemented anemones was significantly higher than that of symbionts from control animals. The mean mitotic index of zooxanthellae in ammonium supplemented anemones increased sharply within 3 days, then decreased at around 8 days and increased again under prolonged ammonium enrichment up to 21 days. This was not unexpected since a previous experiment had shown an increase in division rates of zooxanthellae in tentacles when anemones were incubated in ammonium enriched sea water (section 4.3.2). In this previous experiment a similar pattern in mitotic index was also observed over the time course of the experiment.

When expulsion rates, normalised to animal dry weight, were plotted against corresponding zooxanthella mitotic index values of both treatment and control animals, there was a positive correlation between the expulsion rate and zooxanthella mitotic index (Figure 5.5). No correlation was found between symbiont population density and expulsion rate. For expulsion of zooxanthellae to be an effective mechanism by which the population size is controlled, the numbers of zooxanthellae expelled must be substantial enough to account for a large proportion of those cells added to the population by division. In chapter 8, the numbers of zooxanthellae expelled are compared to the numbers added through division. The ratios, thus obtained, are also discussed and compared with other reported data in Chapter 8.

Viable symbionts (taken as those cells which did not take up methylene blue stain), dividing, and degenerate symbionts were present in all samples of expelled material (see Table 5.8 and 5.9). Degenerating symbionts (those cells in the later stages of senescence) were observed under the light microscope as those cells which were readily stained with methylene blue. Transmission electron micrographs showed these cells to be misshapen, lacking in lipid or starch accumulations and possessing large numbers of calcium oxalate crystals. These observations agreed with the observations made by Taylor (1968) of zooxanthellae found in the mesenterial filaments which he described as being in an advanced stage of senescence.

The observation that viable symbionts are expelled would indicate that there is no selection for zooxanthellae in a state of degeneration, as suggested by Taylor (1968). In the present experiments, degenerate zooxanthellae, as described above, were observed in expelled samples but never observed in electron micrograph sections of tentacles from animals kept in the light with weekly feeding (Taylor 1969b; pers.obs.). They were, however, observed to make up the majority of the symbionts within the mesenterial filaments (Smith, 1939; Taylor 1969b;

pers.obs.). This suggests that, under conditions of light and feeding, symbionts do not degenerate within host gastroderm cells, but that the degeneration is accelerated after expulsion from the gastrodermis. The conditions described here are different from those where zooxanthellae have been observed to degenerate within host gastroderm cells. Such conditions include increased temperatures and very high incident light intensities, as occurred during early afternoon low tides in inter-tidal corals (Brown *et al*, 1995), and prolonged starvation in the light (Yonge & Nicholls, 1930b).

Dividing cells were present in every sample of expelled zooxanthellae, and made up between 3% and 15% of the total counted. The mean division rate of symbionts in expelled samples was higher than that of symbionts within tentacles. These findings agree with those predicted by Suharsono & Brown (1992) who observed a greater zooxanthella division rate when using tentacle homogenates than when using tentacle sections, and suggested that this difference was due to the inclusion within the homogenate sample of symbionts expelled into the coelenteron. They suggested that the release of zooxanthellae into the coelenteron may initiate their division since they were no longer within host tissues and free from host restraints on division.

Samples of expelled material collected after 1-2 hour incubations contained mostly viable zooxanthellae within host gastroderm cells. Previously, host gastroderm cells containing symbionts have only been observed in samples of expelled material released during exposure to high or low temperature stress (Muscatine *et al*, 1991; Gates *et al*, 1992; Brown *et al* 1995). Host cells, including nematocysts, gastroderm cells without symbionts and mesoglea cells were also observed in samples of expelled material and may suggest that cells of the gastrodermis are continually shed and presumably replaced along with the zooxanthellae contained within them. The observation that cells from the mesoglea were present in the expelled material, some of which contained zooxanthellae, may implicate the role of the mesenteries in zooxanthella expulsion as suggested by several authors (Smith, 1939; Taylor 1968).

1. Summary

2. Expulsion rates were highly variable between animals and over time.

3. Expulsion rates increased with increasing light intensity between 50 and 300μE.m^{-2}s^{-1}.

4. Expulsion rates increased with duration of exposure to ammonium enrichment over 21 days. Without exposure to ammonium, expulsion rates fell gradually over the same period.

5. A positive correlation was found between the expulsion rate normalised to animal weight and the mitotic index of zooxanthellae within tentacles of all animals sampled. No correlation was found between the specific expulsion rate and the zooxanthella population density.

6. Symbionts do not appear to degenerate *in hospite* under normal conditions, but expulsion from host cells into the coelenteron may increase the onset of degeneration. Although expelled cells had a higher division rate than symbionts within host cells, this does not provide conclusive evidence of any selection for zooxanthellae in a particular stage of the life cycle.

7. It seems likely that there may be more than one mechanism by which zooxanthellae are expelled. Zooxanthellae may be expelled directly from the gastroderm within intact gastroderm cells. Observations together with published reports indicate, however, that the mesenteries also play a part in zooxanthella expulsion.

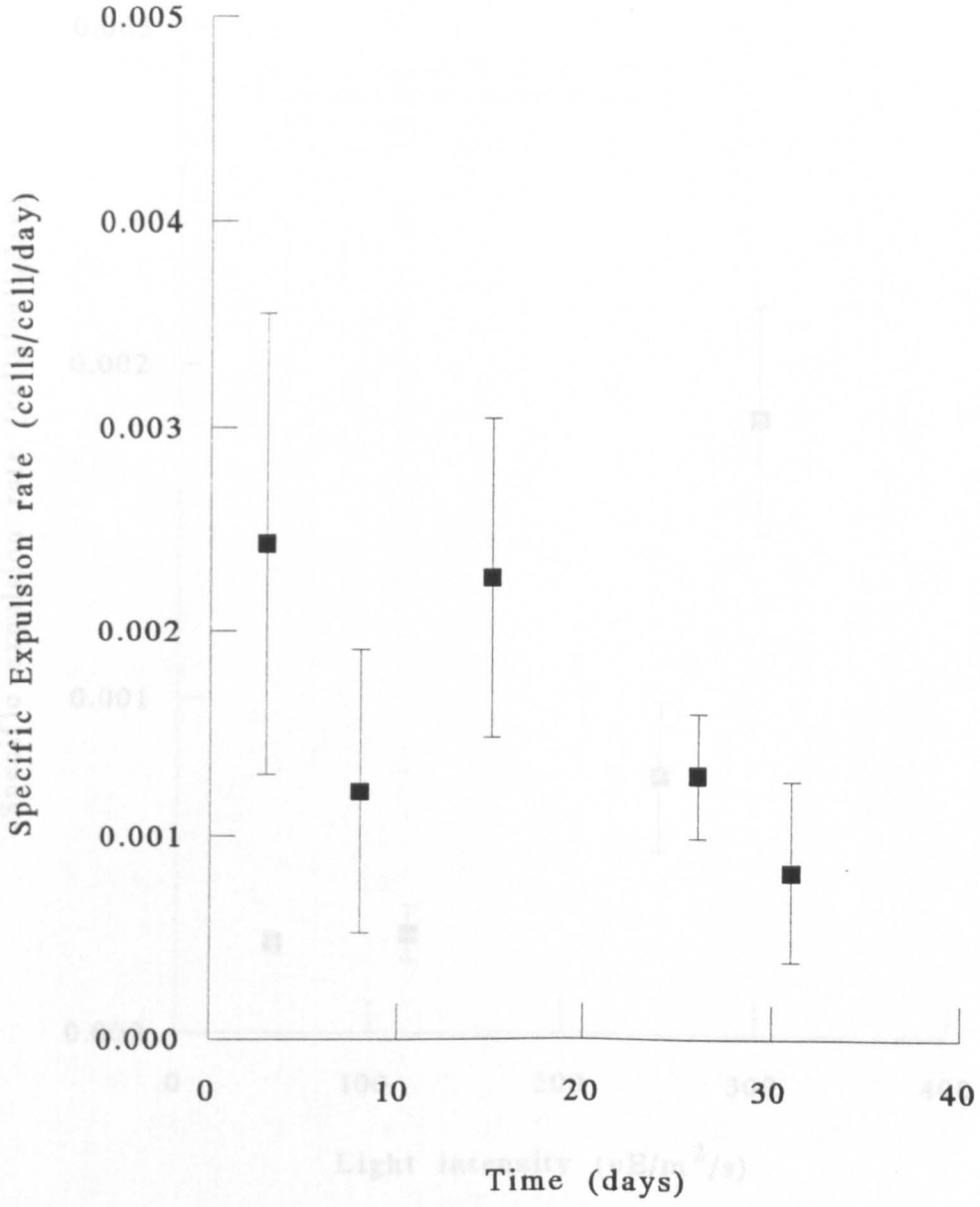


Figure 5.1 Mean specific expulsion rates of symbionts of *A. viridis* maintained unfed over 31 days at 300μE.m^{-2}.s^{-1}, on a 12 hour light:12 hour dark cycle at 11$\pm$1^{o}C. Points are means $\pm$ s.e. (n=5). Sampling interval for each animal was 3 days except for day 8 (4 days), and day 26 (2 days).

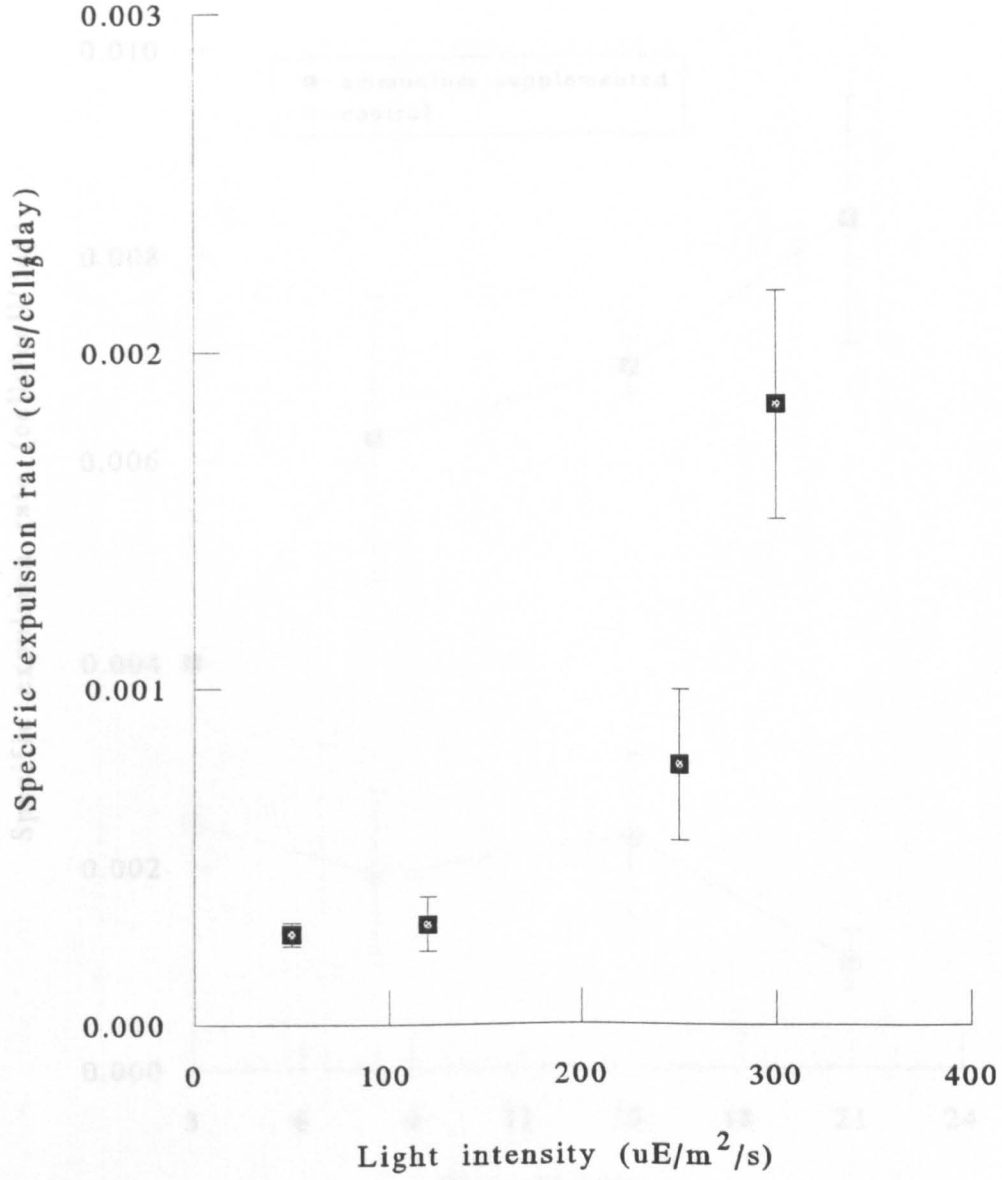


Figure 5.2 Mean specific expulsion rates of zooxanthellae of *A. viridis* at 4 light intensities. Points are means ± s.e (n=5). 4 values were obtained for each animal. Tukey tests for pair-wise comparisons showed a significant difference between rates at $300\mu E.m^{-2}s^{-1}$ and all other light intensities ($p<0.05$); values at 50, 120 and $250\mu E.m^{-2}.s^{-1}$ were not significantly different.

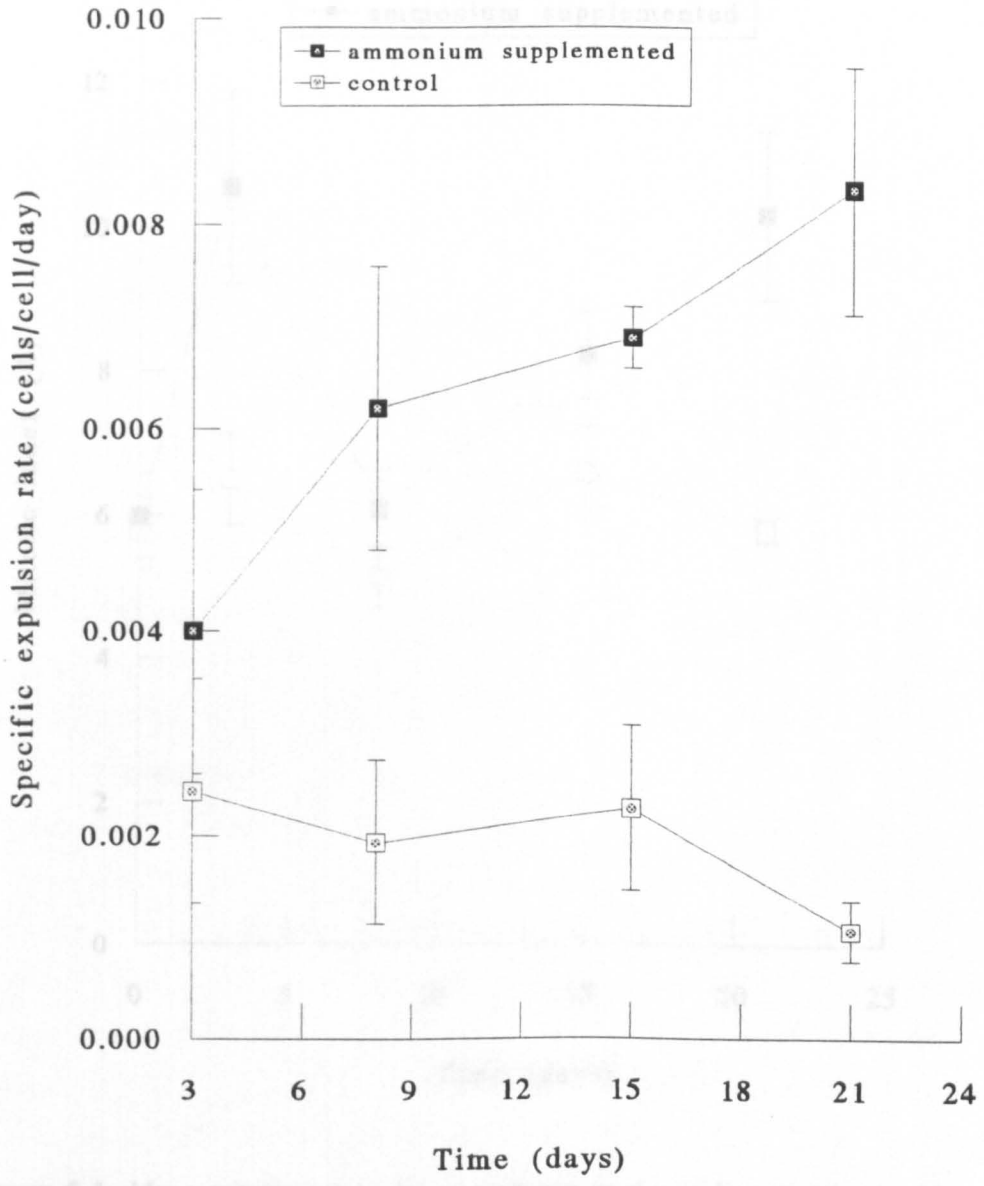


Figure 5.3 Specific zooxanthella expulsion rate from *A. viridis* maintained without feeding at 300μE.m^{-2}.s^{-1} on a 12 hour light:12 hour dark cycle at 11±1°C for 21 days in control sea water or sea water enriched to 20μM ammonium. Points are means ± s.e (n=5).

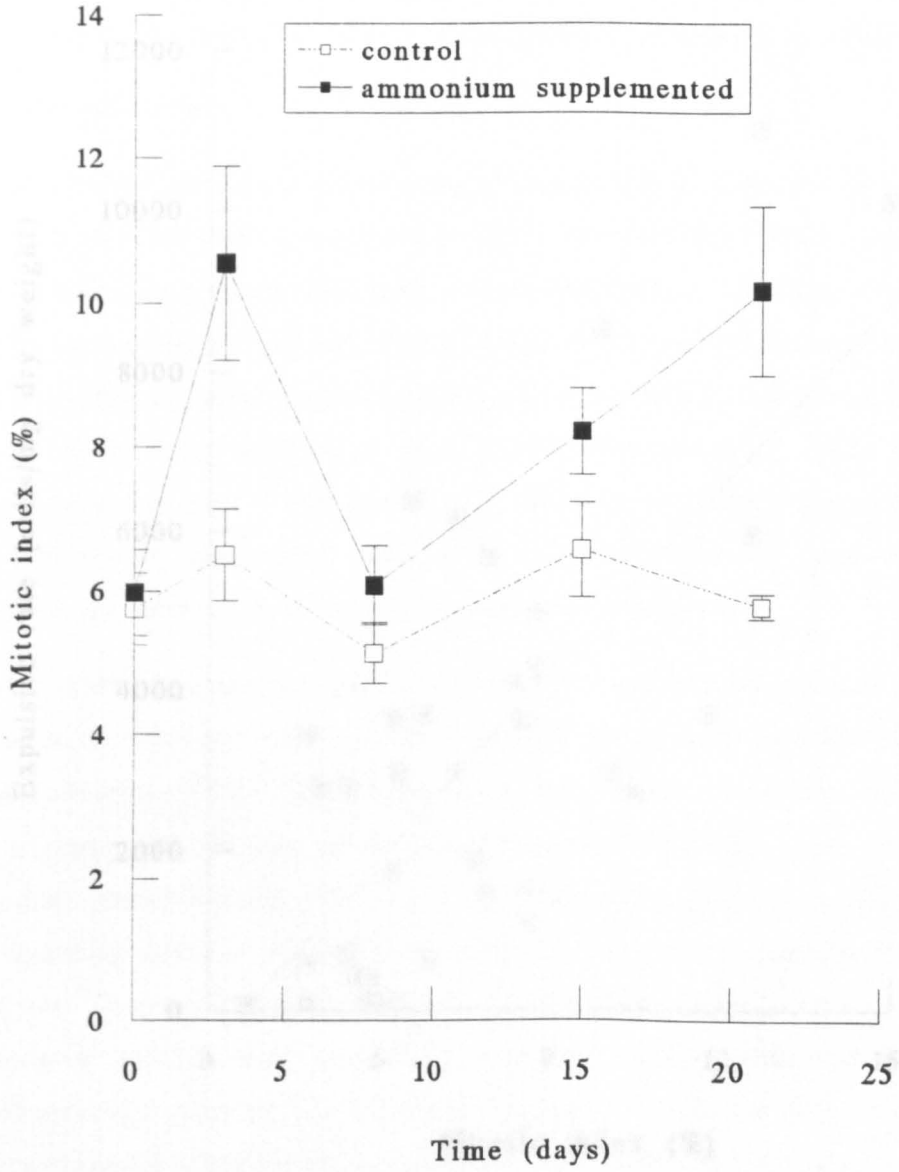


Figure 5.4 Mean mitotic index of zooxanthellae in *A. viridis* maintained without feeding at 300μE.m^{-2}.s^{-1} under control and 20μM ammonium supplemented treatments ± s.e. (n=5). A two way ANOVA showed a significant difference between the ammonium supplemented and control groups.

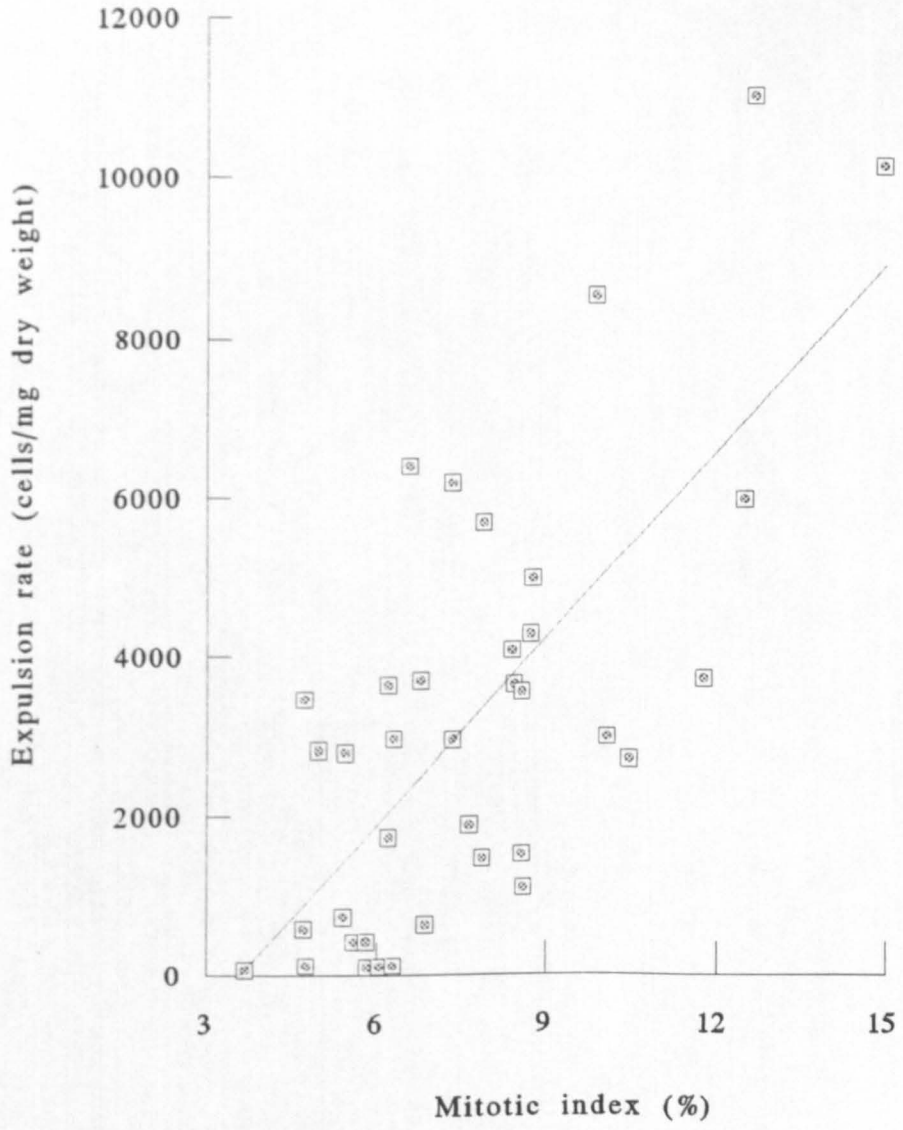


Figure 5.5 Expulsion rates normalised to dry weight plotted against the corresponding zooxanthella mitotic index in tentacles of *A. viridis* maintained at 300μE.m^{-2}.s^{-1} and at 11± 1°C.

Regression analysis:

$$y = -2770 + 776.9\,x \quad r^2 = 50.2\% \quad (n=37)$$

CHAPTER 6

EFFECTS OF LONG TERM DARKNESS AND SUBSEQUENT LIGHT ON THE ZOOXANTHELLA POPULATION AND MITOTIC INDEX IN *A. VIRIDIS*.

6.1 Introduction

When symbiotic anthozoans are subjected to long periods of darkness, a gradual paling of the tissues is observed until they appear completely white (Smith, 1939; Franzisket, 1970). This loss of colour is normally associated with the expulsion of symbionts (Yonge & Nicholls, 1930b; Steen & Muscatine, 1987; Davies, 1988). During coral "bleaching" due to temperature stress, the loss of colour may be brought about either by a loss of zooxanthellae (Jaap 1988; Glynn, 1988; Hoegh-Guldberg & Smith 1989a; Muscatine *et al*, 1991), the loss of algal photosynthetic pigment or both (Reese *et al*, 1988; Kleppel 1989). Franzisket (1970) observed the presence of unpigmented cells within completely bleached corals after 90 days of darkness, and assumed that these were unpigmented symbionts.

Davies (1988) observed that under continuous darkness, the zooxanthella population within tentacles of symbiotic *Anemonia viridis* was rapidly reduced in a few days, but did not entirely disappear; 20% of the original population remained after 6 days, while around 4% of the original population remained for at least 5 months. The change in rate of loss from a rapid to a more gradual loss after 6 days may suggest that some physiological change takes place in the remaining symbiont population that facilitates their long term existence within the host in darkness. An example of this has been found in symbiotic *Hydra* when kept in darkness. Cell division of *Chlorella* was initially inhibited but after a period of about 10 days the symbionts recommenced division provided the host continued to feed. However, in *Hydra* containing symbionts which originated from *Paramecium*, division of the algae did not recommence and they eventually disappeared. This difference was correlated with the fact that the former have an inducible glucose uptake system in the dark, whereas the latter do not (McAuley, 1985; 1986a). Alternatively, the change in the rate of loss may be induced by a reduction in competition for resources occurring as symbiont population density decreases.

In some cnidarians, symbionts are still present after 160 days of darkness (Yonge & Nicholls, 1930b; Kevin & Hudson, 1979), probably long after their starch and lipid stores have been exhausted. They must, therefore, feed heterotrophically (Steen, 1986), deriving amino acids and other nutrients from the host. Carroll & Blanquet (1984a) demonstrated the ability of isolated symbionts, from *Cassiopeia xamachana*, to take up amino acids, particularly alanine,

independently of photosynthesis. Whether this parasitic mode of life in darkness supports division of zooxanthellae, as observed in *Chlorella* symbiotic in *Hydra* by McAuley (1985, 1986a) has not been investigated.

It is not known whether the reduction in the population of symbionts in the dark is due to the increase in the rate of expulsion or to the decrease in the rate of division. However, it could be inferred that the flux of nutrients to the symbionts during darkness is too low to sustain a normal rate of division, and it is this which results in the population decline. Yonge & Nicholls (1930b) noted that the population of zooxanthellae fell when corals were starved for 52 days in the light, although no quantitative measurements were made. They, however, suggested that the host's lowered metabolic activity during prolonged starvation led to the expulsion of zooxanthellae.

In the previous chapters, it was shown that the growth rate of zooxanthellae of *Anemonia viridis* could be enhanced by exposure to high light and increased concentrations of ammonia in the sea water. This suggested that symbiont growth rate is nutrient limited. However, a further restraint on symbiont growth rates may be the space available within the host cell (see Chapters 1 and 4). If the symbiont population is controlled by "space" available for growth, then algal division would only occur during or after host cell division, (McAuley, 1981a). The rate of symbiont division would, therefore, be limited by the rate of host cell division. In Chapter 4, an inverse relationship was found between host size (dry weight) and zooxanthella mitotic index. If space is a limiting factor, it might be expected that growth rates of zooxanthellae in partially aposymbiotic hosts with lower symbiont population densities would be higher than in symbiotic hosts. Even if this were observed, it would not necessarily indicate that the physical attributes of "space" are important. Cook & D'Elia (1987) suggested that growth rate of symbionts is controlled by competition for nutrients. An increase in growth rate may just be correlated with, and not dependent on, an increase in space, as a result of lowered competition for essential nutrients, such as nitrogen.

Partially aposymbiotic anemones may provide a useful model for investigating the conflicting hypotheses of growth rate control. Near completely or completely aposymbiotic *Aiptasia pulchella* and *A. tagetes* can regain their normal population after a period of 3 to 7 weeks, when inoculated with isolated zooxanthellae (Trench 1971b; Kinzie & Chee, 1979; Schoenberg & Trench, 1980; Berner *et al*, 1993). From the inverse relationship between the symbiont mitotic index and population density found in chapter 4, it would be expected that algal growth rates in symbiotic anemones would be lower than those in partially aposymbiotic

anemones due either to less competition for nitrogen, or due to a greater amount of space available for growth. If "space" is a controlling factor, the growth rate of symbionts in partially aposymbiotic anemones in the light might be greater than that of algae in symbiotic anemones when both groups are supplied with ammonium in excess of demand. If availability of nitrogen alone limits symbiont growth rates, then providing excess nitrogen may remove this limitation within symbiotic anemones such that symbiont growth rates in symbiotic anemones would be increased to levels similar to those in partially aposymbiotic hosts.

In addition, the latter hypothesis suggests that symbiont division in partially aposymbiotic animals is not limited by availability of nitrogen and, therefore, it could be argued that growth rates of symbionts in partially aposymbiotic anemones would not be increased by ammonium enrichment. Growth rates of zooxanthellae based on mitotic index data, however, have not been measured over the time course of such experiments involving aposymbiotic hosts.

Experiments were carried out to answer the following questions:

1. Does loss of algal photosynthetic pigment contribute to the loss of colour in anemones in darkness?
2. Is the rate of loss of zooxanthellae in darkness higher in the potentially lower nutrient levels associated with starvation of the host?
3. Is the rate of division, as measured by the mitotic index, lower during prolonged darkness compared to in the light, and is the rate lower in starved anemones?
4. Are the growth rates of symbionts of partially aposymbiotic hosts in the light higher than those in symbiotic hosts?
5. Is the division rate of partially aposymbiotic hosts determined by intracellular space or are the symbionts nitrogen limited and hence able to increase their division rate when stimulated with inorganic nitrogen?
6. If both partially aposymbiotic and symbiotic hosts are no longer nitrogen limited following incubation in 20μM ammonia, is the division rate of algae within aposymbiotic hosts still higher?

6.2 Materials and Methods

6.2.1. Rate of loss and rate of division of zooxanthellae in animals maintained in darkness

In addition to attempting to evaluate the role of intracellular nutritional conditions on division rate and population density of zooxanthellae, this experiment also served to provide the partially aposymbiotic hosts required for the subsequent experiments on regrowth in the light. Since the majority of gastroderm cells in tentacles contain ≤ 3 symbionts (section 3.3.2), maintenance in darkness was continued until the population was about 33% of its original size, at which point it was assumed that most gastroderm cells would contain one symbiont. Furthermore, the opportunity was taken to document the change in the algal chlorophyll *a* content in darkness, since paling of the tissues of stressed symbiotic cnidarians is also associated with loss of algal photosynthetic pigment.

Twenty animals, of approximately equal size, previously fed weekly, were maintained in a running, aerated sea water aquarium at $11 \pm 1^{o}C$ in complete darkness for 66 days. During this time, the animals were fed twice weekly on mussel tissue. A further 20 animals were maintained unfed in an identical aquarium. Two tentacles were taken from 10 animals selected at random from each treatment on days 0, 2, 6, 11, 16, 29, 42 57 and 66. Zooxanthella counts were made, and protein and chlorophyll content per sample were measured (section 2.2.1 and 2.2.5). A further two tentacles were removed from 5 of the animals from each treatment and fixed in 3:1 alcohol:acetic acid for mitotic index counts (section 2.2.3). Sampling was carried out at the same time on days 0, 6, 16, 29, 57 and 66.

6.2.2 Rate of population increase and division rate of zooxanthellae in partially aposymbiotic hosts in the light.

In order to test whether increased availability of "space" affects the division rate of symbionts, the mitotic index of zooxanthellae from the partial aposymbiotic anemones, produced in section 6.2.1 by maintenance in darkness for 66 days with feeding, were compared with that of zooxanthellae from control symbiotic hosts, which had been maintained with weekly feeding at $300\mu E.m^{-2}.s^{-1}$. Ten partially aposymbiotic animals, selected at random, were placed in one half of a partitioned tank together with 10 control symbiotic anemones, and maintained for 35 days at $300\mu E.m^{-2}s^{-1}$ without feeding. Two tentacles were removed from each anemone, and treated as in section 6.2.1 for zooxanthella population density measurements on days 0, 12, 17, 26 and 35 in the light. A further 2 tentacles were removed

from 5 of the animals in each group, selected at random, for mitotic index measurements on days 0, 4, 8, 12, 17, 26 and 35 (section 2.2.3).

The remaining 10 aposymbiotic anemones, which had been fed twice weekly, were placed with 10 control fed symbiotic anemones which had previously been maintained under a light intensity of $300\mu E.m^{-2}.s^{-1}$ in the other half of the above mentioned tank. This half of the tank was supplied with sea water continuously enriched with ammonium to a concentration of $20\mu M$ (section 3.2.1). Two tentacles were removed from each anemone and treated as in section 6.2.1 for zooxanthella population density measurements on days 0, 12, 17, 26 and 35. A further 2 tentacles were removed from 5 of the animals, selected at random from each group, for mitotic index measurements as above (section 2.2.3).

6.2.3. Ultrastructure of symbionts from animals maintained in prolonged darkness.

After 66 days in darkness, one animal from the dark starved group, one animal from the dark fed group and one animal maintained at $300\mu E.m^{-2}.s^{-1}$ were narcotised in 7% magnesium chloride for 30 minutes. Two tentacles were removed from narcotised animals and fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer for 60 minutes. The fixative was replaced with 2 changes of sodium cacodylate buffer made up using the procedure given in section 2.2.6. over 24 hours. The tentacles were cut into 2 mm sections with a razor blade and post fixed in 1% osmium tetroxide for 1 hour. This was followed by 3 rinses in distilled water, each for 10 minutes. The sections were cut into 1mm long fragments and stained for 0.5-1 hour in 0.5% uranyl acetate in distilled water in the dark, and then dehydrated in a series of increasing strength alcohols, for 10 minutes in each alcohol. The final change of dried absolute alcohol was replaced with 3 changes of propylene oxide (epoxy propane).

The samples were transferred to a 1:1 mix of propylene oxide and Spurr's resin or araldite and left over night on a rotamixer. The samples were embedded in fresh resin and left to harden for 2 days in an oven at $60^{o}C$ before sectioning. Sections were stained in uranyl acetate and lead citrate each for 5 minutes and observed under a transmission electron microscope.

6.3 Results

6.3.1 Rate of loss and rate of division of zooxanthellae in animals maintained in darkness.

6.3.1.1 Effect of darkness on population density and chlorophyll content

Figures 6.1a and b and Tables 6.1 and 6.2 show the effect of feeding and starvation on the population density and number of zooxanthellae within tentacles of *Anemonia viridis* maintained in continuous darkness for 66 days. ANOVA tests were carried out on the data for the number of zooxanthellae per tentacle, the zooxanthella population density in tentacles and the animal protein content per tentacle.

There was a significant fall in the population density (expressed as μg protein) in tentacles of both fed and starved animals in darkness over the experiment (Two way ANOVA $F=9.35$ $p<0.001$), and a significant difference in population density between fed and starved animals (Two way ANOVA $F=13.33$, $p<0.001$). When tested using covariance analysis, the slopes (regression coefficients) for fed and starved animals were not statistically different ($p>0.05$). The population density in fed animals fell rapidly over the first 16 days from 3042 to 1800 cells.μg protein^{-1}, then fell more gradually over the remainder of the experiment to a population density of 40% of the initial density. In starved animals, the population density remained at around 3000 cells.μg protein^{-1} for the first 11 days in darkness, before decreasing to 45% of the initial population density after 66 days in the dark.

Table 6.1 Changes in the zooxanthella population density (cells.μg protein^{-1}) ± s.e of mean ($n=10$) in tentacles of *A. viridis* maintained in darkness for 66 days with or without twice weekly feeding.

Day	Fed twice weekly		Starved	
	population density	%	population density	%
0	3042.0 (264)	100	3074.0 (107)	100
2	2659.0 (184)	87	2867.3 (52)	93
6	2543.3 (218)	84	2912.3 (238)	95
11	2042.6 (198)	67	2970.8 (199)	97
16	1796.4 (144)	59	2316.9 (212)	75
29	1770.8 (216)	58	2216.0 (151)	72
42	1582.6 (114)	52	2225.4 (197)	72
57	1367.0 (128)	45	1450.8 (190)	47
66	1220.2 (94)	40	1397.0 (182)	45
Regression coefficient	- 23.0		- 24.9	
significance of slope	$p < 0.001$ significant		$p < 0.001$ significant	

The number of zooxanthellae per tentacle had decreased significantly in starved animals (one way ANOVA F=2.98, p=0.01), but not in fed animals (one way ANOVA F=1.9 p=0.073) after 66 days in darkness. Covariance analysis showed that fed animals had significantly more zooxanthellae per tentacle than starved animals.

Table 6.2 Changes in the zooxanthella population per tentacle ± s.e of mean (n=10) in *A. viridis* maintained in darkness for 66 days with or without twice weekly feeding. Regression coefficients with different superscripts were statistically different ($p<0.05$) when tested using analysis of covariance.

Day	Fed twice weekly		Starved	
	population number $x10^5$	percentage remaining	population number $x10^5$	percentage remaining
0	10.94 (1.6)	100	10.819 (1.3)	100
2	10.477 (0.9)	96	9.198 (1.2)	85
6	9.313 (0.9)	85	8.800 (1.0)	81
11	9.727 (0.8)	89	8.958 (0.9)	83
16	8.475 (1.0)	77	8.004 (0.7)	74
29	8.040 (0.7)	73	6.408 (0.8)	59
42	7.777 (0.7)	71	6.170 (0.8)	57
57	6.750 (0.8)	62	4.340 (0.6)	40
66	5.982 (1.1)	55	4.290 (0.6)	40
Regression coefficient	- 6631 [a]		- 8238 [b]	
significance of slope	p = 0.024		p<0.001	

Table 6.3 shows the mean animal protein content per tentacle during dark treatment.

Table 6.3 Average protein content per tentacle of *A. viridis* maintained in continuous darkness for 66 days ± s.e. (n=10). Regression coefficients (slopes) with different superscripts were statistically different ($p<0.05$) when tested using analysis of covariance.

Day	Fed twice weekly	starved
0	359 (40)	352 (42)
2	394 (26)	321 (44)
6	366 (37)	309 (37)
11	470 (61)	301 (43)
16	452 (63)	345 (31)
29	454 (45)	289 (32)
42	492 (51)	277 (34)
57	494 (41)	279 (34)
66	490 (45)	307 (21)
Regression coefficient	+ 1.8 [a]	- 0.67 [b]
significance of slope	p = 0.007 significant	p = 0.076 n.s.

Covariance analysis showed that animals which had been fed twice weekly in darkness had significantly more animal protein per tentacle than starved animals in darkness (Table 6.3).

Therefore, the discrepancy between the population density expressed on a per tentacle and a per μg protein basis resulted from differences in the protein content per tentacle between fed and starved animals during the period in darkness. Thus, the mean total zooxanthella population per tentacle was significantly greater in fed animals, but because the protein content per tentacle in fed animals was higher than that in starved animals, the mean population density, measured as cells.μg protein^{-1}, was no different in fed animals to that of starved animals.

Figure 6.2 and Table 6.4 shows the changes in chlorophyll *a* for dark-maintained animals.

Table 6.4 Chlorophyll *a* content in tentacles of *A. viridis* maintained in darkness for 66 days. Values in brackets are standard errors of the mean (n=10). Regression coefficients with different superscripts were statistically different ($p<0.05$) when tested using analysis of covariance.

	Chlorophyll *a* per tentacle (μg)		Chlorophyll *a* per zooxanthella (pg)	
Day	fed twice weekly	starved	fed twice weekly	starved
0	2.19 (0.25)	2.60 (0.31)	1.54 (0.22)	1.54 (0.21)
29	1.05 (0.18)	0.95 (0.34)	1.19 (0.17)	0.73 (0.26)
42	0.66 (0.17)	0.43 (0.08)	0.96 (0.04)	0.85 (0.15)
57	0.50 (0.11)	0.31 (0.07)	1.27 (0.16)	0.73 (0.26)
66	0.48 (0.06)	0.21 (0.07)	1.20 (0.17)	0.32 (0.05)
Regression coefficient	- 0.027 ^{a}	- 0.033 ^{b}	- 0.0027 ^{a}	- 0.018 ^{b}
significance of slope	$p < 0.001$	$p < 0.001$	$p = 0.438$ n.s.	$p < 0.001$

Regression and covariance analysis showed that there was a significant fall in the amount of chlorophyll *a* per tentacle over the dark period in both fed and starved animals (Table 6.4), and a significant difference in chlorophyll *a* per tentacle between the groups, with fed animals having more chlorophyll *a* per tentacle than starved animals. Chlorophyll *a* content per zooxanthella in tentacles of fed animals did not change during the experiment (slope not significant $p = 0.438$), but fell significantly in starved animals (slope significant $p<0.001$), resulting in a significant difference between fed and starved animals. The above results show that although total chlorophyll *a* per tentacle in fed animals fell significantly during the dark from 2.191 to 0.45μg, the remaining zooxanthellae maintained their chlorophyll *a* content at a mean of 1.22 pg. chlorophyll a per zooxanthella. Starvation in darkness, however, reduced the chlorophyll a content per zooxanthella significantly.

6.3.1.2 Effect of darkness on zooxanthella mitotic index.

Figure 6.3 and Table 6.5 show the effect of feeding and starvation on the mitotic index of zooxanthellae in tentacles of *A. viridis* maintained for 66 days in darkness. Differences in the mitotic index were tested using two way ANOVA tests. There was no significant difference in zooxanthella mitotic index between fed and starved groups ($F = 1.03$, $p = 0.32$), and no significant change with time in either regime in darkness ($F = 0.38$, $p = 0.86$)

Table 6.5 Mitotic index values of zooxanthellae from anemones maintained with or without feeding in continuous darkness for 66 days ± s.e. of mean (n=5).

Day	Fed twice weekly	starved
0	3.85 (0.8)	2.84 (0.2)
6	3.85 (1.8)	3.82 (1.1)
16	5.90 (1.8)	3.16 (0.7)
29	4.33 (1.7)	3.77 (1.4)
57	3.82 (1.5)	1.83 (0.6)
66	2.22 (0.8)	1.77 (0.6)
Mean	3.99 (0.5)	2.87 (0.4)
One way ANOVA	F=0.19 p=0.96	F=0.72 p=0.62

Zooxanthellae of animals maintained in the dark with twice weekly feeding continued to divide at a similar rate to zooxanthellae from animals at the start of dark treatment, which had means of 3.9% and 2.8% for fed and starved animals respectively. There was a large amount of variation about the mean mitotic index in all samples from both fed and starved groups. Zooxanthellae from one fed animal consistently maintained a mitotic index of between 9 and 11% for the first 29 days of the experiment.

Interestingly, in the samples from fed and starved animals taken after 29 days in darkness, which had high mitotic index values of between 10 and 13%, there was a high proportion of tetrads (cells with 4 nuclei), making up on average 25% of the total dividing cells.

6.3.2. Rate of population increase and rate of division of zooxanthellae in partially aposymbiotic hosts in the light.

6.3.2.1 Effect of light without ammonium enrichment.

In this experiment, symbiotic anemones were compared with partially aposymbiotic anemones to assess whether there was a difference in the division rate of zooxanthellae. The partially aposymbiotic anemones had previously been maintained for 66 days in continuous darkness with twice weekly feeding. Table 6.6 and Figure 6.4a shows the change in numbers and

population density of zooxanthellae in tentacles of partially aposymbiotic anemones maintained in the light. The zooxanthella population density of partially aposymbiotic animals in unenriched sea water increased from 1220 to 2410 cells.μg protein^{-1} which represented an increase from 40% to 79% of the original pre-dark treatment population density after 35 days of light. Zooxanthella numbers increased from 5.98 to 8.79 x 10^5 cells per tentacle in partially aposymbiotic anemones - an increase from 55% to 80% of the pre-dark treatment numbers.

Table 6.6 Changes in the zooxanthella population density and zooxanthella population in partially aposymbiotic *A. viridis* kept in the light ($300\mu E.m^{-2}.s^{-1}$) without feeding for 35 days, after maintenance in darkness for 66 days with twice weekly feeding. Figures in brackets are standard errors (n=10).

Day	No. of zooxanthellae per tentacle x10^5	% of initial population	Zooxanthella density (cells/µg protein)	% of initial density
0	5.98 (0.9)	55	1220 (118)	40
12	7.09 (0.6)	65	1692 (143)	55
17	7.75 (0.6)	67	1992 (199)	65
26	8.16 (0.7)	71	2028 (183)	66
35	8.79 (0.7)	80	2413 (185)	79
Regression coefficients	+ 0.08 x 10^5		+ 32.0	
significance of slope	p = 0.002		p = 0.005	

Thus, after 35 days at $300\mu E.m^{-2}.s^{-1}$ the anemones had not recovered their original population density and numbers.

Table 6.7 Mitotic index of zooxanthellae ± s.e. of mean (n=5) from *Anemonia viridis* maintained at $300\mu E.m^{-2}.s^{-1}$ without feeding. (Aposymbiotic hosts were previously maintained with feeding in darkness for 66 days and symbiotic hosts were previously maintained at $300\mu E.m^{-2}s^{-1}$ with feeding.)

Day	Partially aposymbiotic	Symbiotic
0	2.7 (0.5)	4.2 (0.4)
4	6.8 (1.2)	4.3 (0.5)
8	4.2 (0.1)	-
12	5.0 (0.9)	3.6 (0.3)
17	4.3 (0.1)	2.6 (0.3)
26	4.0 (0.1)	3.1 (0.3)
35	5.5 (0.6)	3.8 (0.3)
Mean	4.64 (0.5)	3.6 (0.3)
ANOVA	F=3.1 p=0.03	F=1.19 p=0.38

The mitotic index of zooxanthellae in tentacles of partially aposymbiotic and symbiotic animals during the period in the light are given in Figures 6.4b and Table 6.7. The mean

symbiont mitotic index of zooxanthellae in anemones maintained under control conditions rose to 6.8% on the second day but did not increase further. The mitotic index of zooxanthellae from symbiotic anemones did not change significantly over the experimental period (one way ANOVA $F=1.19$, $p=0.38$). The mitotic index of zooxanthellae in partially aposymbiotic animals was significantly greater than that of zooxanthellae in symbiotic animals over the experimental light period (two way ANOVA: treatment factor $F=7.8$ $p=0.009$).

6.3.2.2 Effect of ammonium enrichment on partially aposymbiotic & symbiotic hosts.

In this experiment, symbiotic anemones were compared with partially aposymbiotic anemones to assess whether intra-cellular space *per se* is important in determining growth rate of zooxanthellae. The partially aposymbiotic anemones had previously been maintained for 66 days in continuous darkness with twice weekly feeding. Data for the zooxanthella population density and total zooxanthellae per tentacle of anemones maintained in the light with 20μM ammonium enrichment for 35 days are given in Table 6.8 and Figure 6.5a.

Table 6.8 Changes in the population density and zooxanthella population in tentacles (± s.e of mean n=10) of partially aposymbiotic *A. viridis* kept at 300μE.m^{-2}.s^{-1} with 20μM ammonium enrichment for 35 days. Animals had been previously maintained in darkness for 66 days with twice weekly feeding.

Day	No. of zooxanthellae per tentacle x10^5	% of initial population	Zooxanthella density (cells/µg protein)	% of initial population density
0	5.98 (0.9)	55	1220 (182)	40
12	6.68 (0.9)	61	1820 (223)	60
17	10.46 (1.6)	96	2621 (271)	86
26	13.73 (0.9)	125	3888 (159)	127
35	15.33 (1.9)	140	4613 (349)	151
Regression coefficient	+ 0.455 x 10^5		+ 98.2	
significance of slope	$p < 0.001$		$p < 0.001$	

The zooxanthella population density of partially aposymbiotic animals with ammonium enrichment increased rapidly over the period of light treatment, from 1220 to 4613 cells.μg protein^{-1} which represented an increase from 40% to 151% of the pre-dark treatment densities. The number of zooxanthellae increased from 5.98 to 15.33 x 10^5 cells per tentacle in partially aposymbiotic anemones, which represented an increase from 55% to 140% of the pre-dark treatment numbers (Table 6.9). The number of zooxanthellae per tentacle in symbiotic anemones maintained in sea water enriched with 20μM ammonium also increased as did the zooxanthella population density (Tukey tests $p<0.05$).

Table 6.9 Changes in the population density and zooxanthella population in tentacles of symbiotic *A. viridis* kept in the light ($300\mu E.m^{-2}.s^{-1}$) without feeding with $20\mu M$ ammonium enrichment. Animals were previously maintained at $300\mu E.m^{-2}.s^{-1}$ with weekly feeding. Standard errors in brackets (n=10).

Day	Zooxanthellae per tentacle $x10^5$	Zooxanthella density (cells/µg protein)
0	16.3 (2.0)	4317 (144)
12	25.1 (2.1)	6029 (241)
17	21.5 (0.6)	5909 (410)
26	17.4 (2.3)	4806 (263)
35	23.2 (2.9)	6164 (267)
ANOVA	F=2.46 p=0.046	F=4.36 p=0.003

The mitotic index of zooxanthellae in tentacles of partially aposymbiotic and symbiotic animals maintained with ammonium enrichment in the light are shown in Figure 6.5b and Table 6.10. After 8 days at 300 $\mu E.m^{-2}s^{-1}$ with ammonium enrichment the mean mitotic index in tentacles of partially aposymbiotic hosts rose sharply to 40%, before decreasing to 9% after 26 days. The mitotic index of zooxanthellae from partially aposymbiotic anemones was significantly greater than that of symbiotic anemones over the light period, although values from both treatments had risen significantly during the experiment ($F=39.3\ p<0.001$).

Table 6.10 Mitotic index values of zooxanthellae from partially aposymbiotic and aposymbiotic *A. viridis* maintained at $300\mu E.m^{-2}.s^{-1}$ maintained in sea water enriched with ammonium to $20\mu M$.

Day	Partially aposymbiotic	Symbiotic
0	3.8 (1.0)	2.7 (0.5)
4	28.7 (2.0)	8.5 (1.2)
8	40.0 (1.9)	5.9 (0.7)
12	18.6 (0.9)	8.8 (0.9)
17	12.1 (2.0)	4.9 (0.6)
26	9.49 (1.0)	5.5 (0.8)
35	9.8 (0.7)	5.4 (0.2)
ANOVA	F=33.4 p<0.001	F=10.48 p<0.001

6.3.2.3 Comparison of division rates of zooxanthellae in partially aposymbiotic anemones with and without ammonium enrichment

In section 6.3.2.2 it was shown that under ammonium enrichment, which it was assumed would create in both partial aposymbiotic and symbiotic hosts, a condition in which zooxanthellae were no longer nitrogen limited, the algae with more intracellular space had higher mitotic indices, suggesting that space is limiting in symbiotic forms. To examine the effect of nitrogen availability, when "space" is not limiting, the rate of increase of the

zooxanthella population and the mitotic indices were compared in partial aposymbiotic hosts under control and 20μM ammonium enriched conditions. The data are derived from Figures 6.4a and 6.5a for the population comparison, and from Figures 6.4b and 6.5b for the mitotic index comparisons. Figure 6.7 a and b compare the population of zooxanthellae per tentacle and the population density per unit protein. Covariance analysis showed that ammonium enrichment significantly increased the zooxanthella number per tentacle (Table 6.11), while ANOVA and Tukey tests showed that ammonium significantly increased the zooxanthella mitotic index (ANOVA $F=19.02$ $p<0.001$; Tukey $p<0.05$) over the 35 days. These results indicate that when space is not limiting availability of nitrogen does limit the rate of division.

Table 6.11 Results of regression analyses carried out on the change in zooxanthella population density and numbers in tentacles of partially aposymbiotic anemones.

	Number of zooxanthellae		Population density (cells per μg protein)	
	Control	Ammonium enriched	Control	Ammonium enriched
Slope	$+0.008 \times 10^5$	$+0.455 \times 10^5$	+ 32.0	+ 98.2
significance of slope	$p = 0.002$	$p < 0.001$	$p = 0.005$	$p < 0.001$
difference in slopes	$p < 0.05$ significant		$p < 0.05$ significant	

Calculation of growth rates from changes in the zooxanthella population

Growth rates were estimated from changes in the population with time by using the general growth equation: $\ln_t = \ln N_o + \mu t$ (see Appendix 3)

where μ is the specific growth rate (cells.cell^{-1}.d^{-1}). Values are presented in Table 6.12.

Table 6.12 Changes in zooxanthella numbers and mitotic index in tentacles of partially aposymbiotic *A. viridis* introduced into the light (300μE.m^{-2}.s^{-1}). The "calculated growth rate" was estimated from the change in the no. of zooxanthellae in tentacles.

Day	Control			20μM Ammonium enriched		
	No. of zooxanthellae per tentacle ($\times 10^5$)	Calculated growth rate cells.cell^{-1}d^{-1}	Mitotic index (%)	No. of zooxanthellae per tentacle ($\times 10^5$)	Calculated growth rate cells.cell^{-1}d^{-1}	Mitotic index (%)
0	5.98 (0.9)		4.1 (0.5)	5.98 (0.9)		3.8 (1.0)
4	-		6.8 (0.2)	-		28.7 (2.0)
8	-	0.014	4.2 (0.1)	-	0.009	40.0 (1.9)
12	7.09 (0.6)		5.0 (0.9)	6.68 (0.9)		18.6 (0.9)
17	7.75 (0.6)	0.018	4.3 (0.1)	10.46 (1.6)	0.0898	12.1 (2.0)
26	8.16 (0.7)	0.006	4.0 (0.1)	13.73 (0.9)	0.030	9.5 (1.0)
35	8.79 (0.7)	0.008	5.5 (0.7)	15.33 (1.9)	0.0115	9.8 (0.7)

In anemones maintained in control sea water, the maximum calculated growth rate of zooxanthellae was 0.018 cells.cell^{-1}.d^{-1} which occurred between days 12 and 17. Under ammonium enrichment, initial growth rates averaged 0.009 cells.cell^{-1}.d^{-1} over the first 12 days, then rose to a peak of 0.0898 cells.cell^{-1}d^{-1} between 12 and 17 days then declined to a mean of 0.0224 cells.cell^{-1}d^{-1} up to 35 days in the light. Thus, under both groups the maximum calculated growth rate of symbionts in partially aposymbiotic hosts occurred between 12 and 17 days after re-introduction into the light. Maximum mitotic index values of zooxanthellae in tentacles, however, occurred earlier between days 4 and 8 after re-introduction into the light.

6.3.3. Ultrastructure of symbionts from animals maintained in prolonged darkness.

Differences in ultrastructural appearance between light and dark maintained, and between fed and starved animals in the dark, based on electron micrographs are given in Table 6.13.

Table 6.13 Features of zooxanthellae from light maintained and dark maintained anemones.

Feature	light maintained	dark maintained fed	dark maintained starved
Chloroplasts	Compacted towards outer edge of cell. Lamellae tightly packed	less compacted towards edge of cell; lamellae tightly packed.	Chloroplasts more oval in section (less flattened). Thylakoid lamellae separated, and vacuolated.
Pyrenoid	present, surrounded by starch.	present; surrounded by starch.	no starch sheath
Starch / lipid	abundant	starch present but not abundant, lipid absent	both absent in all cells
Membrane/cell wall	visible, complete	visible, complete	visible, complete; separated from host vacuolar membrane possibly due to shrinkage of zooxanthella.
Approximate thickness of gastroderm layer	4-5 zooxanthellae thick	3-4 zooxanthellae thick	1-2 zooxanthellae thick
Accumulation body	sometimes absent, varying in size	almost always present, large	almost always present, large
Calcium oxalate crystals	sometimes absent, never very numerous	always present, taking up approx. 10-20% of area of section.	always present, taking up approx. 25-60% of area in section.

Animals maintained in the dark for 66 days with twice weekly feeding were structurally similar in appearance to animals maintained with feeding in the light, although there was a small proportion of senescent symbionts in the fed animals maintained in darkness. However, in animals maintained in the dark without feeding, the majority of the symbionts were showing many signs of abnormality.

These features included an irregular shape probably caused by cell shrinkage, an absence of any lipid or starch storage vacuoles and an abundance of calcium oxalate crystals. There were several large membrane bound bodies with calcium oxalate crystals, or holes in the sections where the crystals had floated out during sample preparation. The starch sheath around the pyrenoid was very reduced or absent. The chloroplast was less compressed and took up a larger part of the cell volume than of zooxanthellae from light maintained animals. The thylakoid membranes of the chloroplasts appeared separated and vacuolated. In some very senescent cells, very few organelles could be distinguished with the exception of the chloroplast, which had begun to lose its internal structure.

6.4 Discussion

6.4.1 Rate of loss and rate of division of zooxanthellae in animals maintained in darkness

From previous work (e.g. Davies, 1988) it was expected that the population of zooxanthellae would decline in darkness. However, it became clear that selection of the wrong normalising unit to express population size could skew the results. Since the starved animals lost weight during the experiment, the mass of protein in a tentacle declined. Normalising to unit protein which was itself decreasing during the experiment, therefore, masked the true rate of reduction in the zooxanthella population in the starved group. Thus, fed animals maintained in continuous darkness had a lower symbiont population density than starved animals, since host protein per tentacle was higher in fed animals in continuous darkness compared to that of starved animals. A similar observation was found when animals fed in the light were compared with animals starved in the light (section 3.3.3.2). Thus, although the number of symbionts was higher in fed animals, the symbiont density was lower due to relatively more animal protein per tentacle.

6.4.1.1 Effect of continuous darkness on zooxanthella numbers and density within tentacles

The rate of loss of zooxanthellae from anemones in continuous darkness decreased gradually over 66 days, after which time tentacles from animals fed twice weekly and animals starved contained 55% and 40% respectively of the total number of zooxanthellae in animals maintained in the light. These rates of loss were much lower than the initial rate of loss of zooxanthellae from *Anemonia viridis* observed by Davies (1988), who found that 21% of zooxanthellae remained in tentacles after just 10 days of continuous darkness without feeding.

When symbiotic anemones were maintained in continuous darkness with twice weekly feeding, zooxanthellae were being lost from the host but the amount of chlorophyll *a* per zooxanthella in the resident population remained unchanged at a mean of 1.22 pg. chlorophyll *a*. Thus, the observation that feeding in darkness does not lead to a loss of chlorophyll per symbiont suggests that in animals fed in continuous darkness, zooxanthellae are expelled or digested before the chlorophyll breaks down. Starvation in darkness, however, reduced the chlorophyll *a* content per zooxanthellae as well as reducing the total number of zooxanthellae. Thus the nutritional status of the host influences zooxanthella chlorophyll *a* content.

The number of zooxanthellae in tentacles of starved animals was significantly lower after 66 days in darkness, while in fed animals, the symbiont population had not decreased significantly. Kevin & Hudson (1979) also found that numbers of zooxanthellae in fed colonies of *Plesiastrea urvillei* had not fallen significantly after 48 days in darkness.

From these experiments, therefore, two phenomena require discussion: 1) The decline in the population in darkness, and 2) the faster decline which occurs under starvation of the host. As shown earlier, the population level at any time, is a result of the processes of gain and loss of zooxanthellae. No information is available on changes in the rate of loss in darkness. An increase in the population results from cell division which has been shown to be influenced by the availability of nutrients. Zooxanthellae are clearly pre-adapted to survival within the host when autotrophic nutrition ceases. They have the ability to survive in hosts maintained in darkness for up to 160 days (Yonge & Nicholls, 1930b; Kevin & Hudson, 1979; Thorington & Margulis, 1981; Davies 1988). Presumably, during such times, the algae initially draw upon their own carbon reserves and reduce their internal amino acid pool. No information is available on how long these will sustain the symbionts' metabolic demands. However, under continued darkness, they must become dependent on a flux of nutrients from the host, and at this stage, could be regarded as intracellular parasites. Mechanisms available for heterotrophic feeding have been described, including specific transporters for amino acids (Carroll & Blanquet, 1984a). In *Chlorella* during darkness, McAuley demonstrated an inducible glucose uptake system. Since the algae are parasitic during sustained darkness, they constitute a metabolic cost to the host. This would explain why symbiotic *Hydra* grow more slowly in darkness than aposymbiotic *Hydra* when both groups were given equal feeding (Douglas & Smith, 1983). Similarly, Steen (1986) found that aposymbiotic *Aiptasia pallida* when starved in darkness suffered a lower mortality rate than symbiotic anemones treated similarly. The experiments described here on *A. viridis* show that the zooxanthellae benefit nutritionally from host feeding when they can not photosynthesise.

Rather unexpectedly, it was found that the rate of division of the zooxanthellae, as indicated by the mitotic index, did not decline significantly in darkness. Furthermore, there was no significant difference in the mitotic index of the algae from fed and starved hosts. The algae would be dependent on a continuous supply of nitrogen for cell growth and division to occur and this implies a flux of amino acids is available from the host. Thus the observation that zooxanthellae are dividing would indicate that a sufficient flux of nutrients was available to them.

From the foregoing it would appear that since the rate of division does not decline significantly during prolonged darkness, the fall in the zooxanthella population must be explained in terms of an increased rate of loss. The benefit to the host of reducing the metabolic burden of its intracellular parasites is obvious, but the mechanism is not known. It is possible that the rate of expulsion increases. Alternatively, digestion of zooxanthellae by the host has still not been investigated. Furthermore, during starvation in darkness, the rate of decline of the symbiont population is faster than when the anemones are fed. The metabolic burden would be greater during starvation, suggesting that the loss process may be directly related to relative metabolic costs of the algae in darkness.

Muscatine *et al* (1989) found that feeding in the light made no difference to the mitotic index of zooxanthellae from *Stylophora pistillata,* and concluded that the symbionts derived no nutritional benefit from host feeding. However, in the present experiments with *Anemonia viridis*, fed and starved animals maintained in darkness continued to divide for up to 66 days. In continuous darkness, the symbionts cannot photosynthesise and uptake of inorganic nutrients is prevented under prolonged absence of light (Wilkerson & Muscatine 1984; Davies 1988), suggesting, therefore, that the symbionts take up nutrients directly from the host cytoplasm. Trench (1987) suggested that the supply of organic and inorganic compounds from the host may be important to the survival of symbionts under light limitation, although by how much these compounds enhance algal growth is not known.

The division rate of the zooxanthellae in the dark may be enhanced when the symbiont population has been reduced, through decreased competition for nutrients as has been suggested happens in the light. A small symbiont population, as occurs after prolonged absence of light, would have a lower total nutritional requirement such that their total nutritional demand would be small and more easily supplied, thus allowing maintenance and division under prolonged darkness to continue in the remaining population within the host.

Since it appears that zooxanthellae must derive some benefit from host feeding, it would follow that in a starved animal, fewer metabolic host products would become available for the symbionts' use than in a fed animal, such that zooxanthellae in a starved animal may be nutrient limited (Falkowski *et al*, 1993). In a 12 hr light:12 hr dark cycle, the mitotic index of zooxanthellae from fed animals was higher than that of zooxanthellae in starved animals (section 4.3.2.3). However, the difference in the symbiont mitotic index between fed and starved animals over 66 days in darkness was not significant. This could, perhaps, be attributed to the large amount of variation in the mitotic index of zooxanthellae in both fed and starved animals. In fed animals this may have been due to differences in the amount and the nutritional content of food relative to the size of each animal. However, in the starved animals, the causes of variation in the mitotic index between animals would not be due to different sized food rations. Unexpectedly high mitotic index values of 10-13% were recorded in particular animals in the starved group maintained for 29 days in darkness. In starved animals the production of ammonium from host protein metabolism may be increased temporarily as the host may break down structural proteins for respiration. This may provide sufficient nitrogen for symbiont division and may partly explain the high zooxanthella mitotic index values in starved animals.

Evidence from comparisons of growth and mortality rates of aposymbiotic and symbiotic hosts in the dark with identical feeding or starvation suggest that the host is unable to prevent metabolic costs incurred by the symbionts. However, this evidence appears to be inconsistent with that from studies involving incubation of isolated zooxanthellae with host homogenates which suggest that the host has the ability to suppress or depress the passage of photosynthates and organic nutrients to symbionts (Carroll & Blanquet, 1984b), thereby controlling their growth rate.

A high proportion of the dividing cells after 32 days in darkness in both fed or starved samples with high division rates were tetrads. Trench (1993) reports the observation of tetrads in *Zoanthus* sp., *Xenia* sp. and *Tridacna maxima*. Tetrads may, perhaps, arise from an increase in the duration of cell plate formation and in the cell separation phase or a deficiency in certain substances necessary for complete cell synthesis. Experiments with the free living alga *Synechoccus* sp. showed that the duration of the paired cell stage increased significantly relative to other cell stages at slow growth rates, and that in darkness some cells arrested in the doublet stages (Vaulot, 1992).

6.4.2 Rate of population increase and rate of division of zooxanthellae in partially aposymbiotic hosts in the light.

The partially aposymbiotic anemones had been produced by maintenance in darkness for 66 days with feeding. The zooxanthella population had fallen to 40% of the initial level. After 35 days of exposure to the light, the population had regrown to 80% of the initial level. In the light the division rate, as indicated by the mitotic index, increased from a pre-dark treatment mean of 4.0% to a mean of 4.6%. This is not a big increase, therefore it seems likely that the population increase was achieved also in part by a reduction in the rate of symbiont loss.

The mitotic index of the symbionts from partially aposymbiotic hosts was significantly higher than those of symbiotic anemones maintained under identical conditions of light. This could be explained in terms of either increased intracellular space available to the symbionts of the partially aposymbiotic hosts or to lower competition for available inorganic nitrogen.

Aposymbiotic or near completely aposymbiotic *Aiptasia pulchella* can regain their normal population after a period of 3 to 7 weeks when inoculated with isolated zooxanthellae (Trench, 1971b; Kinzie & Chee 1979; Schoenberg & Trench, 1980). Berner *et al* (1993) followed the regrowth of the zooxanthella population in partially aposymbiotic *Aiptasia pulchella*, and estimated zooxanthella growth rates from changes in density to be 0.4 cells.cell^{-1}d^{-1} during the most rapid growth, and 0.02 cells.cell^{-1}d^{-1} after 3 weeks when the population was near the maximum reached (≡ normal growth rate of symbionts within symbiotic hosts). Uninoculated controls exposed to identical temperature and photoperiod exhibited relatively low rates of spontaneous repopulation of 0.08 cells.cell^{-1}d^{-1} between days 9 and 15. These calculated growth rates are much higher than those observed for zooxanthellae from partially aposymbiotic *Anemonia viridis* maintained both with and without ammonium enrichment which achieved growth rates of 0.018 and 0.0898 cells.cell^{-1}d^{-1} respectively. The former were kept at a temperature of 24±1°C whilst *Anemonia viridis* were maintained at 11±1°C and the higher temperature may partly explain the higher rate of regrowth.

When partially aposymbiotic hosts were incubated in 20μM ammonia at 300μE.m^{-2}.s^{-1}, zooxanthella population in tentacles increased to 140% of the pre-dark treatment levels. The mitotic index increased from 3% to 29% on day 4 and to 40% on day 8, before falling back to 9% by day 17.

The mitotic index and population of zooxanthellae in tentacles of symbiotic anemones increased during incubation in 20μM ammonium, as reported in earlier experiments (Chapters

3 and 4). However, throughout the 35 days of the experiment, the mitotic indices of algae in partially aposymbiotic anemones were significantly higher than those of symbiotic hosts.

When algal division rates in partially aposymbiotic hosts maintained in control and ammonium enriched sea water were compared, mitotic index values of zooxanthellae maintained in ammonium enriched sea water were significantly higher than those maintained in control sea water (Figure 6.6) As a result of the increased rate of division, the rate of repopulation of tentacles was significantly increased (Figure 6.7). In this comparison, the increased growth rate in 20μM ammonia is a direct indicator of limitation of algal growth by lack of nitrogen in the anemones maintained in unenriched sea water.

The above experiment, however does not enable a determination of which particular factor, of those discussed earlier, associated with low symbiont densities gives rise to higher symbiont division rates. Cook *et al* (1988) suggested that competition for nitrogen, produced from host metabolism, was greater at high symbiont densities and led to a suppression of zooxanthella growth rates. However, if competition for nitrogen was the only factor limiting to symbionts at high population density, as suggested by Cook *et al* (1988), then comparing growth rates of animals with a low and with a high symbiont density under ammonium enrichment would have produced a different result to that described above. Division rates of zooxanthellae within control symbiotic anemones maintained with ammonium enrichment were, in fact, still lower then those of aposymbiotic anemones under the same ammonium treatment, suggesting that nitrogen may not be the only limiting factor. In addition, ammonium enrichment dramatically increased the zooxanthella mitotic index within partially aposymbiotic anemones from a maximum of 5.8% in the group maintained without enrichment to 40% in the ammonium enriched group. This observation that ammonium enrichment can increase the mitotic index of zooxanthellae and can increase the rate of recovery of the zooxanthella population in partially aposymbiotic anemones compared to those without ammonium enrichment, suggests that the nitrogen supply from host catabolism to the zooxanthellae at a low density might still be limiting.

The pattern of mitotic index in partially aposymbiotic anemones maintained with ammonium enrichment was interesting. The mitotic index of zooxanthellae from ammonium enriched animals rapidly increased to 40% within 8 days of light treatment then decreased almost as rapidly within the next 9 days. The fall in division rate occurred when the population level was only 61% of the original number. In comparison, the highest rate of increase in cell numbers and cell density from counts of cell numbers (Table 6.11), occurred between 12 and

17 days after transfer in to the light. Thus, there was a delay of 6 days between the peak in mitotic index and the largest rate of increase in the zooxanthella population. During the time when the mitotic index was at a maximum, the population had not increased likewise. It is possible, that during the first 12 days of light, symbionts are being lost from the animal, either through expulsion or digestion.

Observations of tentacles from animals maintained in darkness showed that degeneration of zooxanthellae was occurring within host tissues of both starved and fed hosts, but particularly in the starved animals. Animals maintained in the dark with feeding contained zooxanthellae which were mostly healthy in appearance. Yonge & Nicholls (1930b) observed degenerating zooxanthellae *in situ* in corals starved in the light, implying that the degenerative process in the symbionts began before their expulsion from the host. The results of this study and that of Yonge & Nicholls (1930a) suggest that host starvation, whether in the light or in darkness, causes degeneration of symbionts within the host tissues but that host feeding enables the prolonged existence of zooxanthellae within the host.

Summary

1. 40% and 55% of zooxanthellae remained in tentacles of starved and fed anemones respectively, after 66 days incubation in continuous darkness. The final zooxanthella population densities in tentacles of fed and starved animals were similar, at 40% and 45% of initial levels respectively, after 66 days in continuous darkness.

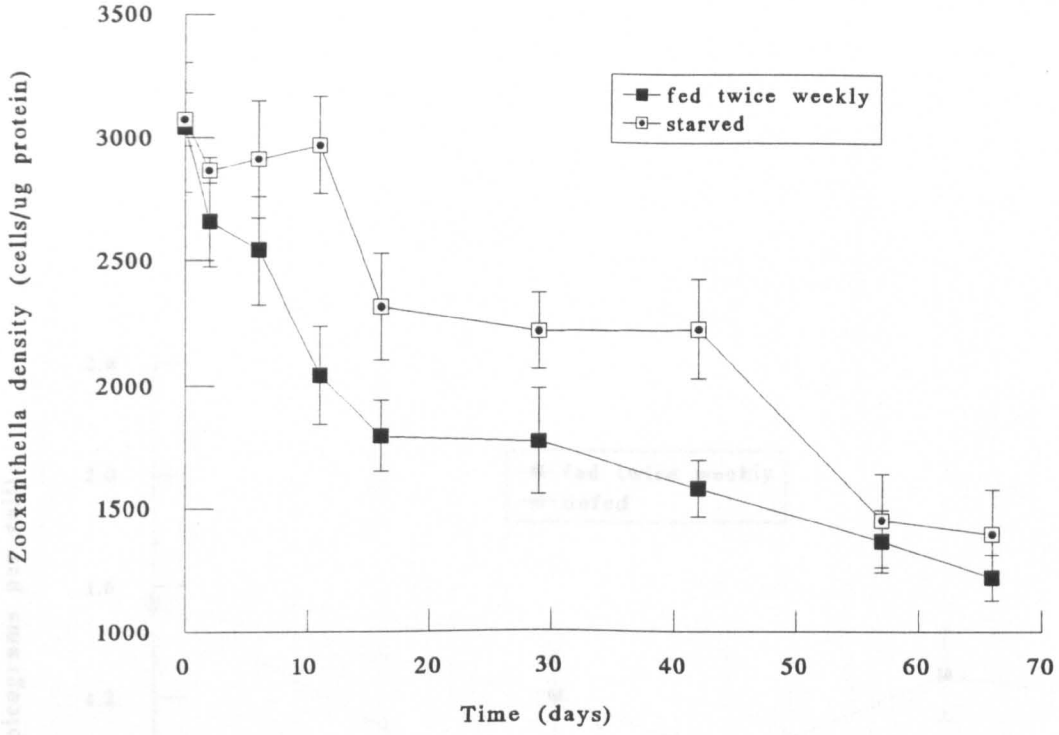
2. The gradual loss of colour in fed anemones in continuous darkness over 66 days, was due to a gradual loss of zooxanthellae, and not as a result of a decline in chlorophyll *a* per cell. In starved animals, the loss of colour was due to both the loss of zooxanthellae and a reduction in the amount of chlorophyll *a* per cell.

3. Division rates of symbionts from fed and starved animals, measured as the mitotic index, had not changed significantly over 66 days in continuous darkness. It is suggested that the zooxanthellae were deriving nutrients from the host to support this division in darkness.

4. Regrowth of zooxanthellae in tentacles of partially aposymbiotic anemones (with 55% of the initial zooxanthella population) maintained in the light for 35 days was enhanced by the addition of ammonium to the sea water.

5. Zooxanthella growth rates in partially aposymbiotic anemones were greater than those in symbiotic anemones when both groups were maintained with ammonium enrichment and these results suggest that nitrogen is not the only factor limiting to zooxanthella growth at high symbiont population densities.

a)



b)

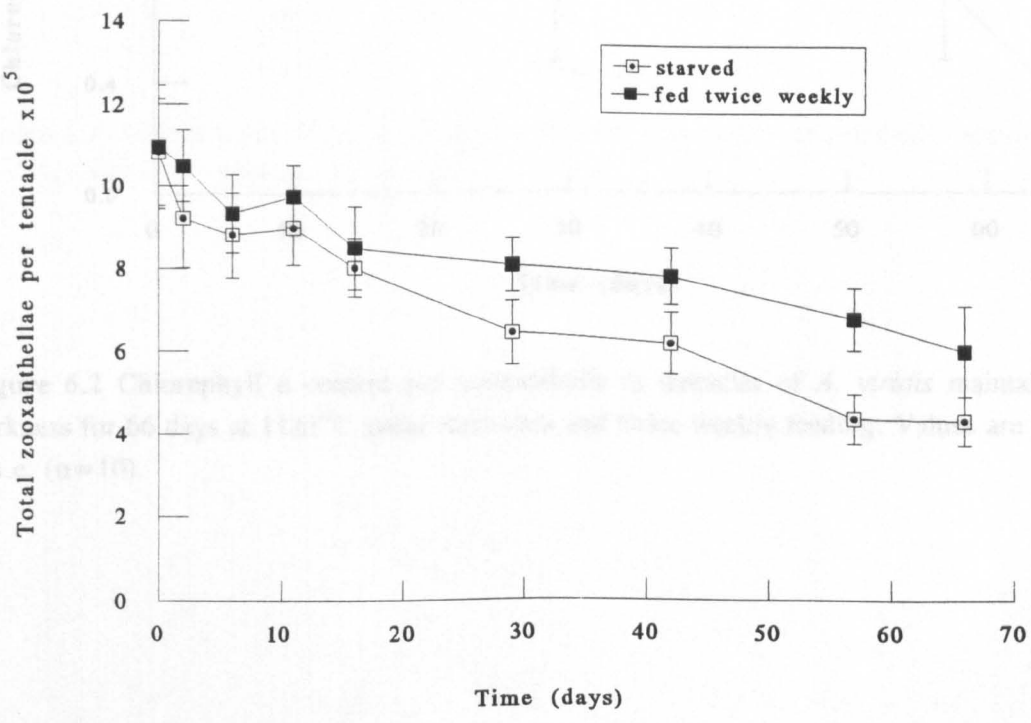


Figure 6.1 Changes in a) population density and b) total numbers of zooxanthellae in tentacles of *A. viridis* maintained either starved or fed twice weekly for 66 days in darkness. Values are means ±s.e. (n=10).

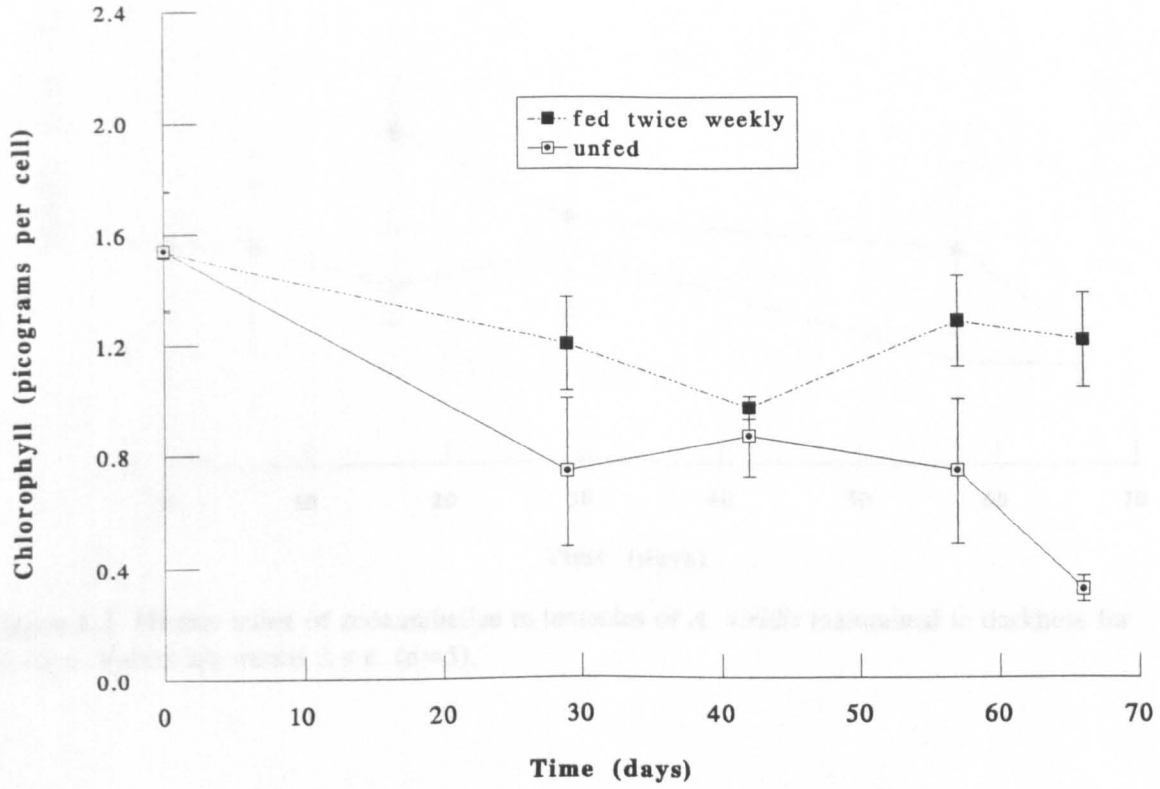


Figure 6.2 Chlorophyll *a* content per zooxanthella in tentacles of *A. viridis* maintained in darkness for 66 days at 11±1°C under starvation and twice weekly feeding. Values are means ± s.e. (n=10).

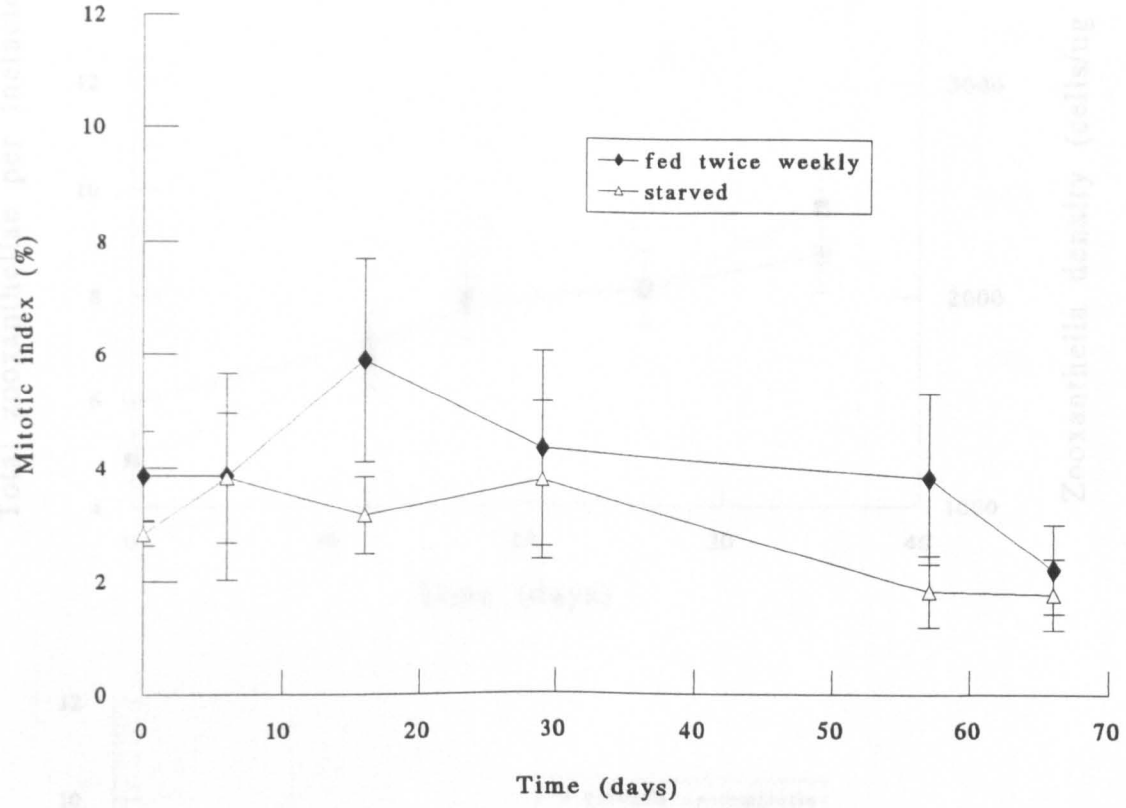
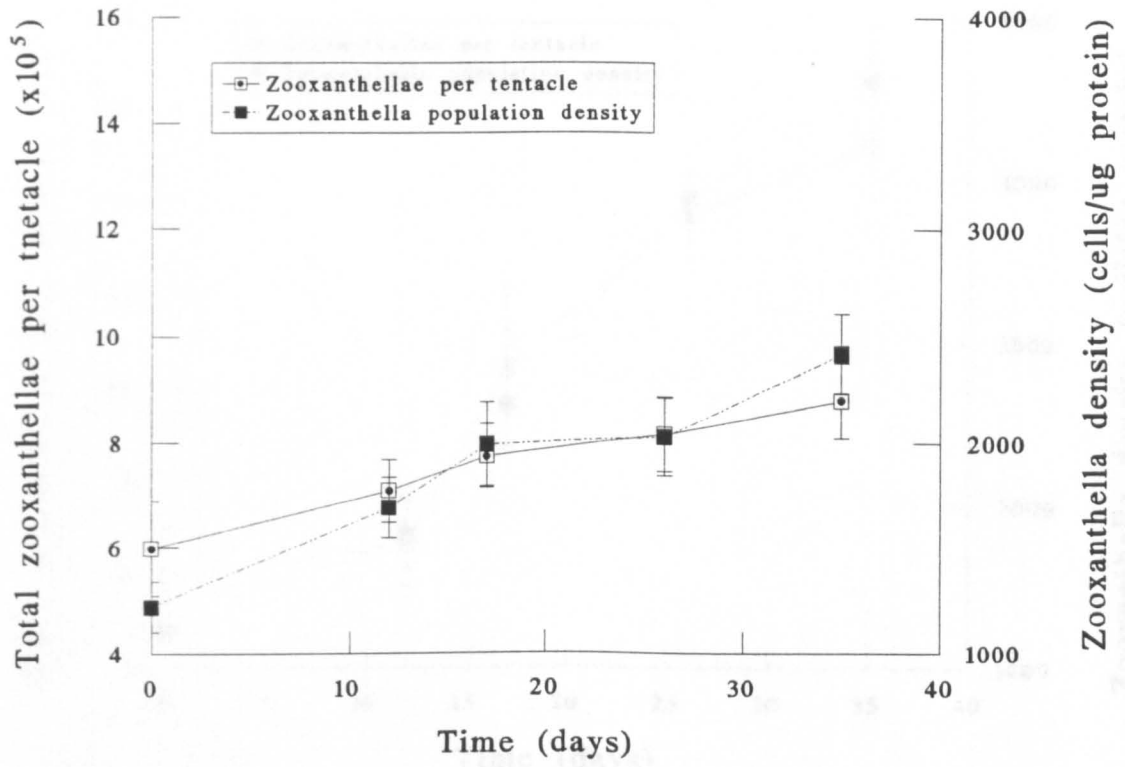


Figure 6.3 Mitotic index of zooxanthellae in tentacles of *A. viridis* maintained in darkness for 66 days. Values are means ± s.e. (n=5).

a)



b)

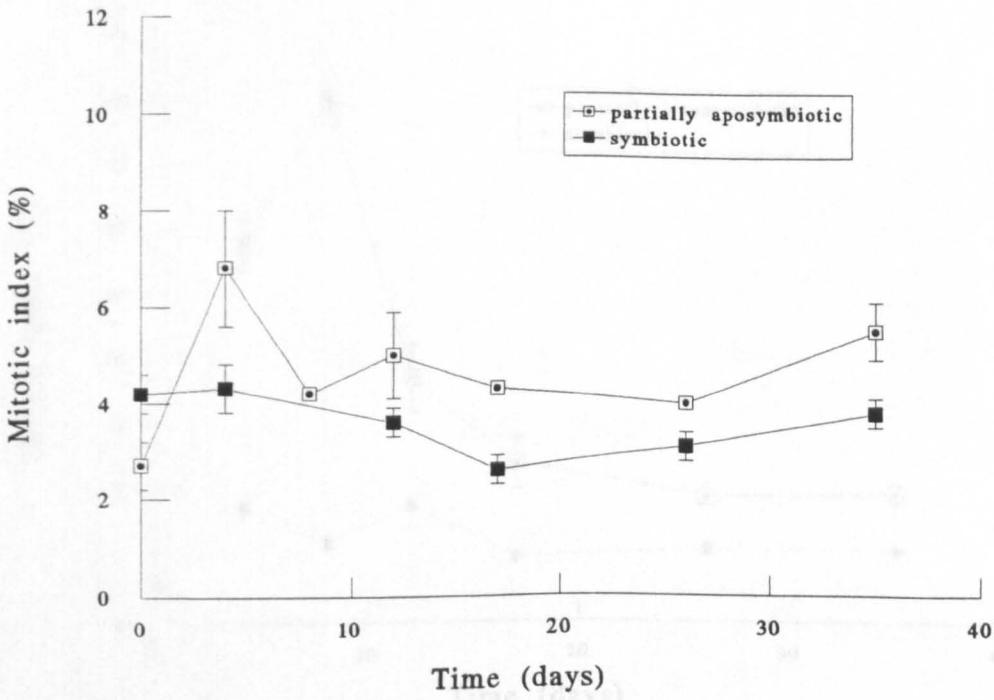
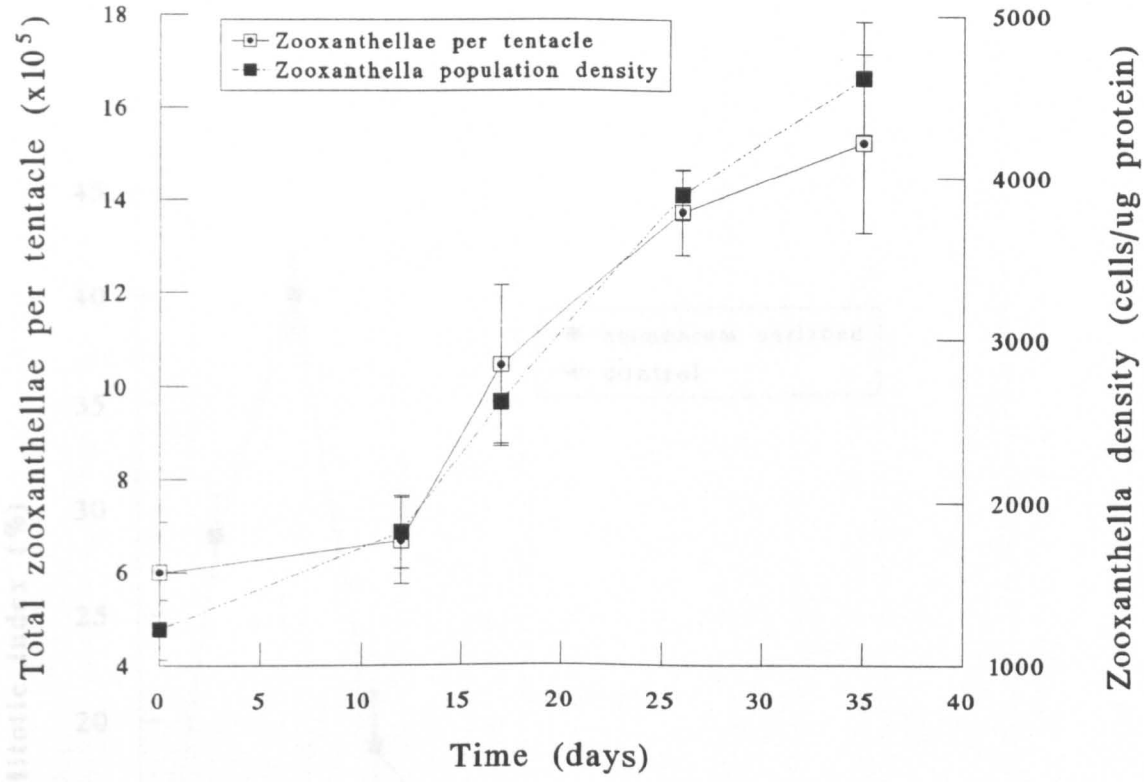


Figure 6.4 a) Changes in zooxanthella population density and zooxanthellae per tentacle in partially aposymbiotic *A. viridis* ± s.e (n=10) and b) mitotic index ± s.e. (n=5) in tentacles of partially aposymbiotic and symbiotic *A. viridis*. Animals were maintained for 35 days at $300\mu E.m^{-2}.s^{-1}$ at $11\pm1^{o}C$ in control sea water without feeding. Partially aposymbiotic hosts had been maintained in darkness for 66 days with weekly feeding. Symbiotic hosts had been maintained at $300\mu E.m^{-2}.s^{-1}$ with feeding.

a)



b)

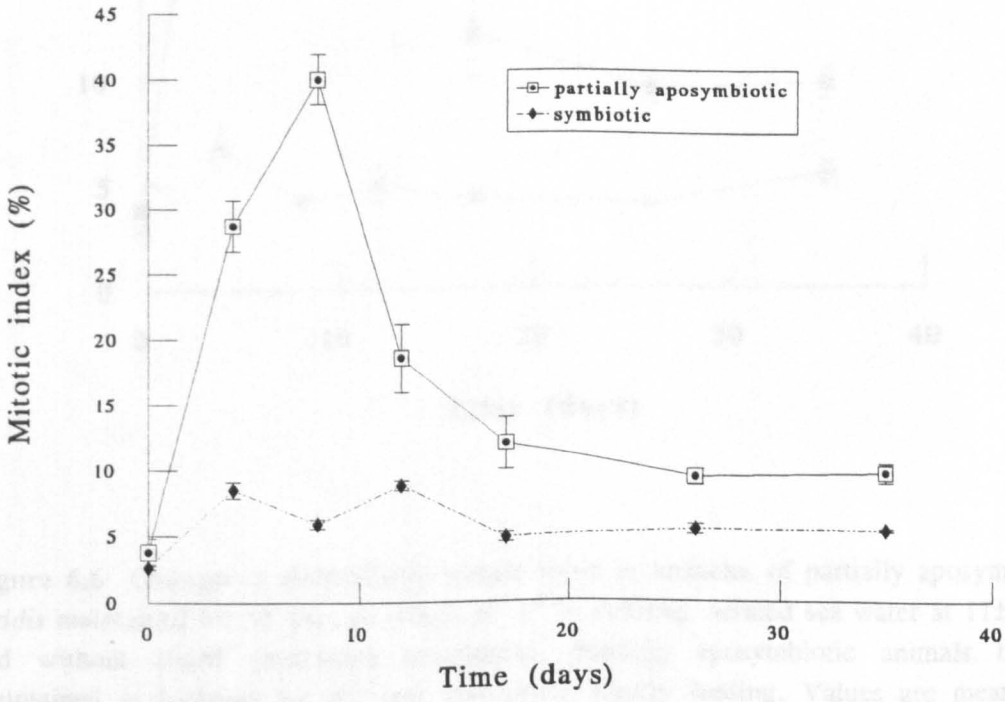


Figure 6.5 a) Changes in zooxanthella population density and zooxanthellae per tentacle in partially aposymbiotic *A. viridis* ± s.e (n=10) and b) mitotic index ± s.e. (n=5) in tentacles of partially aposymbiotic and symbiotic *A. viridis*. Animals were maintained for 35 days without feeding at 300μE.m^{-2}.s^{-1} at 11±1°C in sea water enriched to 20μM ammonium. Partially aposymbiotic hosts had been maintained in darkness for 66 days with twice weekly feeding. Symbiotic hosts had been maintained at 300μE.m^{-2}.s^{-1} with feeding.

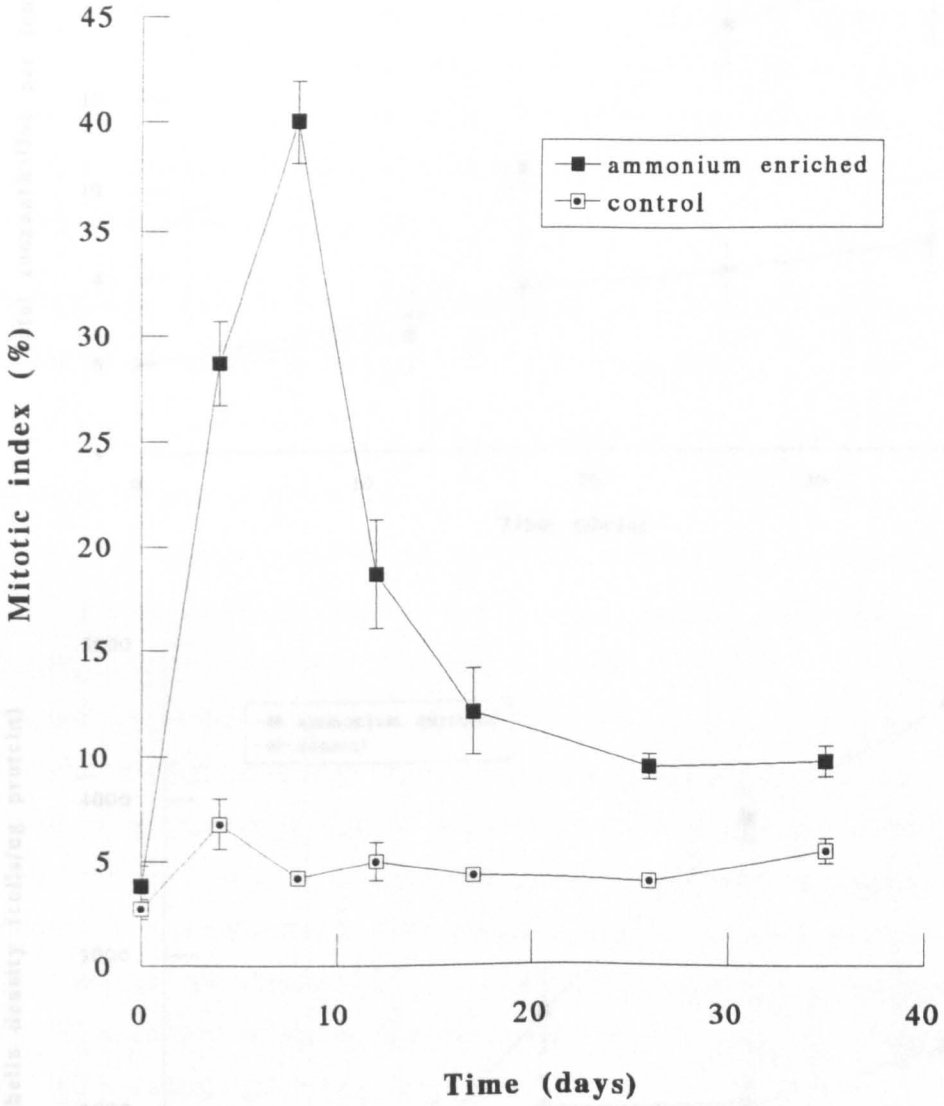
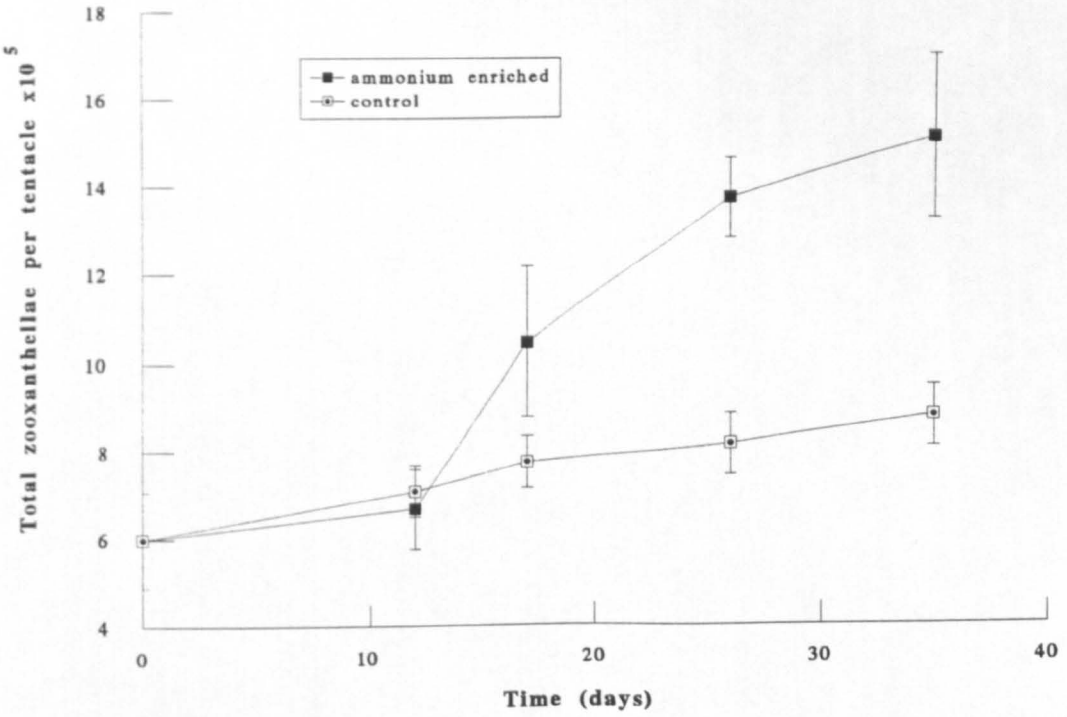


Figure 6.6 Changes in zooxanthella mitotic index in tentacles of partially aposymbiotic *A. viridis* maintained for 35 days at 300μE.m^{-2}.s^{-1} in running, aerated sea water at 11±1°C with and without 20μM ammonium enrichment. Partially aposymbiotic animals had been maintained in darkness for 66 days with twice weekly feeding. Values are means ± s.e. (n=5).

a)



b)

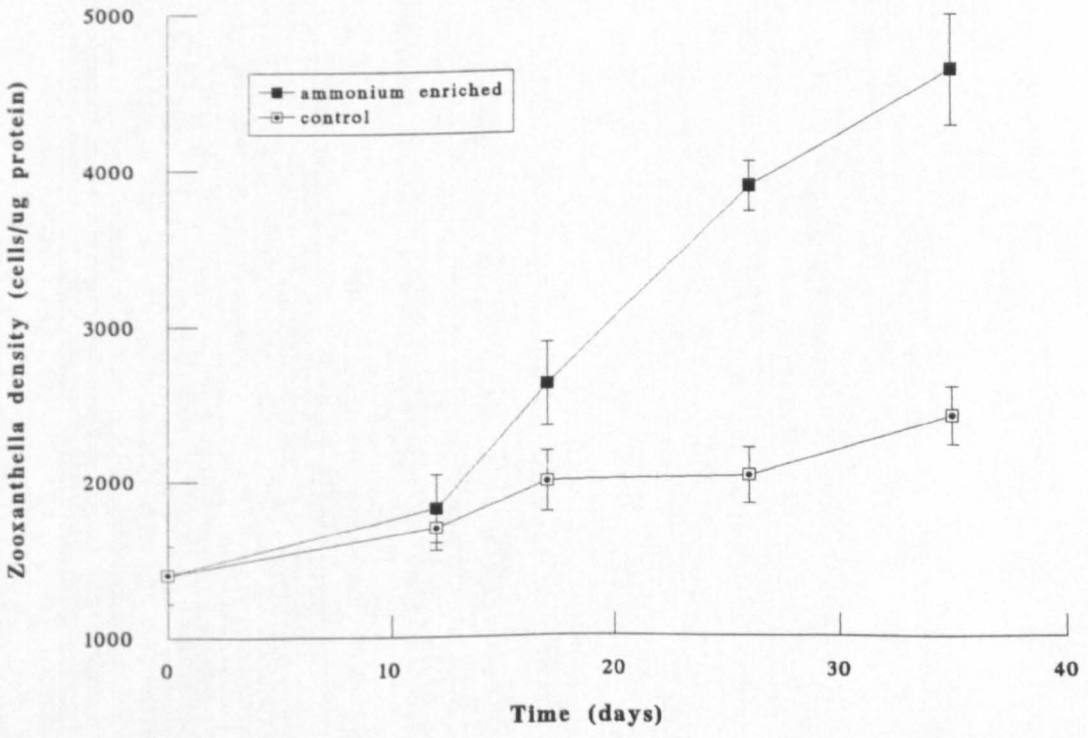


Figure 6.7 Changes in a) zooxanthellae per tentacle and b) zooxanthella population density in partially aposymbiotic *A. viridis* maintained for 35 days at 300μE.m^{-2}.s^{-1} at 11±1°C in control sea water or sea water enriched with ammonium to 20μM. Partially aposymbiotic animals had been maintained in darkness for 66 days with twice weekly feeding. Values are means ± s.e (n = 10).

CHAPTER 7

THE CALCULATION OF SPECIFIC GROWTH RATES.

In chapter 4, the mitotic index was used to investigate the relative effect of ammonium and light intensity on the division rate of zooxanthellae *in hospite*. To attempt a direct comparison with expulsion rates, the mitotic index needed to be converted to a specific growth rate. However, the estimation of population growth rates from mitotic index values, based on previous published methods (see Wilkerson *et al*, 1983) proved to be problematic when dealing with symbiotic associations.

Phytoplankton biologists use a relationship based on the observed mitotic index to calculate μ, the specific growth rate derived by McDuff & Chisholm (1982):

$$\mu = 1/t_d * \ln (1 + f) \qquad (I)$$

where μ has units of cells.cell^{-1}d^{-1},

t_d = duration of nuclear and cell division ending in cell separation (days)

f = number of dividing cells as a fraction of the total number of cells at any one time (mitotic index). For derivation of equation see Appendix 3.

However, very often the duration of t_d is unknown and the value of f for some species of phytoplankton may be zero, at certain times of the day, if division is synchronous. A simpler method of determining μ could be devised from the above equation if the population does show synchronous division. A synchronously dividing population is recognisable as a plateau shaped curve when the mitotic index, measured every 1 or 2 hours over a 24 hour period is plotted against time. From a plot of this type, it can be seen that the maximum mitotic index recorded is likely to include all cells undergoing division in one day, providing the time taken for a cell to undergo division (t_d) is less than 1 day.

Thus, equation (I) can be simplified to equation (II):

$$\mu = 1 / t * \ln (1 + f_{max}) \qquad (II)$$

where t = total sampling duration in days. Since consecutive measurements of the mitotic index are usually taken over 1 day, t usually equals 1 day.

f_{max} = maximum number of cells undergoing division as a fraction of the total and is assumed to include all cells undergoing division in one day.

By making t = 1 day, this is assuming that the duration of cell division (t_d) is less than 1 day and that cells do not undergo more than one division in a day.

Alternatively, μ can be estimated from sequential observations

$$\mu = (1/n*t_d) \sum_{i=1}^{n} \ln(1+f_i) \qquad \text{(III)}$$

where n = number of consecutive samples (for derivation see McDuff & Chisholm, 1982).

Vaulot (1992) considered the problem for cases when the assumptions stated above were not valid. He derived an equation (IV) to calculate μ when $t_d \geq 24$ hours in, for example, slow growing algae, and when division is only moderately phased.

$$\mu = 1/t * \ln [(1+f_{max})/(1+f_{min})] \qquad \text{(IV)}$$

where f_{max} = maximum fraction of cells undergoing division in one day

f_{min} = minimum fraction of cells undergoing division in the same day

Asynchronous division

For the f_{max} approach (equation II) to be true it must be the case that all dividing cells can be recognised in at least one of the consecutive samples taken over that day. This approach is therefore, only applicable to populations showing moderately synchronous division where the cycle can be clearly divided into dividing and non dividing periods, where $t_d < 1$ day, and when cells do not undergo division more than once in a day. Dinoflagellate symbionts *in hospite*, on the whole, do not appear to show this type of division pattern (section 4.4.1). Two notable exception to this are the symbiont populations of *Mastigias* sp. (Wilkerson *et al*, 1983) and *Myrionema ambionense* (Fitt & Cook, 1989) which both displayed synchronous division.

Fitt & Trench (1983a) observed that *Symbiodinium microadriaticum* displayed synchronicity in their division when cultured in a 14hr:10hr light:dark cycle. However, they showed that the daily division pattern changed as the cultures aged, such that the alternating pattern of peaks of division and troughs of no division, occurring during the exponential growth phase, became less pronounced as the population entered the lag or stationary phase. In the lag phase, division appeared to be continuous throughout the day. This may be as a result of an increase in the duration of cell division to a period greater than 24 hours (the entraining period).

The patterns of mitotic index of symbionts from *Anemonia viridis* observed in this study, did not show division patterns with distinct peaks under all conditions measured, since dividing cells were present in variable numbers throughout the 24 hour period (section 4.3.1),

suggesting that division was either asynchronous or that the duration of t_d was much larger than that of zooxanthellae from *Mastigias sp.*

As cycles become less tightly phased or as t_d becomes close to or greater than 24 hours, estimates of specific growth rates, based on equation (II), may become less accurate. For *Anemonia viridis*, therefore, a knowledge of the magnitude of t_d is required to enable calculations of μ using equation (I).

Estimation of t_d

McDuff & Chisholm (1982), in their derivation of the specific growth rate, defined t_d as the combined duration of mitotic stages ending in cell separation. Simplified, these stages consist of a nuclear division phase followed by cytokinesis which involves the development of a cell plate between the two newly formed cells before separation occurs. However, techniques to measure the mitotic index (f) differ as to which of these stages of dividing cells are identified. The widely adopted "doublet" method only counts cells as dividing if cell plates are visible - a feature which appears towards the end of cell division. Estimates of specific growth rates of dinoflagellate symbionts have all been based on mitotic index counts of doublet cells (e.g. Wilkerson *et al*, 1983). Estimates of t_d based on this method are really only estimates of the period when the cell plate is visible and may, therefore, be underestimating the total duration of mitosis. Use of nuclear stain, such as Feulgen's, allows the additional identification of cells with 2 nuclei. This stage occurs earlier in the division cycle, and would, therefore, improve the estimate of t_d.

Weiler & Chisholm (1976), estimated t_d for phytoplankton populations by measuring the time elapsed between the point at which an increase in number of cells with 2 nuclei occurs and the time when the number of 'doublet' cells begins to decrease (the time when the number of recently divided cells appears). This method, however, is only applicable to populations displaying moderately phased or tightly phased division. Therefore, an alternative approach was required to estimate t_d for the population of symbionts in *A. viridis*.

The application of the Feulgen's stain to zooxanthella populations of associations where synchronous division patterns can be demonstrated, would enable estimates of t_d to be made using the method above. Estimates made in this way may improve the accuracy of growth rate estimations in those symbiotic populations which do display synchronous division. This method may also be applied to symbiotic associations to investigate the effects of environmental factors on the duration of t_d of zooxanthella populations *in hospite*.

Wilkerson *et al* (1983) calculated the duration of t_d for the zooxanthellae of *Mastigias* sp., which displayed synchronous division. They then used this value of t_d to calculate μ for zooxanthellae in other host species using equation (I). Firstly, the specific growth rate of symbionts from *Mastigias* was obtained using equation (II) from counts of cells with cell plates, and substituted into equation (III) to obtain t_d. This value of t_d (11 hours) was then used in equation (I) to estimate growth rates of symbionts from *Stylophora pistillata* and *Aiptasia pulchella*. Specific growth rates have been estimated for a variety of species, whose zooxanthella populations show asynchronous division, by using the above value of t_d, and making the assumption that it is representative of symbiotic populations in other host species. This value for t_d has been used subsequently by several workers (Muscatine *et al*, 1984; Hoegh-Guldberg *et al*, 1987; Cook *et al*, 1988; Wilkerson *et al*, 1988; Muscatine *et al*, 1989).

Stambler & Dubinsky (1987) found that the pattern of division of symbionts within *Anemonia viridis* maintained at 22°C was synchronous over 24 hours, and calculated a value for t_d of 15.5 hours using the above method of Wilkerson *et al* (1983) based on counts of cell plates to obtain the mitotic index (f).

Changes in the cell cycle induced by environmental variables.

The application of the value for t_d of 11 hours for zooxanthellae from *Mastigias* sp., to calculating μ for zooxanthellae from other host species (e.g. Muscatine *et al*, 1984; Hoegh-Guldberg *et al*, 1987; Cook *et al*, 1988; Wilkerson *et al*, 1988) is based on the assumption that t_d does not vary between host and symbiont species, and under different environmental conditions. There is some evidence from phytoplankton studies to suggest that t_d is not constant and can vary with temperature (Olson *et al* 1986), generation time and light (Weiler & Eppley, 1979; Vaulot, 1992). Smith & Hoegh-Guldberg (1987), however, reported that differences in growth rates were not due to changes in the duration of cell division. They suggested that growth rates were controlled by changes in the duration of the G1 phase which occurs before DNA synthesis. They did, however, find evidence to suggest that t_d varies between host species (Smith, & Hoegh-Guldberg, 1987).

Estimations of t_d from division patterns of *S. microadriaticum* in different phases of the culture cycle, made by Fitt & Trench (1983a), suggest that the duration of t_d increases beyond 24 hours as cultures reach the lag phase.

Brown & Zamani (1992) reported that the mitotic index, measured from nuclear counts, was 3-5 times the mitotic index based on counts of cells with cell plates and inferred from this that the duration of the two phases of cell division did not change in relation to each other.

Brown & Zamani (1992) suggested that nuclear division was not necessarily linked to cell plate formation and cell division, and that different stages of the cell cycle were susceptible to certain environmental factors. Data from the present study show that the proportion of cells with 2 nuclei relative to the proportion of cells having cell plates is not constant (Appendix 4), indicating that one or both of the two phases of the cell cycle change in relation to each other. Under ammonium enrichment, there was a significantly greater proportion of dividing cells showing two nuclei (Appendix 4 graphs 4a and 4e).

Vaulot (1992) found that the duration of the paired cell stage in *Synechoccus* increased significantly relative to other cell stages at slow growth rates. Some cells arrested in G2 and in the doublet stages in darkness (Vaulot, 1992). Calculations based on consecutive mitotic index measurements of cultured symbionts from *Aiptasia tagetes*, made by Fitt & Trench (1983a), showed that when cultured symbionts were maintained in continuous darkness, the duration of t_d increased as division became less phased and more continuous throughout the day. In *Gymnodinium nagasakiense*, however, the duration of mitosis was found to be proportional to the generation time (Videau & Partensky, 1990).

If t_d does vary with environmental conditions, then its value would need to be determined under each different set of environmental conditions used before assumptions can be made in the calculation of specific growth rates.

In this study, high mitotic index values of above 20% measured under ammonium enrichment, coincided with the appearance of large numbers of tetrads (cells with 4 nuclei or 4 cell plates). Incubation in continuous darkness for 29 days (section 6.3.1) also resulted in the appearance of large numbers of tetrads. These tetrads, also observed by Trench (1993), may arise as a result of a shorter duration of nuclear division relative to cell division, or a decrease in the duration of the gap phases G1 and G2, such that the second nuclear division of daughter cells precedes cytokinesis of the daughter cells.

Measuring the mitotic index of unphased populations.

Brown & Zamani (1992) suggested that the use of a nuclear stain to count dividing cells may be preferred to counting cells with cell plates in unstained samples of zooxanthellae. They pointed out that in unstained samples, the occurrence of two non-dividing cells slightly

overlapping be mistaken for a cell showing cell plate formation. Thus, the use of Feulgen's stain in determining mitotic index may reduce the frequency of misidentification.

Errors may be introduced into calculations of specific growth rate for zooxanthellae under particular conditions if a value of t_d is used which was obtained from hosts kept under different conditions. For example, the value of t_d estimated under a certain set of conditions may be underestimated in different conditions where the doublet phase may be prolonged under certain circumstances (e.g. the effect of darkness on *Synechoccus* sp.), and this will lead to an overestimate of μ.

An additional problem is that of identification. Some cells, while appearing to have two nuclei, may also show signs of cell plate formation making this method subject to error in identification and interpretation. In the present study, peaks in the frequency of cells with 2 nuclei, cells with cell plates and recently divided cells were not evident.

The value of t_d will be different according to which stages in the cell division process were identified. Thus the value of t_d derived from counts over 24 hours of doublet cells carried out on unstained samples will be different to that derived from counts over 24 hours of stained cells in which the nuclei are visible. If t_d has been derived by one method it cannot be then applied to calculate specific growth rates where the mitotic index has been estimated using a different method.

Ideally, the stage which is least variable in duration of the replicative process would give the most accurate estimate of the specific growth rate.

Calculation of growth rates.

In this study, several methods have been used to calculate specific growth rates from mitotic index data obtained from section 5.3.3 in Chapter 5. All calculations for specific growth rate depended on an estimate of t_d (McDuff & Chisholm, 1982: Appendix 3). Since division of *Anemonia viridis* in this study did not show a definite pattern over 24 hours (section 4.4.1), the mitotic index data of Stambler & Dubinsky (1987) and Suharsono (1990) were used to calculate t_d. These two studies both record mitotic index data, measured as the proportion of doublet cells, for *A. viridis* over 24 hours and found synchronous patterns of division with distinct maximum and minimum values. The former study was carried out at 22°C, while the latter was carried out at 15°C. Since the two studies obtained mitotic index values from counts of unstained cells (i.e. doublet cells only), the following estimates of specific growth rate could only be made from mitotic index data obtained in this study (Table 7.2 and 7.3) of the

proportion of stained cells appearing as doublets, since the duration of t_d calculated here is specific to the doublet cell stage.

t_d was estimated using two methods, both of which were based on mitotic index values measured over 24 hour periods from the studies mentioned above:

Method 1

In estimates (1) and (3), f_{max} values derived from 24 hour mitotic index data from Stambler & Dubinsky (1987) and Suharsono (1990) respectively were substituted into the equation:

$$\mu = 1 / t * \ln (1 + f_{max}).$$

The value of μ obtained was substituted into the equation for sequential observations:

$$\mu = (1/n*t_d) \sum_{I=1}^{n} \ln(1+f_i)$$

and solved for t_d.

Method 2

In methods (2) and (4), f_{max} and f_{min} derived from 24 hour mitotic index data from Stambler & Dubinsky (1987) and Suharsono (1990) respectively were substituted into the equation:

$$\mu = 1/t * \ln [(1+f_{max})/(1+f_{min})] \qquad \text{where } t = 1 \text{ day}$$

The value of μ obtained was substituted into the equation for sequential observations given above and the equation was solved for t_d.

Table 7.1 Values for the f_{min} and f_{max} and the duration of t_d estimated from mitotic index counts measured over 24 hour periods. Mitotic index values were obtained by counting the number of doublets (cells with cell plates). † from McDuff & Chisholm (1982) ‡ from Vaulot (1992).

Method used to calculate μ	Mitotic index data from Suharsono (1990)			Mitotic index data from Stambler & Dubinsky (1987)		
	f_{max}	f_{min}	t_d	f_{max}	f_{min}	t_d
$\mu = 1/t * \ln (1+f_{max})$ †	0.045	0.012	13.7 h	0.03	0.005	15.5 h
$\mu = 1/t * \ln [(1+f_{max}) / (1+f_{min})]$ ‡	0.045	0.012	18.8 h	0.03	0.005	17 h

where: f_h = mitotic index based on counts of cells with cell plates only (expressed as a decimal)
f_{max} = maximum mitotic index from counts from samples taken every 2 h over 24 hours.
t_d = duration of cell division
f_{min} = minimum mitotic index value observed in 24 hour period used by Vaulot (1992).

Mitotic index data measured over 24 hours under ammonium enrichment are not available therefore the calculated values of t_d were applied to both control (Table 7.2) and ammonium enriched (Table 7.3) animals.

The values of t_d obtained were then used in the equation;

$$\mu = 1/t_d * \ln (1 + f)$$

to calculate growth rates for data recorded in this study.

Table 7.2 and 7.3 show estimates of growth rates from mitotic index data (from section 5.3.3) for animals maintained in sea water enriched with ammonium and control sea water.

Table 7.2 Table of growth rates, estimated using each of 4 methods, based on the mean mitotic index from counts of 4 samples taken from tentacles from each animal. Animals were maintained at 11±1°C and at a light level of 300μE.m^{-2}.s^{-1}. Samples were taken over 21 days. The doubling time range was estimated using the highest and lowest values of μ. (1) (2) (3) and (4) correspond to the 4 methods used.

control	mean mitotic index ($x10^2$)		μ (1) t_d =15.5h	μ (2) t_d =17h	μ (3) t_d = 13.7h	μ (4) t_d = 18.8h	doubling time (d) of population
Animal	f_{a+b}	f_b	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	
1	7.46	4.00	0.061	0.055	0.069	0.05	10-14
2	5.95	3.45	0.053	0.048	0.059	0.043	12-16
3	4.82	2.68	0.041	0.037	0.046	0.034	15-20
4	5.13	2.97	0.045	0.041	0.051	0.037	14-19
5	5.82	3.56	0.054	0.049	0.061	0.045	11-15

Table 7.3 Table of estimated specific growth rates. Animals were maintained in sea water enriched to 20μM ammonium at 11±1°C and at a light level of 300μE.m^{-2}.s^{-1}.

	mean mitotic index (%)		μ (1) t_d =15.5h	μ (2) t_d =17h	μ (3) t_d = 13.7h	μ (4) t_d = 18.8h	doubling time (d) of population
Animal	f_{a+b}	f_b	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	$\ln(1+f_b)/t_d$	
6	8.60	4.37	0.066	0.060	0.075	0.055	9-13
7	7.18	3.85	0.058	0.053	0.066	0.048	10.5-14
8	10.6	4.56	0.069	0.063	0.078	0.057	9-12
9	7.74	3.70	0.056	0.051	0.064	0.046	11-15
10	8.28	4.4	0.067	0.061	0.075	0.055	9-12.5

The values for specific growth rates given in Tables 7.2 and 7.3 will be compared with expulsion rates, measured in the same animals, in Chapter 8.

All estimates of t_d given in Tables 7.1 are greater than those estimated from zooxanthellae of *Mastigias* sp. by Wilkerson *et al* (1983) of 11 hours, and of *Myrionema amboinense* of 9 hours calculated from data of Fitt & Cook (1989), and Smith & Hoegh-Guldberg (1987) who estimated a value of 8.8 hours for zooxanthellae in *Stylophora pistillata*. A value of 14.7 hours was estimated for zooxanthellae from *Pocillopora damicornis* by Smith & Hoegh-Guldberg (1987).

By using f_{min} in the calculation, the value of t_d was increased which led to lower specific growth rates.

CHAPTER 8

DISCUSSION

It has often been reported that the symbiont population density or host:symbiont biomass ratio within cnidarians remains stable under constant conditions, and that when these conditions change, this ratio changes. This phenomenon has been demonstrated in the *Hydra-Chlorella* association, where the numbers of symbionts per digestive cell were counted (Muscatine *et al*, 1975; Muscatine & Pool, 1979; Smith, 1987), but less frequently in anthozoans.

Environmental conditions under natural circumstances, however, are rarely constant. Under certain conditions the biomass ratio may move away from the "optimum" and become disadvantageous to the host. For example, when symbiotic *Hydra* was maintained with nitrogen enrichment under constant light, the symbiont population density, measured as numbers of *Chlorella* per host digestive cell, increased dramatically and led to the death of the host (Blank & Muscatine, 1987). In most cases, however, the effects of a change in the symbiont:host biomass ratio are more gradual and not quite so dramatic. A high symbiont population density, resulting from ammonium enrichment, was correlated with a decline in host skeletal growth (Stambler *et al*, 1991). Symbiont population density was found to be inversely related to chlorophyll content and gross photosynthetic capacity per symbiont (Hoegh-Guldberg & Smith, 1989b; Falkowski *et al*, 1993), such that at high symbiont population densities, the photosynthetic rate per symbiont was reduced and the host would receive less translocated fixed carbon per symbiont. When symbiotic and aposymbiotic *Hydra* were maintained in continuous darkness and fed equal amounts, the *Chlorella* symbionts became a metabolic cost to the host, and as a result, symbiotic cnidarians grew more slowly than aposymbiotic cnidarians (Douglas & Smith, 1983). In several symbiotic associations maintained under prolonged darkness, however, the population of symbionts is gradually reduced and despite their initial metabolic cost, the host is able to live and grow whilst maintaining a very small symbiont population, provided it is given a supply of food.

Despite changing environmental conditions, however, the host:symbiont relationship is generally a stable, albeit, dynamic, one. For example, when *Anemonia viridis* was starved in the light, the animals lost weight but there was a corresponding fall in the zooxanthella population, such that the density did not change significantly (Chapter 3). In addition, a change in light intensity did not bring about a change in the zooxanthella population density in *A. viridis* (Chapter 3). Several studies, including this one (see Chapter 6), have demonstrated that the observed ratio of symbiont:host biomass can be restored in experimentally induced aposymbiotic hosts if they are returned to conditions in which they were previously maintained (Schoenberg & Trench, 1980; Berner *et al*, 1993).

It can be seen that an increase or decrease in the symbiont population density due to changing environmental conditions can be brought about by changes either in the zooxanthella population or in the host biomass. A change in the population may occur due to changes in division rates or expulsion rates or both. The equation below illustrates this idea that the steady state symbiont population is determined by the inputs and losses to the population.

Rate of change in the zooxanthella population = (division rate x population) - (rate of loss x population)

This study investigated the effects of different environmental variables on the association between *Anemonia viridis* and its symbionts and attempted to follow the changes in the zooxanthella population and the above rates.

Zooxanthellae in tropical symbioses live in a high light: low nitrogen environment and are therefore considered to be nitrogen limited (Cook & D'Elia, 1987) and thought to obtain nitrogen from host metabolism to meet their requirements (Rahav *et al* 1989; Falkowski *et al*, 1993). The evidence for the nitrogen limitation hypothesis came initially from the observations that there was a significant increase in the symbiont population density in corals after 2 weeks of ammonium enrichment (Muscatine *et al*, 1989; Stambler *et al* 1991; Muller Parker *et al* 1994). Other studies, however, report that nitrogen enrichment did not have a big effect on population densities (Hoegh-Guldberg & Smith 1989b; Stambler *et al* 1994).

The symbiont population density in tentacles of *Anemonia viridis* increased after 14 days exposure to 20μM ammonia, although the magnitude of the increase was small relative to other studies. However, in a longer term experiment, the zooxanthella population density normalised to animal protein and total dry weight, in *A. viridis* maintained with ammonium enrichment for 80 days increased only temporarily, before decreasing back to pre-experimental levels. The change in the population density was a result of changes in the total zooxanthella population, host protein content and animal weight. Overall, the zooxanthella population had increased by 4% while the host weight had increased by 11% and host protein content had increased by 19%

The studies of Muscatine *et al*, 1989, Stambler *et al* 1991, and Muller Parker *et al* 1994, which showed an increase in zooxanthella population density under ammonium enrichment, measured symbiont densities as cells per coral surface area, while the studies of Hoegh-Guldberg & Smith 1989b and Stambler *et al* 1994, measured population densities as cells per mg host protein. Normalising to coral surface area or host protein content does not take into account total animal tissue growth. Using the former normalisation does not give any indication of an increase in the size or depth of polyp, while using the latter assumes that the proportion of host biomass made up of protein remains constant.

With the exception of Belda *et al* (1993) who carried out a study on *Tridacna gigas*, and the present work, no other studies have measured growth of host animal soft tissue. Belda *et al* (1993) reported that ammonium enrichment over a 3 month period led to a doubling in host tissue weight and a similar increase in the zooxanthella total population, such that the symbiont population density measured as cells per tissue weight did not change significantly. When the data of Muscatine *et al* (1989) for host protein concentration of *Stylophora pistillata* were used to calculate population densities per unit host protein, there was no effect of ammonium enrichment when compared with control colonies. Similarly, Muller Parker *et al* (1994) found that when population densities were measured per unit host protein there was no significant difference between the population density of ammonium enriched and control corals after 4 weeks. Discrepancies in the two methods of measuring population densities in corals suggest that there are differences in the rate of animal tissue growth relative to skeletal growth under ammonium enrichment, such that ammonium enrichment may not affect the symbiont:host biomass ratio, but does affect the host soft tissue weight:skeletal weight ratio, and hence symbiont weight: skeletal weight ratio.

The studies carried out by Muscatine *et al* (1989) on *Stylophora pistillata* and Belda *et al* (1993) on *Tridacna gigas* report an increase in host protein concentration per coral surface area and per host weight respectively in animals supplied with ammonium enriched sea water. Furthermore, under ammonium enrichment the C:N ratio of host tissue was found to decrease in *S. pistillata* and *T.gigas* (Muscatine *et al* 1989; Belda *et al* 1993). The present study of *Anemonia viridis* similarly showed an increase in protein concentration per animal weight under ammonium enrichment. These results might suggest that the host, too, may be nitrogen deficient. In addition, the data might be interpreted as evidence for the translocation of organic nitrogen from symbionts to host.

Further evidence supporting the idea that zooxanthellae were nitrogen limited came from the observation that ammonium enrichment significantly increased the mitotic index of zooxanthellae in *Aiptasia pallida* (Cook *et al,* 1988). However, other ammonium enrichment studies of varying duration (e.g. Muscatine *et al*, 1989; Belda *et al*, 1993) report no difference in mitotic index to that of control animals. The mitotic index of zooxanthellae within *Anemonia viridis* increased with increasing ammonia concentration up to concentrations far greater than might be expected in temperate coastal waters (Chapter 4). Enrichment at a high light intensity had a greater effect on the mitotic index than at a very low light intensity. Enrichment of sea water to 20μM ammonia significantly increased the mitotic index for up to 35 days. After that time, the initially rapid peak in mitotic index began to subside but division rate still remained elevated for a further 45 days.

Thus in *Anemonia viridis*, under ammonium enrichment, the division rate of zooxanthellae in *A. viridis*, measured as the mitotic index, increased significantly for a period of 80 days,

while the population density remained largely unchanged and the total population changed by only 4%. The discrepancy in the large number of zooxanthellae unaccounted for implied that zooxanthellae were being removed from the population.

Ammonium enrichment over 80 days led to a significant increase in host weight. These results show for the first time in symbiotic anthozoans that the flux of nitrogen compounds from zooxanthellae to host is sufficiently high to permit host growth. The form in which these compounds might pass from symbiont to host is not known.

Host feeding over 80 days led to a 46% increase in host weight but only a 7% increase in the total zooxanthella population. This resulted in a significant decrease in the zooxanthella population density. Feeding stimulated the zooxanthella mitotic index compared to starved animals. The increase in mitotic index was less rapid than the increase under ammonium enrichment, however the long term increase in the mitotic index of zooxanthellae from fed *Anemonia viridis* was not significantly different to the effect of ammonium enrichment. The net increase in the symbiont population with weekly feeding was similar to that of animals maintained with ammonium enrichment. Fed animals increased in weight substantially more than did ammonium enriched animals, however, the protein:weight ratio in host tissues and protein content per symbiont increased only in ammonium enriched animals.

The data of Muscatine *et al* (1989) also showed that feeding had a similar effect to ammonium enrichment on both host and symbiont parameters of population density, host protein, symbiont mitotic index, chlorophyll per symbiont and C:N ratios, although, in general, the values for fed animals were slightly lower than for ammonium treated animals but higher than for starved animals.

During starvation in the light, animals lost weight and showed a decrease in the total zooxanthella population and a decrease in animal protein concentration. Because of these changes, the zooxanthella population density did not change significantly. During starvation the mitotic index remained at around 4% throughout the 80 days. Since there was no change in the mitotic index it was assumed that the fall in the population was brought about by an increase in the rate of loss of zooxanthellae. Expulsion of symbionts (Chapter 5) probably only accounts for a small proportion of the decrease in the population within *A. viridis*. However expulsion rates of anemones maintained in the light without feeding for periods longer than 3 weeks have not been investigated.

An increase in light intensity increased the mitotic index (section 4.3.2) and the specific expulsion rate (section 5.3.2), but did not affect the zooxanthella population density in *A. viridis* (section 3.3.1).

During starvation in the dark the mitotic index did not change significantly which led to the conclusion that the fall in the population was brought about by an increase in the rate of loss

of zooxanthellae and not by a decrease in division rate. However, by what mechanism the zooxanthellae are lost is not known.

In addition to the above factors, the host exerts an influence on the symbionts. The symbionts' environment is greatly modified by the host such that it differs from the free-living environment of phytoplankton in the following ways:

1. High symbiont densities within host tissues may lead to increased competition for nutrients (Cook & D'Elia, 1987), as evidenced by an inverse correlation between the mitotic index and population density (see chapter 4), and between chlorophyll concentration and population density (Hoegh-Guldberg & Smith 1989b; Rees, 1991). The experiments in Chapter 6 showed that even at 50% symbiont population density ammonium enrichment had a significant effect on the mitotic index of zooxanthellae in *A. viridis*, implying that nitrogen can become limiting at high symbiont densities.

2. Some of the products of host metabolism, derived from host feeding, may be essential for symbiont growth. Their supply, which is thought to be limited by the feeding rate and hence the rate of turnover by the host, may influence zooxanthella growth (Falkowski *et al* 1993).

3. However, in *Anemonia viridis*, host feeding caused an increase in host tissue growth as well as an increase in zooxanthella mitotic index (Chapter 3). The increase in mitotic index could be attributed to an increase in nutrient supply from the food source, or to the reduction in population density brought about by the addition of new host tissue, thus creating more "space". In *Anemonia viridis*, inverse relationships were found between the zooxanthella mitotic index and population density. As mentioned earlier this may be due to increased competition for nutrients, particularly nitrogen (Chapter 6). However, in addition, the amount of "space" available to each symbiont is increased at low symbiont population densities.

4. The effect of a 'host factor' has been shown to increase photosynthetic capacity, oxygen production, carbon fixation and the pool of amino acids, and cause changes in pH (Gates *et al*, 1995). This may affect the symbionts in a variety of ways such as altering the growth rate and division cycle, bringing about changes in biochemical composition and in the uptake of inorganic nutrients. However, many of these effects are as yet unknown.

Factors affecting zooxanthella losses.

The observations that zooxanthellae are regularly expelled from cnidarian symbioses is in no doubt, but the reasons why they are expelled are far from clear. It may be that expulsion serves different purposes in different symbiotic associations, or that expulsion is a purely random and incidental occurrence, as suggested in chapter 5, or that it is an important method of symbiont transmission in some species (Smith, 1987). One of the objectives in this study was to investigate the magnitude of losses by expulsion in relation to the numbers of

symbionts produced though division in *Anemonia viridis*. Since symbiont division was shown in chapter 4 to vary greatly with several intrinsic and external factors, it was hypothesised that these factors may also affect the rate of zooxanthella expulsion. It was therefore of interest to compare the specific growth rates estimated in Chapter 7 (Table 7.2 & 7.3) with the expulsion rates of the corresponding animals obtained in Chapter 5. However, specific expulsion rates were based on the zooxanthella population of the whole animal, whilst the mitotic index, was estimated from samples obtained from the tentacles only. The values presented in Tables 8.1 & 8.2 were based on the assumption that growth rates of zooxanthellae in tentacles were not different from those of zooxanthellae in the whole body. This was tested by comparing mitotic index obtained from tentacles with those of samples from sections of the whole body. The results (Appendix 6) showed that there was no significant difference between the samples. Tables 8.1 and 8.2 gives expulsion rates of *A. viridis* maintained under control and ammonium enriched conditions (section 5.3.3) and compares these values with the corresponding specific growth rates calculated for each animal.

Table 8.1 Specific expulsion rates and specific growth rates calculated for each animal maintained in control sea water from 4 consecutive measurements over 21 days. Specific growth rates are the mean for each animal of values given in Table 7.2.

Animal	Specific expulsion rate (cells.cell^{-1}d^{-1})	Specific growth rate (cells.cell^{-1}d^{-1})	% of symbionts added through division that were expelled
1	0.00274	0.0588	4.66
2	0.00480	0.0508	9.45
3	0.00095	0.0395	2.41
4	0.00202	0.0435	4.64
5	0.00216	0.0523	4.13
Mean	0.00253	0.04898	5.058

Table 8.2 Specific expulsion rates and specific growth rates calculated for each animal maintained in 20μM ammonium enriched sea water from 4 measurements. Specific growth rates are the mean for each animal of values given in Table 7.3.

Animal	Specific expulsion rate (cells.cell^{-1}d^{-1})	Specific growth rate (cells.cell^{-1}d^{-1})	% of symbionts added through division that were expelled
6	0.00540	0.0640	8.44
7	0.00863	0.0563	15.33
8	0.00836	0.0668	12.51
9	0.00463	0.0543	8.53
10	0.00666	0.0645	10.33
Mean	0.00674	0.0612	11.03

For animals maintained in control sea water, the percentage of symbionts added through division that were expelled ranged from 0.8 to 5.5%, with an average value of 3.4%. In animals maintained in sea water enriched with ammonium, this percentage ranged from 7 to 15% with a mean of 11.2%. Thus, the number of cells expelled relative to those added increased in animals maintained in sea water enriched with ammonium compared with that from control animals.

The percentage of symbionts of the total added to the population that were expelled for animals maintained in control sea water for *Anemonia viridis* (Table 8.1) is similar to that found by Hoegh-Guldberg *et al* (1987) for corals. Hoegh-Guldberg *et al* (1987) measured the expulsion rates of Red Sea corals in the field and found that numbers expelled were approximately 4% of the symbionts added to the population calculated from mitotic indices. They concluded that the numbers of symbionts expelled from corals in unstressed conditions represented an insignificant loss in terms of energy and numbers, and would have little effect on reducing the population.

When maintained in control sea water, the population of zooxanthellae which was initially 2.026×10^8 decreased by 2.56×10^7 symbionts in 35 days (Table 3.11). Given a specific growth rate of 0.04898 cells.cell^{-1}d^{-1} (Table 8.1) and a specific expulsion rate of 0.00253 cells.cell^{-1}d^{-1}, the estimated number of cells added through division would be 9.22×10^8 and the estimated number of cells expelled would be 1.72×10^7 zooxanthellae.

Thus, the estimated total population after 35 days based on the equation on page 143 would be:

$$(2.026 \times 10^8) + (9.22 \times 10^8) - (1.72 \times 10^7) \Rightarrow 11.08 \times 10^8$$

initial population	cells added	cells expelled	population after 35 days

Clearly the population remains much smaller than this. Thus, these values of 3.4% and 11% obtained in this study would appear to be insignificant for expulsion to be a population control mechanism, despite the observation that specific expulsion rate was proportional to specific growth rate of symbionts. There are several possible explanations for this:

1) There may be an additional, more effective mechanism for removing symbionts. The possibility that cells are digested either intra-cellularly or within the coelenteron or mesenteries has not been ruled out. Symbiont digestion as a form of regulation was considered by Trench, (1974; 1979), Muscatine & Pool (1979), and Hoegh-Guldberg & Smith (1989b), but very little work has been done on the subject. Degenerating symbionts within host cells were observed in the mesenteries but not elsewhere in the host tissues of *A.*

viridis maintained in the light (Chapter 3). Similarly, Taylor (1969) observed degenerate symbionts within the mesenteries of *A. viridis*. It is not known whether symbionts thus observed are in the process of lysosomal break down or are the result of autolysis. Fitt & Trench (1983b) suggested that host cells did have the capacity to lyse symbionts through the action of lysosomes, although to what extent this occurs and what effect it has on the population is not known.

2) Not all of the expelled cells were counted. This seems unlikely, unless expelled cells rapidly disintegrate either in the coelenteron or outside the host's body, and become indistinguishable to the counting procedures used in these experiments.

3) Specific growth rates are overestimated using the current measure of the mitotic index. The different methods used gave variable doubling times (Table 7.2 and 7.3). As mentioned in Chapter 7 accurate estimation of specific growth rates relies almost exclusively on being able to determine the duration of particular phases in the cell cycle and being able to predict how the duration of each phase changes under different environmental conditions. Preliminary observations, given in Appendix 4, suggest that the duration of different phases of cell division of symbionts in *Anemonia viridis* changed under different environmental conditions and these data lend doubt to the accuracy of the doublet technique for measuring the mitotic index. Information on the duration of these phases in symbionts is lacking and until such information is available, estimates of growth rate will continue to be unreliable. Furthermore, errors in measuring growth rates may be reduced by improvements in the estimation of the duration of the phases of the cell cycle using measurements of incorporation of labelled nucleotides into DNA.

Until the host environment can be suitably reproduced *in vitro*, the use of cultured symbionts in investigating the phases of the cell cycle, may not be representative of the situation *in hospite*. However, recent work on "host factor" (Gates *et al*, 1995) may see the improvement of symbiont culture techniques in making the symbiont environment *in vitro* more representative of the environment *in hospite*.

Further studies on improving the accuracy of the measurement of symbiont growth rates and losses to the population, and investigations in to the possibility of symbiont autolysis and host digestion may produce a better picture of the dynamics of the symbiont population.

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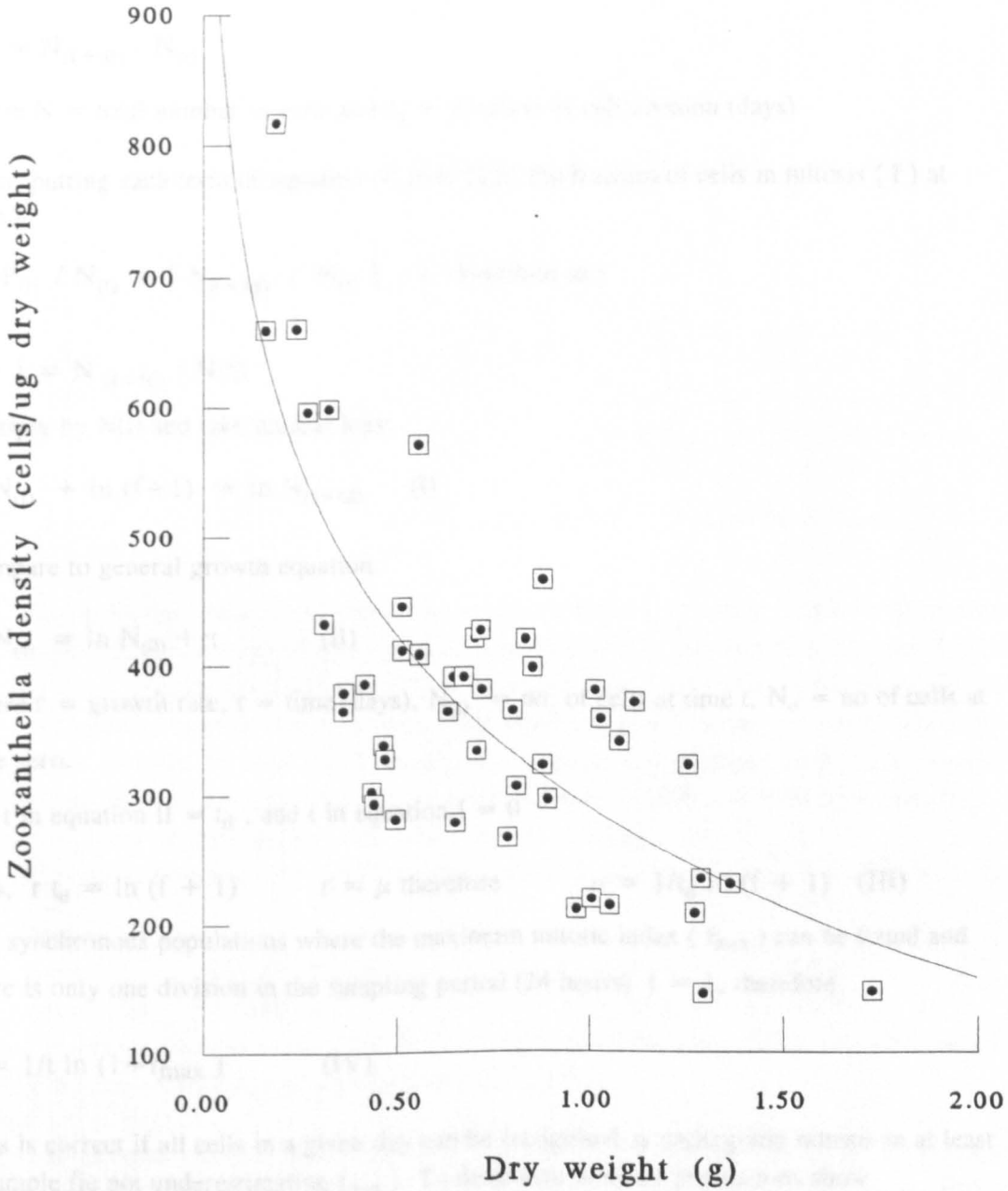
Appendix 1 The distribution and density of zooxanthellae within tissues of *A. viridis*.

Density of zooxanthellae within different tissues of *A. viridis* maintained at 120μE.m^{-2}s^{-1} ± s.e. (n=8).

Tissue type	cells. μg protein^{-1}	Coefficient of variation	% total symbiont number
Mesentery	633.3 (92)	41.2%	7.24 % (1.4)
Column	347.2 (21)	17.1%	4.28 % (0.47)
Base	244.6 (27)	31.2%	1.49 % (0.15)
Oral Disc	1209.5 (71)	16.6%	12.2 % (1.40)
Tentacles	3108.5 (336)	30.6%	74.9 % (2.55)



Appendix 2. Zooxanthella density as cells.µg dry weight^{-1} plotted against dry weight of animals maintained at 300µE.m^{-2}s^{-1} for 80 days. There was no difference between the relationships of starved and fed weekly animals therefore data graphed includes starved animals and animals fed weekly.

$$y = 1610 * 440 \log x \quad (R^2 = 57.8\ \%)$$

Appendix 3 Calculation of Growth Rates of algal populations (from McDuff & Chisholm, 1982).

The number of cells (P) in a sequence of mitotic stages is equal to the increase in the number of cells from the time of observation (t) until the combined duration of mitotic stages (t_d) elapses (or the time taken for the cell in mitosis to become two cells).

$$P_{(t)} = N_{(t+td)} - N_{(t)}$$

where N = total number of cells and t_d = duration of cell division (days)

Then, putting each term of equation (I) over N(t), the fraction of cells in mitosis (f) at time t

$$= P_{(t)} / N_{(t)} = [\, N_{(t+td)} / N_{(t)} \,] - 1 \quad \text{rewritten as :}$$

$$f + 1 = N_{(t+td)} / N(t)$$

multiply by N(t) and take natural logs:

$$\ln N_{(t)} + \ln (f+1) = \ln N_{(t+td)} \qquad (I)$$

Compare to general growth equation:

$$\ln N_{(t)} = \ln N_{(0)} + rt \qquad (II)$$

where r = growth rate, t = time (days), $N_{(t)}$ = no. of cells at time t, N_o = no of cells at time zero.

Let t in equation II = t_d , and t in equation I = 0

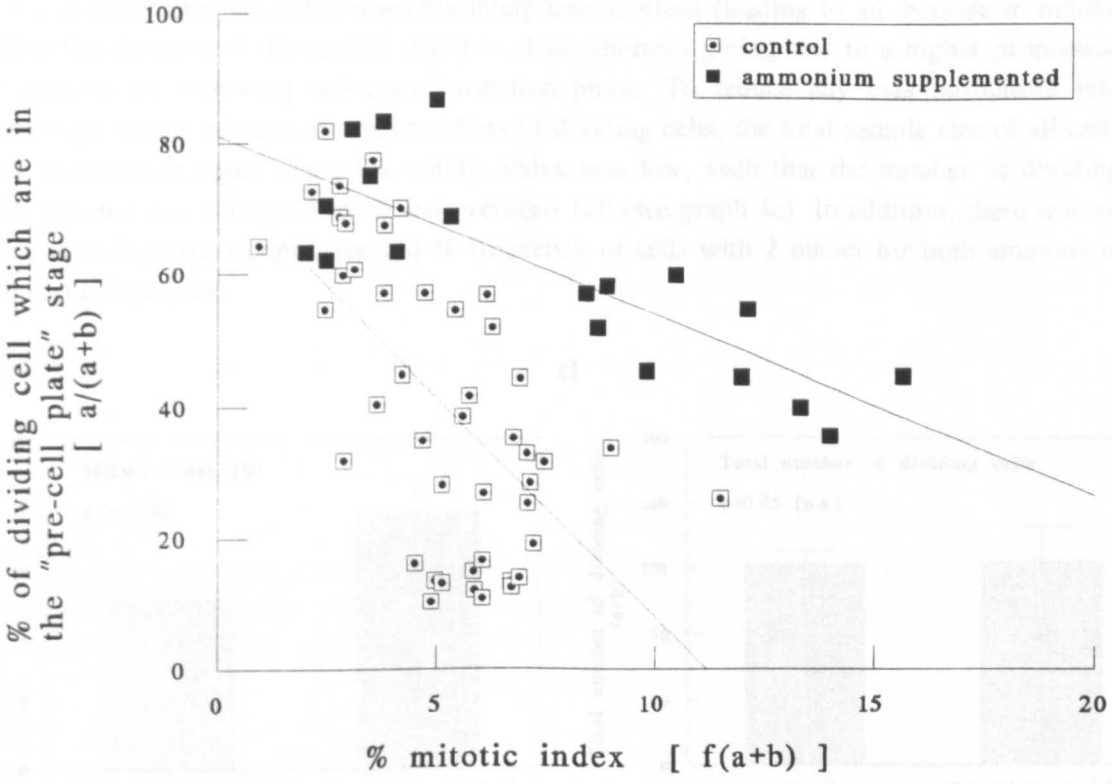
thus, $r\, t_d = \ln (f + 1)$ r = μ therefore $\mu = 1/t_d \ln (f + 1)$ (III)

For synchronous populations where the maximum mitotic index (f_{max}) can be found and there is only one division in the sampling period (24 hours) t = 1, therefore

$$\mu = 1/t \ln (1+f_{max}) \qquad (IV)$$

This is correct if all cells in a given day can be recognised as undergoing mitosis in at least 1 sample (ie not underestimating f_{max}). To determine whether populations show asynchronous or synchronous division, successive samples of the population can be taken to determine the mitotic index over a period (eg 24 or 48 hours). Equation IV, however, is not true for populations where t_d > interval over which all samples were taken (1 day) or the entraining period (normally 24 hours).
Alternatively, μ can be calculated from sequential observations:

$$\mu = (1/n*t_d) \sum_{i=1}^{n} \ln(1+f_i) \qquad (IV) \quad \text{where n = number of samples.}$$



Appendix 4a Relationship between the mitotic index (percentage of all dividing cells stained using Feulgen's stain) of zooxanthellae from tentacles of *Anemonia viridis* and the percentage of those dividing zooxanthellae which have just completed nuclear division but do not show cell plate formation. Animals were maintained at 11±1°C either in control sea water or sea water enriched to 20μM with ammonium sulphate for 14 days. Each point represents one animal from which total counts were made of between 2100 and 3000 zooxanthellae.

a = those dividing cells with 2 nuclei but no cell plate

b = those dividing cells with cell plates visible & both daughter cells formed but still joined.

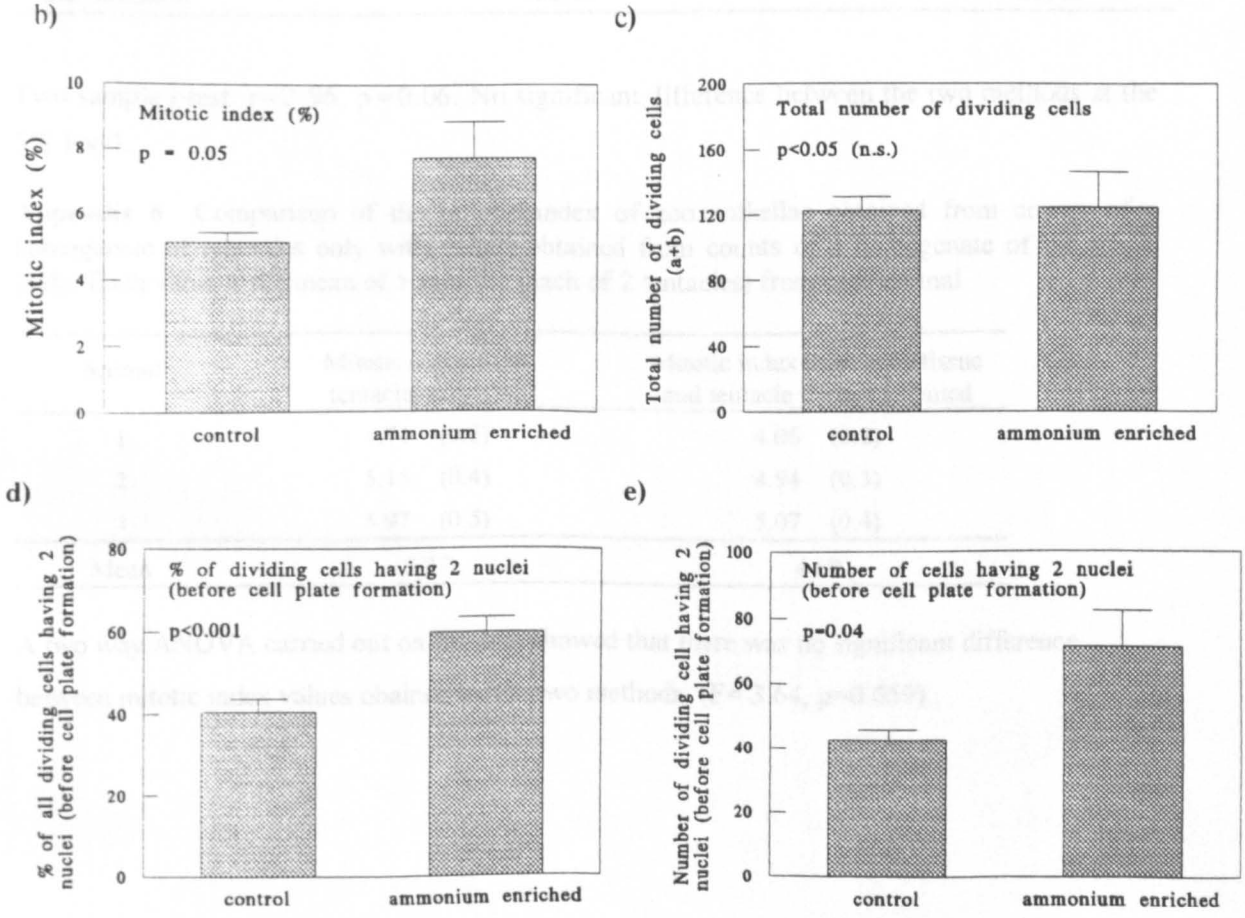
Regression analysis.

control	$y = 75 - 6.7 * x$	$r^2 = 39\%$	$p < 0.001$
ammonium	$y = 81 - 2.7 * x$	$r^2 = 64\%$	$p < 0.001$

Covariance analysis showed that the two regression lines were statistically different in slope ($F = 7.1$ $p = 0.01$) and elevation ($F = 50.9$ $p < 0.001$).

The process of cell division can be separated into nuclear division (karyokinesis) and cytokinesis. An interphase usually separates each complete cell division event. The 2 phases of cell division can be identified using the Feulgen's staining technique (section 2.2). If, as the mitotic index increases, only the duration of the interphase changes while the duration of the nuclear phase and cytokinesis are constant relative to each other, then the regression lines on the graph (4a) would have a slope of zero, but this does not appear to be the case (graph 4a). In anemones in control sea water, as the mitotic index increased, the percentage of dividing cells with 2 nuclei decreased (graph 4a). This appeared to be the result of a decrease in the number of cells with 2 nuclei but no change in the number of cells with cell plates. Assuming that the process of nuclear division is followed immediately by cell division, the

above is interpreted such that when the interphase shortens (leading to an increase in mitotic index) the duration of the nuclear division phase shortens giving rise to a higher proportion of cells in the following cell plate formation phase. To reduce any bias introduced into percentage values because of small numbers of dividing cells, the total sample size of all cells was increased in cases where the mitotic index was low, such that the number of dividing cells counted was not less than 30 and averaged 120 (see graph 4c). In addition, there was no correlation between sample size and % frequency of cells with 2 nuclei for both ammonium and control samples.



Appendix 4 (b - e) When anemones were maintained with ammonium supplementation the zooxanthella mitotic index was greater than in anemones under control conditions as was found in chapter 4. In the zooxanthella samples from ammonium suppplemented animals, there was a higher percentage of dividing cells with 2 nuclei than with cell plates than in samples of zooxanthellae from control animals (graphs a and d). A tentative interpretation of this is that under ammonium supplementation, the duration of the cell plate phase in the zooxanthellae is decreased relative to the duration of the nuclear division phase.

Appendix 5 Comparison of methods for measuring expulsion rates

	volume of stock concentration	total number of cells in 1 ml	volume of stock concentration	total number of cells in 1ml
	0.50ml	2095349	0.20ml	1737500
	0.28ml	1988052	0.10ml	1712500
	0.20ml	1726431	0.05ml	1750000
	0.10ml	2034956	0.04ml	1642835
Mean		1961197		1710709
Std.deviation		162556		23930

Two sample *t*-test: $t=2.96$, $p=0.06$. No significant difference between the two methods at the 5% level.

Appendix 6 Comparison of the mitotic index of zooxanthellae obtained from counts of a homogenate of tentacles only with values obtained from counts of a homogenate of the whole body. Each value is the mean of 5 samples (each of 2 tentacles) from each animal

Animal	Mitotic index from tentacles only (%)	Mitotic index from body tissue and tentacle tissue combined
1	4.74 (0.2)	4.06 (0.2)
2	5.15 (0.4)	4.94 (0.3)
3	5.97 (0.5)	5.07 (0.4)
Mean	5.27	4.69

A two way ANOVA carried out on the data showed that there was no significant difference between mitotic index values obained by the two methods ($F= 3.64$, $p=0.059$).