Nitrogen cycling in the *Anemonia viridis* symbiosis

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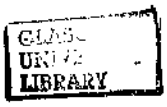

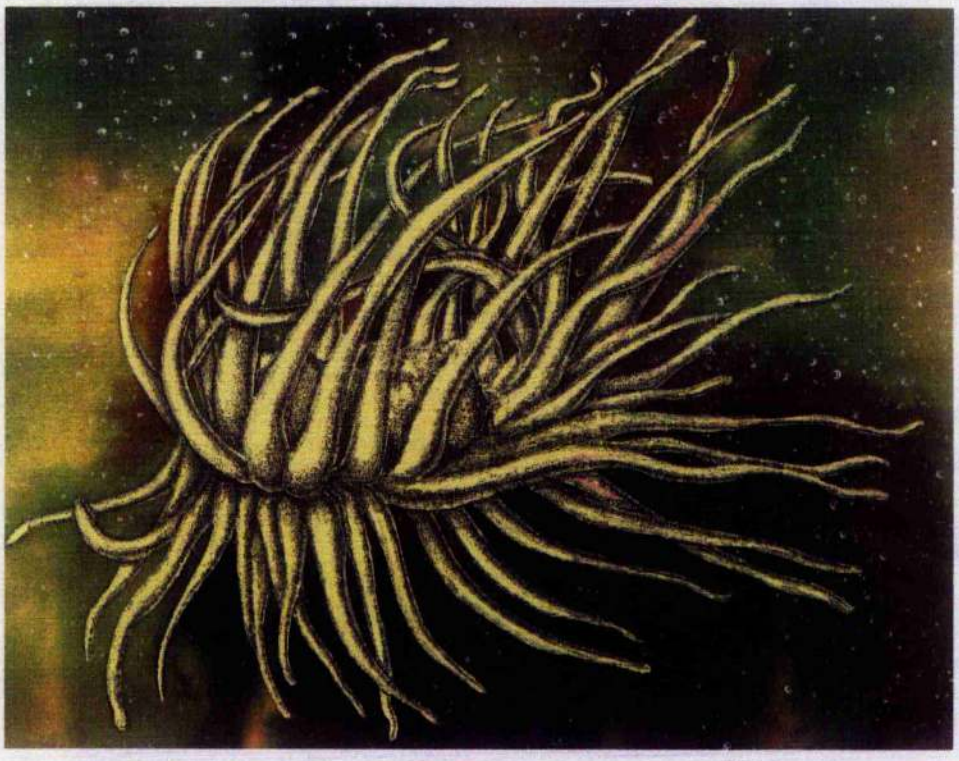

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Abbreviations

Ala	Alanine
AOA	Aminooxyacetic acid
ANCA	Automatic nitrogen and carbon analyser
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APE	Atom % excess
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
AZS	Azaserine
Cys	Cysteine
CF-IRMS	Continuous flow-Isotope ratio mass spectrometry
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DDW	Deionised distilled water
DIN	Dissolved inorganic nitrogen
DON	Dissolved organic nitrogen
DTT	Dithiotreitol
EDTA	Ethylene diaminetetraacetic acid
FAA	Free amino acid
Fd	Ferredoxin
FSR	Fractional synthetic rate
FSW	Filtered seawater
GABA	γ-aminobutyrate
GC-MS	Gas chromatography-Mass spectrometry
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GOGAT	Glutamine 2-oxoglutarate amidotransferase
GS	Glutamine synthetase
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid]
His	Histidine
HMW	High molecular weight

HPLC	High performance liquid chromatography
Ile	Isoleucine
Leu	Leucine
LMW	Low molecular weight
Lys	Lysine
Met	Methionine
MSO	Methionine sulphoximine
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
SSA	Sulphosalicylic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
Ser	Serine
Tau	Taurine
TBDMS	*tert*-butyldimethylsilyl
TCA	Trichloroacetic acid
Thr	Threonine
TLC	Thin layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Abstract

The temperate sea anemone, *Anemonia viridis* forms an endosymbiosis with dinoflagellate algae, or zooxanthellae. Previous studies of this and other symbioses have shown that zooxanthellae photosynthesis can allow them to be autotrophic with respect to carbon. Under environmental conditions where the anemone can satisfy its carbon requirements autotrophically, excretory nitrogen is also retained. In addition to this, *Anemonia viridis* is able to take up ammonium from seawater. Ammonium uptake has been observed in all classes of microalgal-invertebrate symbiosis, but has not been demonstrated in non-symbiotic species or aposymbiotic individuals. Ammonium uptake is also known to be linked with photosynthesis, since it only takes place in the light or after a period of exposure to light. Whether ammonium is recycled between the zooxanthellae and host or is conserved by the host remains unresolved.

Whatever the mechanism by which ammonium is assimilated, it was not known whether ammonium uptake could support growth. To examine this question, symbiotic anemones were maintained for three months in seawater supplemented with 20μM ammonium and weight change was compared with that of control anemones in unsupplemented seawater. During this experiment, anemones in seawater lost weight whereas those in ammonium-supplemented seawater increased in weight. This provided a preliminary indication that ammonium uptake could support growth. To examine this question further, a nitrogen budget was constructed where the only input of nitrogen was from ammonium added to the seawater.

In order to determine this nitrogen budget, it was necessary to develop a system which allowed the nitrogen input to individual anemones to be measured. A flow-through apparatus was produced in which anemones could be maintained under a known input of ammonium for several weeks. During these exposures, this apparatus also allowed the rate of ammonium uptake by the anemones to be measured. The rate of uptake measured was very close to that measured by a previous study, which used a closed-chamber depletion method. Having established that the flow-through apparatus allowed accurate and reproducible measurement of the rate of ammonium uptake, it was then used to estimate a 24 hour nitrogen budget for symbiotic anemones. The total nitrogen content of zooxanthellae and host were measured from a group of control anemones and compared with the nitrogen contents from a second group which had been subjected to a known

input of ammonium using the flow-through depletion apparatus. In the experimental group, the total nitrogen content of both zooxanthellae and host increased after a two week ammonium exposure. While the ammonium recycled within and taken up by the anemones accounted for 68% of growth, this nitrogen input failed to account for all the growth as well as any unmeasured loss of nitrogen. This suggested that another source of nitrogen might have been available to the anemones. Since there was no nitrogen input from holozoic feeding, it was possible that uptake of seawater amino acids could have contributed to the nitrogen budget.

The conversion of inorganic nitrogen as ammonium to organic nitrogen will take place via the free amino acid pool. The free amino acid pools of zooxanthellae and host were found to be very different. That of the host was dominated by just two amino acids, taurine and glycine which could reflect the osmoregulatory role some free amino acids have in sea anemones. The free amino acid pool of zooxanthellae did not contain such high levels of these two amino acids. After 47 days in seawater supplemented with 20μM ammonium, the free amino acid concentration of the zooxanthellae was found to increase twofold. In contrast, the concentration of free amino acids in the host did not show a significant increase.

While microalgae are known to possess enzyme systems to assimilate exogenous ammonium, it has been suggested that the host fraction of algal-invertebrate symbioses may also be capable of this. Host tissue from *Anemonia viridis* was found to contain glutamate dehydrogenase which was most active with the coenzyme NADPH and showed activity comparable with that in other anthozoan hosts. In zooxanthellae, ammonium assimilation is believed to take place via the glutamine synthetase/glutamine 2-oxoglutarate amidotransferase (GS/GOGAT) cycle. Glutamine synthetase was not measured but GOGAT activity was detected in the presence of a ferredoxin analogue. This indicated that GOGAT from zooxanthellae may be ferredoxin-linked *in vivo*.

Ammonium uptake by *Anemonia viridis* was examined in more detail by following the pathway of assimilation using ammonium enriched with the stable isotope ^{15}N. When anemones were incubated with [^{15}N]ammonium at either 10 or 20μM, the zooxanthellae became enriched at up to 17 times the rate of the host, in both cases the enrichment being linear over a thirty minute time period. The rate of zooxanthellae enrichment at 20μM

was twice that at 10μM whereas the rate of host enrichment was not significantly affected by ammonium concentration. When anemones were incubated with [^{15}N]ammonium in the dark, after 12 hours without light, the rate of enrichment was lowered in both zooxanthellae and host. However, only the enrichment of the host was significantly reduced when the light level was lowered from 300 to 150μmol photons.$m^{-2}.s^{-1}$. Zooxanthellae enrichment remained the same at these two light levels.

The low molecular weight material from the zooxanthellae became enriched at 20 times that of the host suggesting that the zooxanthellae were the primary site of assimilation. This was examined in more detail by analyzing the enrichment of amino acids. The amino acids aspartate, glutamate, glutamine, glycine, threonine, alanine, tyrosine, valine, phenylalanine and leucine from zooxanthellae became enriched with ^{15}N. Unfortunately the analysis of amino acids extracted from host tissue was not successful. The amino acid enrichment in the zooxanthellae accounted for 65% of the total low molecular weight material enrichment. Of the amino acids detected, over 90% of the enrichment was accounted for by the three amino acids, glutamate, glutamine and aspartate. The enrichment of the amide group of glutamine was greater than that of the amine group of glutamine or glutamate. This is consistent with the GS/GOGAT cycle as the mechanism of ammonium assimilation.

To examine the flux of ^{15}N from zooxanthellae to host, anemones were pulse labelled with [^{15}N]ammonium and then transferred to an unlabelled chase. Over the time course of the experiments carried out, there was no evidence for a flux of nitrogen from zooxanthellae to host. However, during the chase period the enrichment of low molecular weight material declined and that of high molecular weight material increased, indicating that protein was synthesized in both zooxanthellae and host using ^{15}N from ammonium. Again the low molecular weight material was analyzed in more detail by measuring the amino acid enrichment following a pulse label with ^{15}N. In the zooxanthellae, glutamate turned over most rapidly, followed by aspartate, alanine, glycine and valine (no data are available for glutamine). Unlike these amino acids, the enrichment of the essential amino acids phenylalanine and threonine increased during the chase. It was intended to see whether or not essential amino acids in the host became enriched with ^{15}N. Since these cannot be synthesized by animal cells, this would have provided good evidence for translocation of 'high quality' nitrogen from the zooxanthellae. However, this analysis was unsuccessful. Improved sample preparation should allow successful analysis of the

^{15}N enrichment of the host fraction and shows great potential to answer the question of whether or not zooxanthellae translocate essential amino acids.

In common with other symbiotic cnidarians, *Anemonia viridis* is able to retain excretory ammonium and to take up ammonium from the seawater. Under the laboratory conditions described in this study, ammonium uptake provided a significant input to the anemones but failed to produce a balanced nitrogen budget. While it is possible that both the zooxanthellae and host could assimilate ammonium, the zooxanthellae appear to be the major site of assimilation. Further work is necessary to provide definitive evidence for nitrogen recycling in this symbiosis.

Chapter 1

Introduction

1.1 Algal-invertebrate symbiosis

Symbiosis was defined by de Bary (1879) as *'the living together of differently named organisms'*. In such associations the larger partner is referred to as the host and the smaller partner as the symbiont (Smith & Douglas, 1987). This definition excludes short-term associations, such as insect pollination of flowering plants, but will include all long term associations. The characteristics of such associations vary on a continuum from parasitism to mutualism (Douglas, 1994). Despite the original definition of de Bary, the term symbiosis is commonly accepted as defining a condition where the two symbiotic partners derive a mutual benefit from the association. To be biologically meaningful, such mutual benefits must be defined. In a biological context a mutualistic symbiosis would increase the fitness of both partners when compared to their fitness as individual organisms (Law & Lewis, 1983). However, such benefits may only be evident under certain environmental conditions and are not necessarily an absolute characteristic of symbiosis (Douglas, 1994). *'Practically, in some instances, symbiosis seems to result in mutual advantage. In all cases it results advantageously to one of the parties, and we can never be sure that the other would not have been nearly as well off, if left to itself.'* (Pound, 1893).

Here the intracellular or endosymbiosis formed between invertebrate hosts and dinoflagellate algal symbionts (Dinophyceae) will be considered. Several invertebrate phyla form symbiotic associations with dinoflagellates including the Cnidaria, Protozoa, Porifera, Mollusca, Platyhelminthes and Urochordata (Trench, 1993). The freshwater cnidarian *Hydra* forms a similar endosymbiosis with *Chlorella* (Chlorophyta) algal cells. Historically, symbiotic brown and green algae were termed zooxanthellae and zoochlorellae respectively (McLaughlin and Zahl, 1966). Although neither of these terms has any taxonomic significance (Trench, 1993), the term zooxanthellae will be used here to refer to symbiotic dinoflagellate algae. The phyletic diversity of symbionts is low in comparison to that of invertebrate hosts (Law and Lewis, 1983), but taxonomic diversity has recently been detected within symbiotic algae both in terms of morphological (Trench & Blank, 1987) and genetic differences (Rowan and Powers, 1991; McNally *et al.*, 1994). This shows that the previous classification of zooxanthellae as one species,

Symbiodinium microadriaticum (Freudenthal, 1962), was an inaccurate reflection of symbiont diversity. Indeed, there is now evidence that there can be a number of zooxanthellae taxa within a single host and that their distribution within that host can depend on environmental conditions. For example, within colonies of the coral *Montastrea annularis* the distribution of these taxa is determined by ambient light level (Rowan, 1996).

Marine endosymbioses are most abundant in the shallow coastal waters of the tropics where coral reefs are found. In these well lit, oligotrophic seas zooxanthellae form endosymbiotic associations with a diverse array of invertebrate hosts including anthozoans (corals, anemones and zoanthids), hydrozoans, scyphozoans and bivalve molluscs (tridacnid clams). Although less widespread, such symbioses are also found in temperate regions. Three species of anemone, *Anemonia viridis*, *Anthopleura hallii* and *Cereus pedunculatus*, from British waters form an endosymbiotic association with zooxanthellae (Manuel, 1981). In Britain, these species are restricted to the west coast where Scotland represents the northern limit of their geographical distribution. In this investigation the symbiosis between *Anemonia viridis* and zooxanthellae will be examined.

In symbiosis with Cnidaria, the zooxanthellae are located in the gastrodermal cell layer of the host. Each zooxanthella is enclosed by a series of host-derived membranes forming a symbiotic cell organelle which has been referred to as the symbiosome (Roth *et al.*, 1988). Within a symbiotic individual, the distribution of symbionts is not uniform across the host. In marine cnidarians, zooxanthellae tend to be concentrated in areas which are exposed to light (Day, 1994). In *Anemonia viridis* the zooxanthellae, per unit host protein, are most concentrated in gastrodermal tissue from the tentacles (Beaver, 1996).

In order to be classified as a mutualistic relationship, both the symbiotic partners must derive benefit from the symbiosis. During experiments in which zooxanthellae are incubated with $^{14}CO_2$, either *in hospite* or *in vitro*, some of the carbon fixed by zooxanthellae photosynthesis is subsequently translocated to the host (Muscatine & Cernichari, 1969; Lewis & Smith, 1971; Trench, 1971a, b, c; Muscatine *et al.*, 1972; Trench, 1974). When this carbon flux was quantified, it was found to meet and even exceed the host's respiratory carbon requirement (Davies, 1984; Muscatine *et al.*, 1984; Tytler & Davies, 1986; Edmunds & Davies, 1986). It has been suggested that

zooxanthellae benefit from access to host-derived nutrients. Since nitrogen is often the limiting in marine ecosystems (cf. Ryther & Dunstan, 1971; Roman, 1983) access to nitrogen could play a central role in limiting the growth of both zooxanthellae and host.

1.2 Sources of nitrogen for algal-invertebrate symbioses

The nitrogen available to algal-invertebrate symbioses can be derived from three sources: holozoic feeding, uptake of dissolved organic nitrogen (DON) and uptake of dissolved inorganic nitrogen (DIN).

1.2.1 Holozoic feeding

The importance of holozoic nutrient input to the nitrogen requirements of algal-invertebrate symbioses has received little attention (Davies, 1992) but in corals is believed to represent a small contribution (Johannes *et al.*, 1970; Johannes & Tepley, 1974; Porter, 1974; Edmunds & Davies, 1986). Porter (1976) suggested that coral species rely to different extents on holozoic feeding. Corals with large polyps and a low surface area to volume ratio were proposed to rely more on holozoic feeding, whereas corals with small polyps and a high surface area to volume ratio would be more reliant on photosynthesis. The nutrition of the giant clam *Tridacna gigas* was investigated by Hawkins and Klumpp (1995). This study showed that holozoic feeding was required to maintain juvenile clam growth rates but in larger clams the nitrogen input from ammonium assimilation by zooxanthellae exceeded that from holozoic feeding. The diet of *Anemonia viridis* was investigated by Möller (1978) who found that crustaceans and gastropod molluscs occurred most commonly as prey items. This study did not quantify the contribution of these prey items to the anemone's nitrogen requirements.

1.2.2 Dissolved organic nitrogen uptake

Both symbiotic and non-symbiotic cnidarians are capable of taking up DON from seawater (Stephens 1962; Shick, 1975; Schlichter, 1982; Ferrier, 1991; Wilkerson & Kremer, 1992). Indeed dissolved free amino acids could provide an important nutrient source to the larvae of species which are brooded in the gastrovascular cavity (Chia, 1972). Few of these studies have attempted to quantify the contribution DON could make to nutrition (Wilkerson & Kremer, 1992). Shick (1975) estimated that in the non-symbiotic scyphozoan *Aurelia aurita*, between 0.4 and 1.3% per day of body nitrogen could be supplied by 0.8μM glycine in seawater. Wilkerson and Kremer (1992) calculated that a 500nM amino acid mixture could supply the symbiotic scyphozoan

Linuche unguiculata with 0.5% of its total nitrogen stock per day. In addition, DON from an amino acid concentration of 2μM could satisfy 80% of the respiratory demand of the symbiotic anthozoan *Heteroxenia fuscescens* (Schlichter, 1982).

1.2.3 Dissolved inorganic nitrogen uptake

The ability of algal-invertebrate symbioses to take up DIN was first reported in the studies of Godes (1882) and Pütter (1911). The former suggested that zooxanthellae performed an *'intracellular renal function'* in cnidarians while the latter concluded that the ability of *Aiptasia pallida* to remove ammonium from seawater was a property of the zooxanthellae. The end-product of cnidarian amino acid catabolism is ammonium[1] which is excreted across the whole body surface (Shick, 1991). Ammonium is excreted in this way by non-symbiotic species (Muscatine & D'Elia, 1978; Burris, 1983) and aposymbiotic individuals (Cates & McLaughlin, 1976; Szmant-Froelich & Pilson, 1977; Muscatine *et al.*, 1979; Wilkerson & Muscatine, 1984; Davies, 1988). Symbiotic individuals in the light either show reduced rates of ammonium excretion, or are able to take up ammonium from the external medium (Kawaguti, 1953; Muscatine & D'Elia, 1978; Wilkerson & Trench, 1986; Davies, 1988). There is some evidence that symbiotic corals are able to take up inorganic nitrogen as nitrate (e.g. D'Elia & Webb, 1977; Franzisket, 1973, 1974; Webb and Wiebe, 1978; Wilkerson & Trench, 1986; Marubini & Davies, 1996) but nitrate uptake by symbiotic anemones has not been demonstrated (Wilkerson & Muscatine, 1984; Davies, 1988) even following pretreatment for one month with nitrate (Wilkerson & Muscatine, 1984). In culture, zooxanthellae are capable of taking up nitrate (Wilkerson & Trench, 1986). However, this uptake is only seen in the absence of ammonium which inhibits the activity of nitrate reductase (Syrett, 1981). Since there is no evidence that *Anemonia viridis* is capable of nitrate uptake (Davies, 1988), this introduction will concentrate on ammonium uptake by algal-invertebrate symbioses.

1.3 Nitrogen cycling

The ability to retain and in some cases take up ammonium characterizes algal-invertebrate symbioses and has been referred to as the cycling of nitrogen. Two hypotheses have been put forward to explain this observation.

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[1]The term 'ammonium' refers to the ionized form of ammonia (NH_4^+). Since ammonia occurs primarily in the ionized form at the pH of both body fluids and seawater (Shick, 1991) the term ammonium will be used throughout this thesis to refer to ammonia and ammonium collectively.

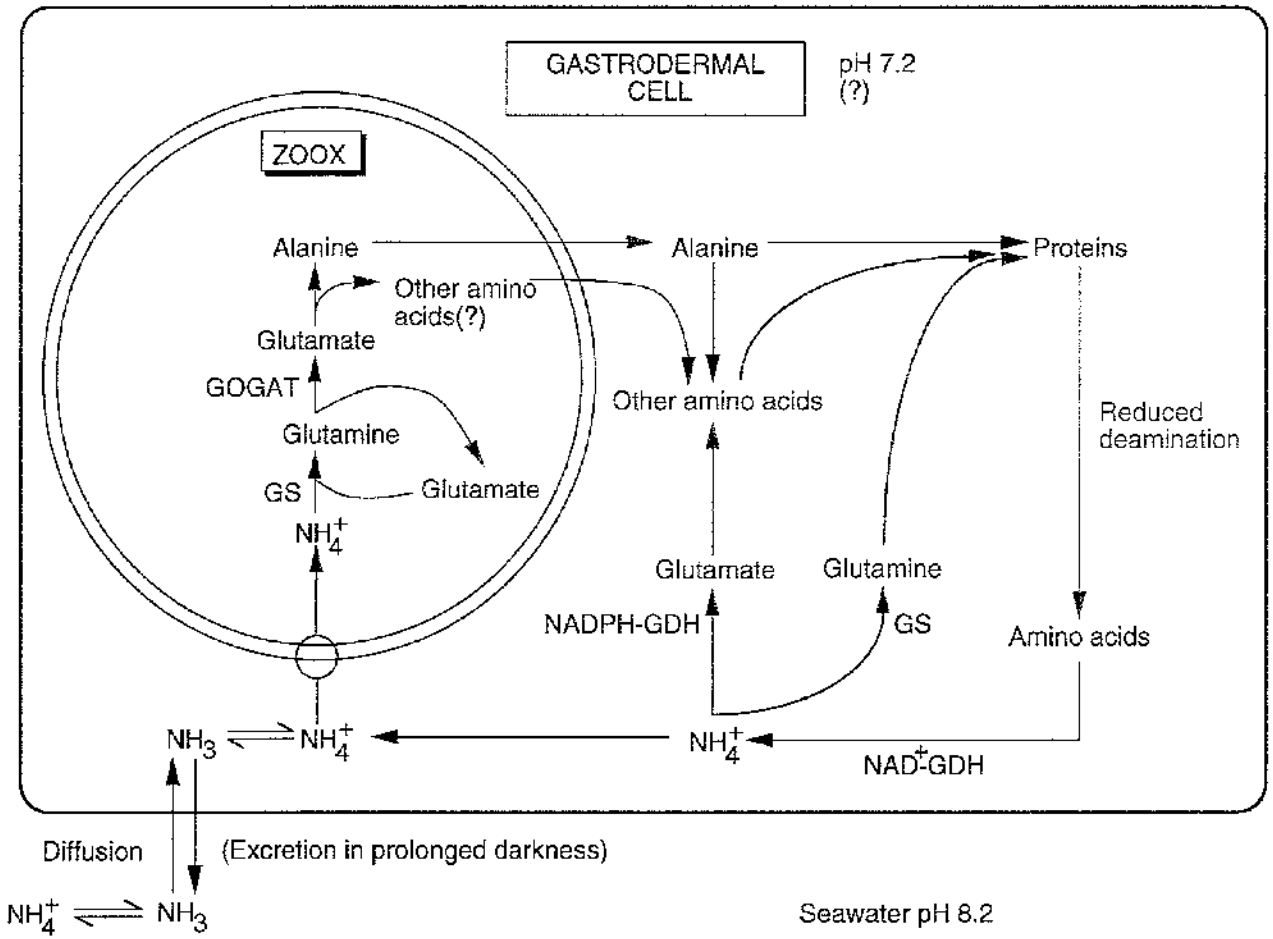


Figure 1
Simplified diagram of the major nitrogen fluxes within a symbiotic gastrodermal cell. In the light ammonium produced by host catabolism is either recycled by the zooxanthellae, as shown in green, or conserved within the host, as shown in red.
Abbreviations: GS, glutamine synthetase; GOGAT, glutamine 2-oxoglutarate amidotransferase; GDH, glutamate dehydrogenase.
(adapted from Davies, 1992)

The first is that of nitrogen recycling which proposes that inorganic nitrogen taken up by a symbiotic individual is assimilated by the zooxanthellae and subsequently translocated to the host as organic nitrogen (such as amino acids). Nitrogen recycling has been described as '*....the corner stone of the concept of nutrient recycling within microalgal-invertebrate symbioses....*' (Trench, 1993). The second hypothesis proposes that nitrogen within a symbiotic individual can be conserved given a supply of translocated carbon skeletons to the host (Rees, 1987). This carbon is provided by translocated photosynthate from the symbionts and allows the host to conserve nitrogen in one of two ways. Firstly, translocated carbon could provide the host with a respiratory substrate allowing it to divert amino acids from gluconeogenesis and so reduce its rate of deamination. This appears to take place in the green *Hydra* symbiosis (Rees & Ellard, 1989) and there is some evidence for nitrogen conservation in corals (Szmant *et al.*, 1990). Secondly, translocated carbon could provide amino group acceptors allowing the host to re-assimilate catabolically produced ammonium and synthesize amino acids. Both these explanations will be considered in detail below and are illustrated diagramatically in figure 1. In order to distinguish between the hypotheses of nitrogen recycling and conservation it would be necessary to determine the pathway by which inorganic nitrogen is assimilated and retained by algal-invertebrate symbioses. Such an interpretation relies on the assumption that ammonium assimilated from seawater follows the same pathway as excretory ammonium which is recycled or conserved within the symbiosis. Therefore the evidence for ammonium assimilation by both zooxanthellae and host will be considered.

1.4 Zooxanthellae DIN assimilation

There are two potential sources of ammonium for zooxanthellae to draw upon, though access to both of these must be via the host cytoplasm. These are the external medium and the end products of host protein and amino acid catabolism. To fulfill the criteria of the definition of nitrogen recycling, excretory nitrogen from the host must be taken up by the symbionts and then translocated back in a form which the host can metabolize.

Zooxanthellae in symbiosis are believed to effect the uptake of ammonium by a 'depletion-diffusion' mechanism (D'Elia, 1977; D'Elia *et al.*, 1983). In this model the zooxanthellae are the site of ammonium assimilation and so deplete the host cytoplasm of ammonium. This creates a concentration gradient and so promotes the diffusion of ammonium from the incubation medium to the host cytoplasm. Evidence in support of

this model is provided by the fact that the ammonium uptake kinetics of freshly isolated zooxanthellae resemble those of the intact association (D'Elia *et al.,* 1983). This suggests that the uptake process observed in isolated zooxanthellae is responsible for the bulk of ammonium uptake in the intact association.

This hypothesis relies upon a diffusive uptake component from seawater which in turn would depend upon a lower ammonium concentration within the cell than in seawater. The available direct estimates of intracellular ammonium concentration have been high (5-50μM, Crossland & Barnes, 1977; Wilkerson & Muscatine, 1984) but may represent an artefact from deamination during tissue preparation. Davies (1988) indirectly estimated that the intracellular concentration of ammonium in starved aposymbiotic *Anemonia viridis* was between 20 and 30μM. This corresponded to the external concentration at which net efflux from these anemones was zero. Since ammonium was assumed to be lost by diffusion, the external and internal concentrations should be equal when there is no net exchange between the two compartments.

However, the depletion-diffusion model of nutrient acquisition has been criticized (Miller & Yellowlees, 1989; Rands *et al.,* 1993). Rands *et al.* (1993) demonstrated that both the membranes surrounding zooxanthellae of *Anemonia viridis* possessed ATPase activity and could therefore carry out selective transport processes. These authors suggested that nitrogen flux to zooxanthellae is not determined by symbiont demand as proposed by the depletion-diffusion model. Instead the selective transporters of the symbiosome membrane and the host's ability to regulate ammonium concentration by altering its rate of deamination (Rees, 1987) could together combine to control this nitrogen flux.

In microalgal cells ammonium is assimilated by the glutamine synthetase/glutamine 2-oxoglutarate amidotransferase (GS/GOGAT) cycle (Syrett, 1981). The pattern of [^{15}N]ammonium assimilation into zooxanthellae both *in vitro* (Summons & Osmond, 1981) and *in vivo* (Summons *et al.,* 1986) gives indirect evidence for the GS/GOGAT cycle as the major route of ammonium assimilation. In addition to this, GS has been detected in zooxanthellae from the anemones *Aiptasia pulchella* (Wilkerson & Muscatine, 1984), *Condylactis gigantea* and the zoanthid *Zoanthus sociatus* (Anderson & Burris, 1987). Further indirect evidence for the role of the GS/GOGAT cycle as the agent of ammonium assimilation *in vivo* is provided by the study of Rahav *et al.* (1989). These authors induced ammonium excretion by incubating the coral *Stylophora pistillata*

with azaserine which specifically inhibits the activity of GOGAT (Wallsgrove *et al.*, 1977). However, GOGAT has not been directly identified in zooxanthellae.

1.5 Host DIN assimilation

Ammonium assimilation by the host would be defined as nitrogen conservation following the scheme outlined in figure 1, and would rely on the provision of carbon skeletons from the zooxanthellae to act as amino group acceptors in the host (Rees, 1987). The role of host tissue in ammonium assimilation has provoked the most debate in the discussion of the mechanism by which symbiotic nitrogen cycling takes place (Rees, 1987; Rees, 1989b; Miller & Yellowlees, 1989). All animal cells conserve some of the ammonium produced by amino acid catabolism. This can be via NADPH-linked glutamate dehydrogenase (GDH) and glutamine synthetase (GS) (Urich, 1990). GDH has been identified in the host fractions of several anthozoan symbioses where its activity is greatest with the coenzyme NADPH (Male & Storey, 1983; Catmull *et al.*, 1987; Dudler *et al.*, 1987; Rahav *et al.*, 1989; Yellowlees *et al.*, 1994). Miller and Yellowlees (1989) argued that the high activity of NADPH-GDH in host material provided strong evidence that the host and not the zooxanthellae was the site of ammonium assimilation.

The host fraction from green *Hydra* possesses high levels of GS which is proposed to assimilate ammonium in this symbiosis (Rees, 1987). Two lines of evidence support this assertion. Firstly, GS activities are greater in host tissue from symbiotic *Hydra* than from aposymbiotic *Hydra* (Rees, 1987). Secondly, the rate of ammonium excretion by aposymbiotic *Hydra* is increased when the GS pathway is blocked using a specific inhibitor, methionine sulphoximine (MSO) (Rees, 1987). However, other interpretations of such inhibitor-based experiments are possible. For instance, since MSO will block the only pathway capable of glutamine production, protein synthesis may be inhibited. Amino acids from protein catabolism will then not be used in protein synthesis and will undergo deamination. This could increase the rate of ammonium production and excretion observed. Recently, GS activity has been measured in the host tissue from several marine symbioses, including the giant clam *Tridacna gigas* (Rees *et al.*, 1994), the coral *Pocillopora damicornis* (Yellowlees *et al.*, 1994) and the anemone *Aiptasia pallida* (Ferrier, 1996). Rees *et al.*, (1994) concluded that in *Tridacna gigas* the host would have the capacity to assimilate ammonium and so play a part in the ammonium uptake shown by this symbiosis.

1.6 Factors affecting DIN uptake

Light is required for ammonium uptake by symbiotic associations (Kawaguti, 1953; Cates & McLaughlin, 1976; Szmant-Froelich & Pilson, 1977; Muscatine *et al.*, 1984; Davies, 1988; Rahav *et al.*, 1989). The rate of ammonium uptake by *Anemonia viridis* is proportional to irradiances between 5 and 300μmol photons.$m^{-2}.s^{-1}$ (Davies, 1988). In darkness, ammonium is only released after several hours (Wilkerson & Muscatine, 1984; Davies, 1988) suggesting that, as in microalgae, a store of carbon skeletons is synthesized which allows ammonium assimilation for a limited time without photosynthesis (Syrett, 1981). A second line of evidence also shows that ammonium uptake is dependent on photosynthesis. Treatment of the coral *Stylophora pistillata* with the photosynthetic inhibitor DCMU caused ammonium excretion under conditions where untreated colonies showed net ammonium uptake (Rahav *et al.*, 1989).

As with other sessile animals, symbiotic anemones and corals rely on diffusion to supply O_2, CO_2 and inorganic nutrients and to remove waste products. A diffusive boundary layer has been shown to develop around corals which is reduced as water velocity is increased (Shashar *et al.*, 1993; Kuhl *et al.*, 1995). Atkinson *et al.* (1994) demonstrated that ammonium uptake by colonies of the coral *Porites compressa* was positively correlated with water velocity. The rate of ammonium uptake is also positively correlated with the concentration of ammonium in the incubation water. The kinetics of this are reviewed by Muscatine (1980) and, as discussed above, have been explained in terms of the 'depletion-diffusion' model of ammonium uptake (D'Elia, 1977; D'Elia *et al.*, 1983).

1.7 Translocation of nitrogen from symbiont to host

Evidence for the transfer of organic compounds from zooxanthellae to host comes primarily from experiments in which either the intact symbiosis or freshly isolated zooxanthellae are incubated with a ^{14}C labelled source of CO_2, in the form of bicarbonate. Initial studies showed that this label became incorporated into both lipid and protein in the host (von Holt & von Holt, 1968a; Muscatine & Cernichiari, 1969; Trench, 1971a). Further analysis showed that small amounts of labelled metabolites were released by freshly isolated zooxanthellae in the light (von Holt & von Holt, 1968b; Trench, 1971b) and not just because the algal cells were lysed (Trench, 1971b). The amount of photosynthate released was greatly increased when freshly isolated zooxanthellae were incubated in a homogenate of host tissue (Muscatine & Cernichiari, 1969; Trench, 1971c; Muscatine *et al.*, 1972). Analysis of this released material has shown that glycerol is the

predominant metabolite present (Trench, 1971b; Muscatine *et al.*, 1972; Schmitz & Kremer, 1977; Hofman & Kremer, 1981). However, the presence of alanine, a non-essential amino acid, has been consistently reported in these studies (Muscatine & Cernichiari, 1969; Trench, 1971b,c; Muscatine *et al.*, 1972). When ^{14}C was supplied as glucose rather than bicarbonate, the predominant product released by freshly isolated zooxanthellae of the coral *Agaricia agaricites* was alanine (Muscatine *et al.*, 1972). Further evidence that alanine forms a part of the translocate from zooxanthellae to host comes from the investigation by Lewis and Smith (1971). Their study used an inhibition technique to promote the release of photosynthate from intact algal-invertebrate symbioses. When a variety of symbiotic associations were incubated with [^{14}C]bicarbonate the rate at which ^{14}C labelled glycerol, glucose and alanine appeared in the medium could be increased by incubation with unlabelled glycerol, glucose or alanine to flood the intracellular pool of these compounds. Various other compounds had little or no effect on ^{14}C release. This was interpreted as evidence that these three compounds were translocated from zooxanthellae to host. In addition to this, incubation with 10mM ammonium increased the release of alanine, suggesting that this inorganic nitrogen was assimilated by the zooxanthellae and could be translocated to the host as amino acids such as alanine.

However, in most of these studies amino acids formed a small part of the translocated material and the predominant amino acid released was alanine, a non-essential amino acid. The only exception to this is the symbiotic acoel flatworm *Convoluta roscoffensis* where the predominant products translocated from the prasinophycean algal symbionts are the amino acids alanine, glutamine, glycine and serine (Muscatine *et al.*, 1974). The lack of evidence for a substantial transfer of nitrogen-containing compounds and essential amino acids from symbionts to host is a major criticism of the hypothesis of nitrogen recycling in cnidarian endosymbioses (Rees, 1987, 1989a,b; Douglas, 1987, 1994). Evidence that essential amino acids are translocated from zooxanthellae to host is very sparse. Markell and Trench (1993) showed that five species of cultured zooxanthellae released protein-containing compounds termed glycoconjugates which were found to contain all the essential amino acids. These authors suggested that if the proteins were digested by the host then these glycoconjugates could provide a source of essential amino acids. Despite the lack of biochemical evidence for a significant transfer of nitrogen-containing compounds from zooxanthellae to host, Falkowski *et al.* (1993), reviewing symbiont population control in corals, postulated that such a flux must exist. Since the

flux of ammonium from host to zooxanthellae appears to exceed the growth demand of the zooxanthellae, Falkowski *et al.* (1993) proposed that translocation of organic nitrogen, including essential amino acids, to the host would prevent the zooxanthellae from accumulating nitrogen.

1.8 Symbiont nutrient status

The ratio of zooxanthellae to host biomass must be regulated, since under constant environmental conditions the zooxanthellae population density is maintained in an equilibrium state with the growth of the host (Taylor, 1969; Muscatine & Pool, 1979). In other words, neither zooxanthellae nor host outgrow the other. Trench (1987) proposed three mechanisms which could produce balanced growth: (1) synchronized division of host and symbiont cells; (2) removal of excess symbiont cells; (3) host control of symbiont division by limiting nutrient availability. The *Chlorella* symbionts of *Hydra* were only found to divide when the host cell was stimulated to divide (McAuley, 1981). There is no such evidence of synchronized host and symbiont division within zooxanthellae symbioses (Davies, 1992). The removal of excess symbionts could be via two mechanisms, namely expulsion or digestion of symbionts. Continuous, low level expulsion of symbionts has been recorded in a number of symbioses (e.g. Steele, 1975, 1976, 1977; Steen & Muscatine, 1987; Stimson & Kinzie, 1991) including *Anemonia viridis* (Taylor, 1969; Beaver, 1996). There is also some evidence for host digestion of symbionts. Janssen and Möller (1981) suggested that acid phosphatases surrounding zooxanthellae from *Anemonia viridis* provided evidence for host digestion of symbionts. Recently, Titlyanov *et al.* (1996) have concluded that the coral *Stylophora pistillata* could regulate its zooxanthellae population by digesting symbionts and expelling their remains. However, it is the third mechanism of producing balanced growth, nutrient availability, which has received the most attention. Cook and D'Elia (1987) proposed that the zooxanthellae population could be regulated by limiting their nutrient supply. The evidence that symbiotic algae are nutrient deficient in terms of nitrogen (Flynn, 1990) is discussed below.

1.8.1 Zooxanthellae population

Addition of ammonium increases the symbiont population density (Hoegh-Guldberg & Smith, 1989; Muscatine *et al.*, 1989; Dubinsky *et al.*, 1990; Stambler *et al.*, 1991; Hoegh-Guldberg, 1994; Muller-Parker *et al.*, 1994) and zooxanthellae mitotic index (Cook *et al.*, 1988; Cook & Fitt, 1989; McAuley & Cook, 1994; Beaver, 1996). This

phenomenon has also been observed in the field where the peak mitotic index of zooxanthellae in symbiosis with *Mastigias* sp. corresponded with the jellyfish host's migration to an ammonium-rich nutricline (Muscatine & Marian, 1982). Heterotrophic feeding by the host increases the mitotic index of zooxanthellae from the marine hydroid *Myrionema amboinense* (Fitt & Cook, 1989; McAuley & Cook, 1994), the sea anemones *Aiptasia pallida* (Cook *et al.*, 1988) and *Anemonia viridis* (Beaver, 1996) but had no effect on the zooxanthellae division rate in the coral *Stylophora pistillata* (Muscatine *et al.*, 1989).

1.8.2 Photosynthesis

Measurement of the change in the maximal quantum yield of chlorophyll fluorescence provides evidence that zooxanthellae photosynthesis may be nitrogen-limited (Falkowski *et al.*, 1993). When algal cells are deprived of nitrogen, the ratio of the maximal variable fluorescence to the minimal fluorescence drops below 1.6, independent of light intensity (Kolber *et al.*, 1988). This decrease is caused by the loss of proteins that transfer excitation energy to the photosynthetic reaction centre (Falkowski *et al.*, 1992; Kolber *et al.*, 1988). Intact corals show changes in the quantum yield of fluorescence of 0.5 to 0.6. This is increased to 0.88 after six weeks at 20μM ammonium suggesting that nitrogen limitation is reduced by treatment with ammonium (Falkowski *et al.*, 1993).

1.8.3 Nitrogen assimilation

Nitrogen-limited algal cells would be expected to show enhanced uptake rates for nitrogenous compounds when transferred to a medium containing nitrogen (Syrett, 1981; Rees, 1991). The comparison between ammonium uptake rates of nitrogen-starved cultured zooxanthellae and freshly isolated zooxanthellae by Gunnerson *et al.* (1988) showed that the latter had lower ammonium uptake rates. This suggests that these freshly isolated zooxanthellae were not nitrogen-limited. Nitrogen-limited microalgae assimilate ammonium in darkness because carbon skeletons produced during photosynthesis remain available as a substrate for assimilation (Syrett, 1981). Ammonium assimilation in darkness has been observed in several symbioses including *Aiptasia pulchella* (Wilkerson & Muscatine, 1984), *Anemonia viridis* (Davies, 1988) and also in freshly isolated symbionts from a variety of marine symbioses (D'Elia *et al.*, 1983). This assimilation in darkness could imply nitrogen-limitation and so contradicts the suggestion by Gunnerson *et al.* (1988) that zooxanthellae are not nitrogen-limited.

1.8.4 C:N ratio

The C:N ratio of nitrogen-limited microalgal cells increases because reserves of carbon accumulate in the absence of assimilated nitrogen. When symbiotic associations are incubated with increased levels of ammonium, the C:N ratio decreases (Muscatine *et al.*, 1989; Snidvongs & Kinzie, 1994) suggesting that these cells were nitrogen-limited. However, these data have been criticized by Rees (1991) for two reasons. Firstly, that any host contamination of zooxanthellae samples could lead to artificial differences in C:N ratios. Secondly, that since symbiotic algae release photosynthate predominantly in the form of carbohydrate or lipid the C:N ratio of these algae will appear nutrient-sufficient, since 'excess' carbon is removed by translocation.

1.8.5 Ammonium enhancement of dark carbon fixation

Ammonium enhances dark carbon fixation in nitrogen-deficient cells (Flynn, 1990). This is because the addition of ammonium to such cells promotes amino acid synthesis, requiring organic acids, and enhanced carbon fixation is needed to replace the organic acids used. Cook *et al.* (1992) showed that the feeding history of the anemone *Aiptasia pallida* modulated the degree to which ammonium enhanced dark carbon fixation. The enhancement was greatest in zooxanthellae from starved anemones and was very low from fed anemones. This suggests that the zooxanthellae from the starved anemones were nitrogen-limited. The same trend with feeding was seen with zooxanthellae from the coral *Madracis mirabilis* (Cook *et al.*, 1994). Zooxanthellae from *Montastrea annularis* containing a high symbiont density had higher ammonium enhancement values than zooxanthellae from colonies which, because of a minor bleaching event, had a lower symbiont density (Cook *et al.*, 1994). These authors proposed that the degree to which zooxanthellae were nitrogen deficient was positively correlated with symbiont density.

1.8.6 Glutamine:Glutamate ratio

The ratio of glutamine to glutamate is an important indicator of nitrogen status in microalgae (Flynn *et al.*, 1989; Flynn, 1990). When the supply of carbon as 2-oxoglutarate limits the rate of the GS/GOGAT cycle (fig. 4.1, page 63) the levels of glutamine increase relative to glutamate causing the ratio of gln:glu to increase (>0.5). If this ratio is low (<0.2) it suggests that the system is limited with respect to nitrogen. This could either be due to a diminished nitrogen supply, or to a physiological nitrogen stress such as a shortfall in a reductant (Flynn, 1990) which would reduce the rate of ammonium assimilation. McAuley (1992) compared several physiological parameters

from the *Chlorella* symbiont of *Hydra* which could indicate nitrogen limitation: gln:glu ratio, N:C ratio and the cellular chlorophyll and protein contents. These indicators suggested that the symbionts may be nitrogen-deficient because their capacity to assimilate nitrogen is reduced by carbon translocation as maltose to the host (Rees, 1989a, 1990; McAuley, 1992, 1996). Under conditions of low DIN availability, zooxanthellae can show low gln:glu ratios. When *Pocillopora damicornis* was maintained under low nutrient conditions the zooxanthellae showed gln:glu ratios which would be characteristic of nitrogen limitation by Flynn's (1990) definition (McAuley, 1994). When this coral was maintained in 20μM ammonium the gln:glu ratio was no longer indicative of nitrogen limitation. The same study made a comparison with zooxanthellae from *Anemonia viridis,* which had a gln:glu ratio higher again suggesting that these symbionts were not nitrogen-limited (McAuley, 1994).

Rees (1991) proposed that the evidence for symbiont nutrient limitation was more consistent with a system where the host restricts nutrient supply to its symbionts (Rees, 1989a). The symbionts suffer from a restricted nutrient supply largely because they transfer photosynthate to the host. This allows the host to reduce its rate of deamination (Rees, 1989a, 1991; Rees & Ellard, 1989) and so limit the ammonium available to the symbionts. Clearly the question of whether or not symbiotic algae are nitrogen-limited *in hospite* does not have a simple and universal answer. From the evidence it appears that under low nitrogen conditions and in the absence of host feeding, some symbiotic algae may be nitrogen-limited.

1.9 Aims of the experimental work

The above evidence suggests that, to a greater or lesser extent, zooxanthellae are limited or 'impoverished' (Davies, 1992) with respect to nitrogen. This presents a clear paradox if the zooxanthellae are indeed translocating significant amounts of organic nitrogen to the host rather than using this limiting nutrient for their own maintenance and growth. The investigations summarised above have shown that microalgal-invertebrate symbioses retain and can take up inorganic nitrogen. There is some evidence that this can contribute to the nitrogen requirements of these organisms.

The experimental work described in chapter 3 was carried out to investigate the quantitative aspects of nitrogen cycling by constructing a nitrogen budget. While it appears that the zooxanthellae are the principle site of ammonium assimilation, it is

possible that a flux of nitrogen from zooxanthellae to host could provide the host with a substrate for growth. In chapter 4, the free amino acid pools, gln:glu ratios and enzymatic mechanisms by which nitrogen cycling could take place were analysed. The final aim of this investigation was addressed in chapter 5. Here the pathway of ammonium assimilation was followed using ^{15}N to see whether the process of nitrogen cycling is dominated by recycling via the zooxanthellae or conservation by the host. Chapter 5 also addressed the qualitative nature of any nitrogen flux from zooxanthellae to host by analysing the amino acid products of ammonium assimilation in both components of the symbiosis.

Chapter 2

Methods

The methods summarised here have been used routinely throughout the experimental work described in this thesis. Where techniques only apply to a particular chapter the methods can be found in the appropriate section of that chapter.

2.1 Animal collection and maintenance

Anemonia viridis (Forskål) (figure 2.1) of the brown colour morph were collected from between 1 and 2m depth in Loch Sween, Argyll, on the west coast of Scotland and maintained in recirculating seawater aquaria at the Division of Environmental and Evolutionary Biology (University of Glasgow) under the following standard conditions:

- temperature of 15°C (± 1°C),
- salinity of 34‰
- illumination of 70μmol photons.$m^{-2}.s^{-1}$
- 12:12 hour daily photoperiod.

Illumination was provided by cool white fluorescent strip lights. Anemones were fed weekly on chopped, frozen mussel (*Mytilus edulis*) tissue. Since individual anemones may have experienced different environmental regimes in the field, the animals were acclimatised to these standard conditions for at least four weeks before use in experiments. Aposymbiotic anemones were used which had been in darkness for between four and five years. These were maintained in darkness under the same standard conditions as symbiotic anemones but were fed twice a week.

2.2 Biomass determination by buoyant weighing

The weight of anemones in seawater was determined by the buoyant weighing technique of Tytler (1982) using an electronic balance (Mettler AJ50). The buoyant weight was converted to dry weight using the following equation for anemones weighed at 15°C (pers. comm. A. Harland):

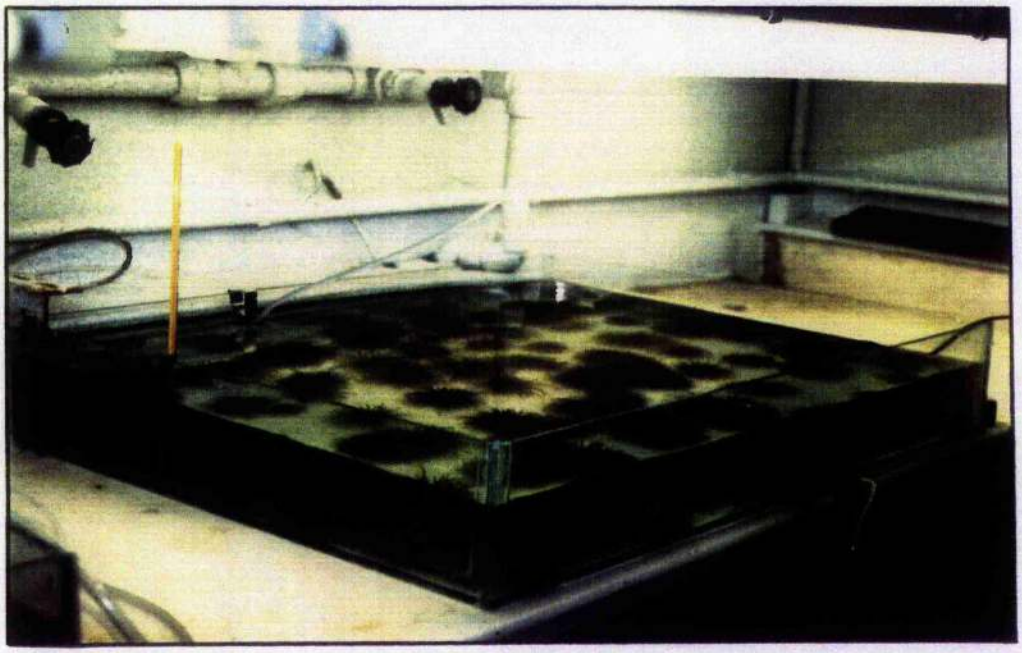
Dry weight = 0.0506 + (5.8824.Buoyant weight)

Figure 2.1

(a) *Anemonia viridis* in the field and (b) the shallow aquarium system used to maintain anemones under standard conditions of illumination, temperature and salinity.

a)

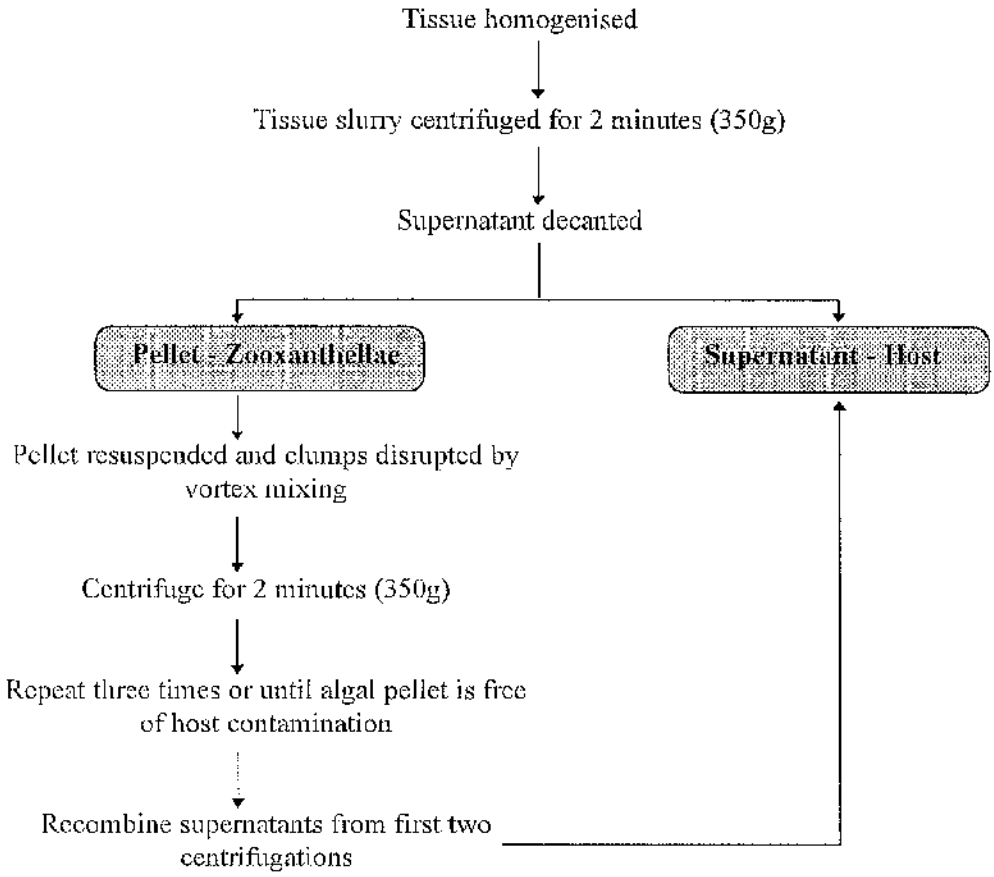
b)



2.3 Separation of zooxanthellae and host material

Zooxanthellae and host material were separated by homogenisation (Ultra-Turrax T25, Janke & Kunkel) followed by repeated centrifugation and washing as described in figure 2.2. This method was adapted from that of Davies (1988). The separation of zooxanthellae and host was carried out in either filtered seawater (0.3µm) or artificial seawater (w/v: 3.1% NaCl, 1.0% $MgSO_4$, 0.002% $NaHCO_3$) (Harvey, 1957). A sample of the algal pellet was examined microscopically after the centrifugation and washing procedure had been repeated three times. If host contamination was still evident the procedure was repeated. However, only the first two supernatants produced were recombined to produce the host extract.

Figure 2.2 Separation of zooxanthellae and host material



2.4 Seawater ammonium analysis

Two ammonium analyses were used in this study. Both are based on the indophenol reaction first described by Berthelot (1859) and reviewed by Searle (1984). The first analysis is based on the method of Solórzano (1969) incorporating the modifications of Liddicoat *et al.* (1975) and was used for analyses where there were less than ten samples to process at any one time. The second method was developed to enable many samples to be processed and analysed together. This was necessary in order to conduct the ammonium uptake experiments described below and in chapter 3. This second procedure used a scaled down method based on that described by Parsons *et al.* (1984) and was carried out using a 96 well microplate format. In both ammonium assay methods all glassware was cleaned of any contaminating ammonia by soaking in 15% hydrochloric acid and rinsing five times in de-ionised water before use. All reagents were of analytical grade and were prepared using deionised distilled water (DDW).

2.4.1 Method 1

This method was employed when fewer than ten samples were to be processed at any one time and was based upon the method of Solórzano (1969).

Reagents

1. Phenol-alcohol: 10g phenol in 75ml absolute ethanol and 25ml acetone.
2. Catalyst: 0.5g potassium ferrocyanide in 100ml DDW (protected from light).
3. Oxidising solution: 0.2g sodium dichloroisocyanurate in a solution of 1.6g sodium hydroxide in 40ml DDW. A solution of 20g trisodium citrate dissolved in 40ml of DDW was added to this and the volume made up to 100ml (prepared each day).
4. Artificial seawater (Harvey, 1957): 31g sodium chloride, 10g magnesium sulphate, 0.02g sodium bicarbonate dissolved in 1000ml DDW.

Procedure

Seawater samples of 1ml volume were taken and stored in capped acid-washed glass vials. If samples needed to be stored until analysis, 40µl of the phenol reagent was added and the samples were frozen at -20°C until analysis (Degobbis, 1973). Following addition of the phenol reagent, the sample was thoroughly mixed and 100µl of the oxidising solution was added. The sample was again mixed and 40µl of the catalyst was added. The samples were then exposed to ultraviolet light for 40 minutes for colour

development to take place. The absorbance of samples and standards prepared in artificial seawater were determined at 640nm against a reagent blank (Phillips PU8720 spectrophotometer).

2.4.2 Method 2

This method was developed in order to process up to 192 samples at a time and is based upon the method of Parsons *et al.* (1984). It involved automating both the preparation and reading of the assay by adopting a 96 well plastic microplate format. The procedure described here incorporates several modifications from the method described above since it was found that the characteristic blue colour of the indophenol reaction produced by method 1 failed to develop in the plastic wells of the microplate.

Reagents

All reagents apart from the catalyst were prepared as described for method 1. The catalyst used in this procedure was that described by Parsons *et al.* (1984) and was prepared by dissolving 0.05g sodium nitroprusside in 10ml DDW (protected from light).

Procedure

A 250µl sample of seawater was added by pipette into each microplate well (Corning). 10µl of the phenol reagent and 30µl of the oxidising reagent were added to each well and the contents were thoroughly mixed using a multichannel pipette. 10µl of the sodium nitroprusside catalyst was then added to each well and the contents were again thoroughly mixed by pipetting. The plates were incubated for 20 minutes in the dark at room temperature and the absorbance of samples and standards recorded at 620nm (Dynatech plate reader). Since no published studies describing microplate-based ammonium assays could be found at the time this assay was developed, the method was cross-calibrated with an established continuous flow analysis method (Kahn, 1994) based on the original procedure of Brown (1973). Ten samples were analysed by both methods and no significant difference was found (Mann-Whitney U test, $p<0.05$, data not shown). A similar technique has since been described by Craggs *et al.* (1994).

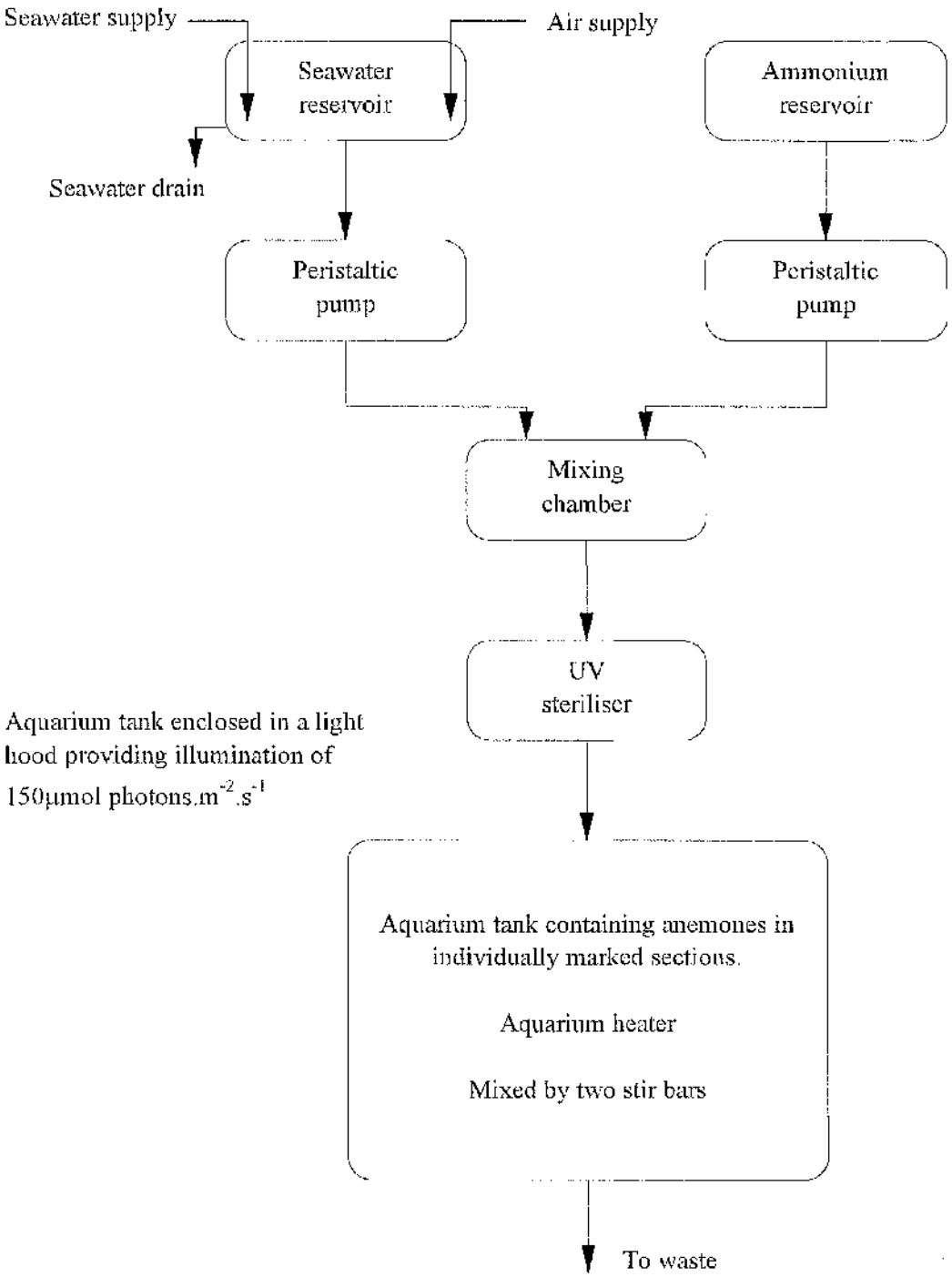
2.5 Ammonium exposure apparatus

Figure 2.3 illustrates schematically the apparatus used to maintain anemones under a constant ammonium exposure for prolonged periods. A 2mM stock solution of ammonium sulphate was prepared in distilled water and added to the ammonium reservoir. This was then attached to a peristaltic pump (Watson-Marlow 101U) which delivered a constant inflow into a glass mixing chamber. A second peristaltic pump (Watson-Marlow 501U) from a seawater reservoir delivered a constant inflow of seawater to the same chamber. Here the seawater and ammonium solutions were thoroughly mixed in a ratio of 100:1 by a magnetic stir bar to give a final ammonium concentration of 20μM. The ammonium-enriched seawater was then passed through a UV steriliser (Tropical Marine Centre) before it entered an aquarium tank containing the anemones. Within this tank an aquarium heater maintained the experimental temperature of 15°C and the water was continuously circulated by magnetic stir bars. The tank containing the anemones was enclosed under a light hood providing an illumination of 150μmol photons.$m^{-2}.s^{-1}$ with a 12:12 hour photoperiod and the whole apparatus was housed in a constant temperature room (8°C). The tank and tubing were cleaned twice a week to prevent algal growth. Seawater samples were taken both from the inflow supplying the tank and from the tank itself and assayed using the method described in section 2.4.1 to confirm the concentration of ammonium to which the anemones were exposed.

2.6 Flow-through ammonium depletion apparatus

A frequently adopted depletion-based method to calculate the rate of ammonium uptake in algal-invertebrate symbioses is to produce a cumulative depletion curve as ammonium from an initial spike is removed from the seawater and taken up by the organism (e.g. Wilkerson & Muscatine, 1984; D'Elia *et al.*, 1983). This curve can then be divided into time periods and the mean concentration and uptake rate calculated for each period. The latter can either be calculated from the tangent to the curve or from the difference in concentrations between the start and end of an incubation time period. However, this method was found to be inadequate to measure ammonium uptake by *Anemonia viridis*, since the variability of the depletion data precluded fitting cumulative depletion curves (Davies, 1988). Davies therefore measured ammonium uptake by *Anemonia viridis* by incubating individual anemones in chambers with seawater spiked to a known concentration of ammonium. A sample of seawater was taken at the beginning of each incubation and a second sample was taken an hour later, and the concentration of ammonium in the samples was measured.

Figure 2.3
Schematic diagram of the flow-through apparatus used to maintain anemones in 20μM ammonium supplemented seawater.



In chambers containing symbiotic anemones in the light the concentration of ammonium was found to decrease over this time period. This depletion was assumed to represent uptake of ammonium by the anemone and was used to calculate the rate of uptake.

This method still suffers from one major drawback. During the one hour incubation the concentration of ammonium to which the anemone is exposed is continually decreasing as it is taken up by the anemone. This means that the anemone is exposed to a continuously diminishing concentration of substrate. The method described here is intended to overcome this problem by supplying each anemone with a constant inflow of ammonium-supplemented seawater. It was reasoned that in such a system the ammonium concentration of seawater flowing out of a chamber containing a symbiotic anemone in the light would decrease until it reached a steady state. The difference between this steady state concentration and that supplying the chamber would therefore represent uptake by the anemone.

Flow-through chambers to contain individual anemones were made using 250ml volume Pyrex glass beakers. The beakers were fitted with glass spouts which overflowed when filled to approximately 200ml. Each chamber was supplied with a glass inflow tube to deliver seawater to the base of the chamber and a second glass tube to deliver a gentle aeration. This aeration was supplied by an aquarium pump and the air was drawn through a 2M sulphuric acid trap to remove any atmospheric ammonia. Preliminary experiments showed that this aeration provided sufficient mixing and did not reduce the concentration of ammonium within the chambers (data not shown). The inflow tubes supplying each chamber were connected to one of ten 'Autoprene' peristaltic pump tubes (Santoprene tubing, Autoclude). The constant inflow of seawater was generated using a ten channel peristaltic pump (Autoclude VL) and came from a seawater reservoir spiked with ammonium sulphate. This apparatus is illustrated schematically in figure 2.4 and shown in figure 2.5.

Anemones of a similar mass were buoyant weighed and transferred to the incubation chambers containing filtered (0.3μm) seawater which had been passed through a UV steriliser for at least 24 hours before use. These animals were allowed at least one hour to attach to the base of the beaker during which time they were maintained under the experimental irradiance and temperature of 15°C.

Figure 2.4
Schematic diagram of the flow-through apparatus used to measure the rate of ammonium uptake from individual symbiotic anemones. Diagram shows one of ten replicate incubation chambers.

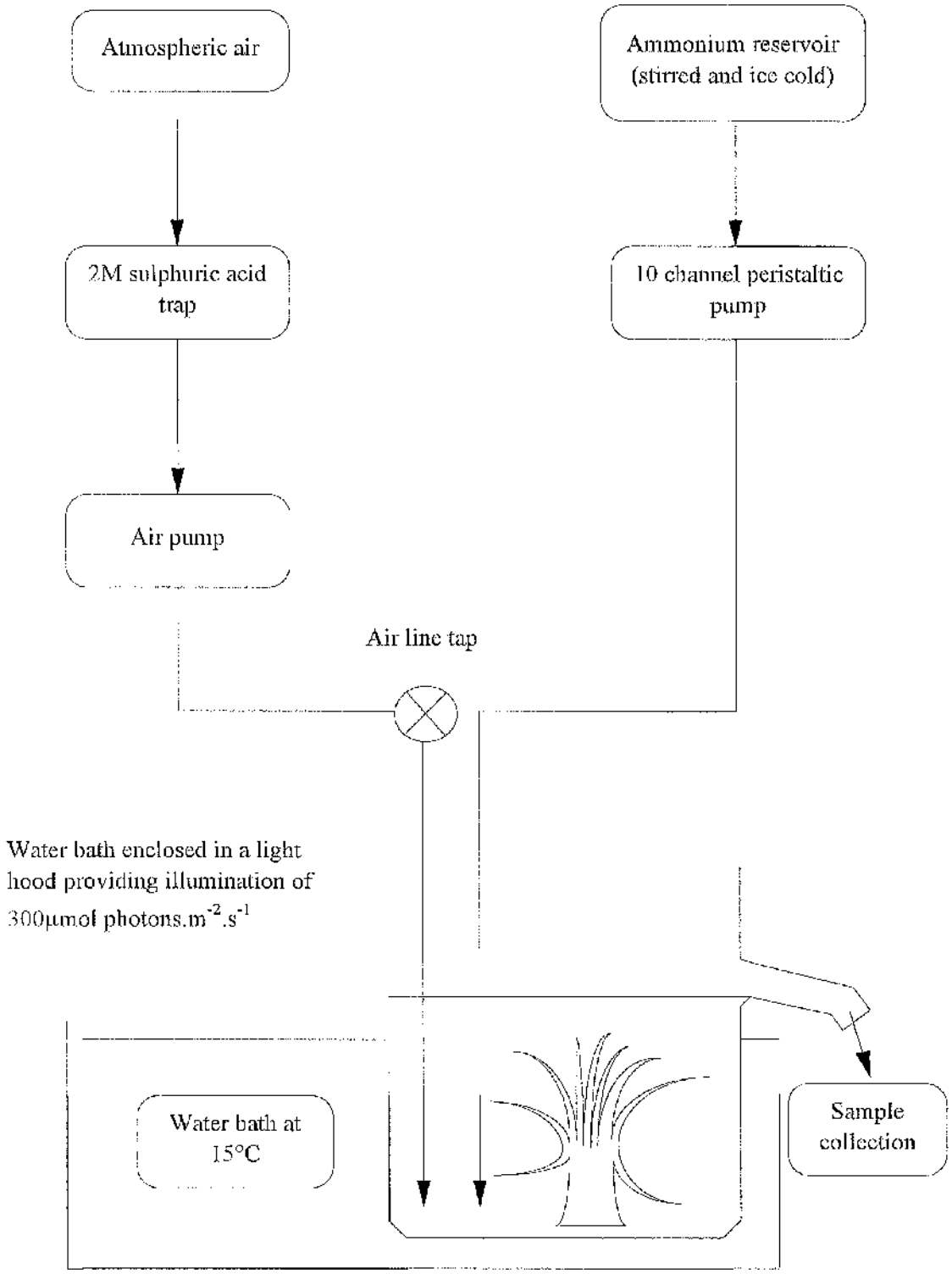
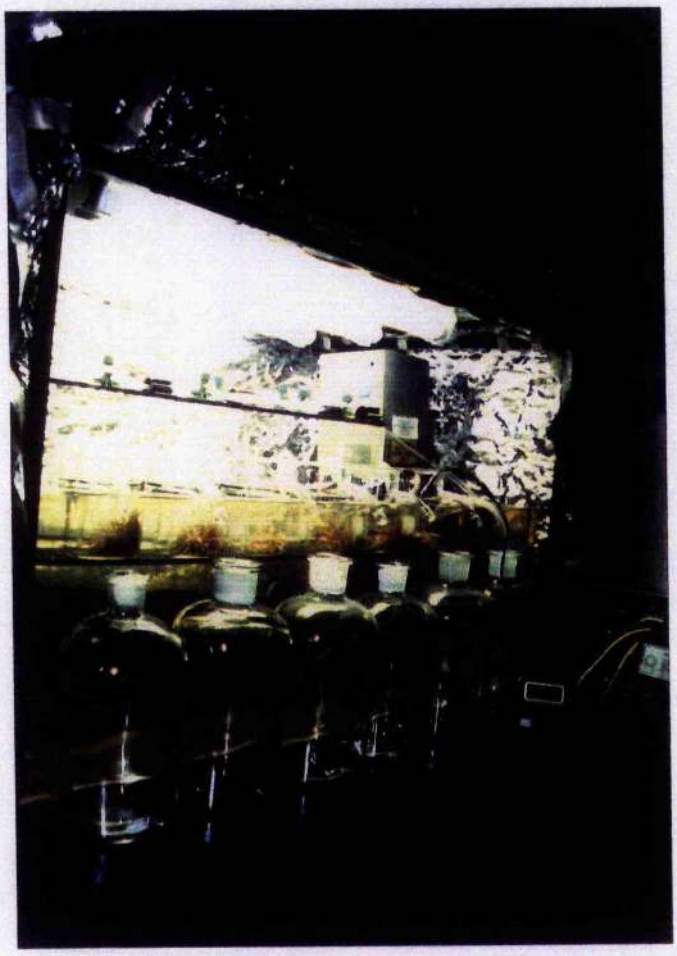


Figure 2.5

Flow-through ammonium depletion apparatus showing overflow chambers containing symbiotic anemones, the light hood and constant temperature water bath used to measure the rate of ammonium uptake.

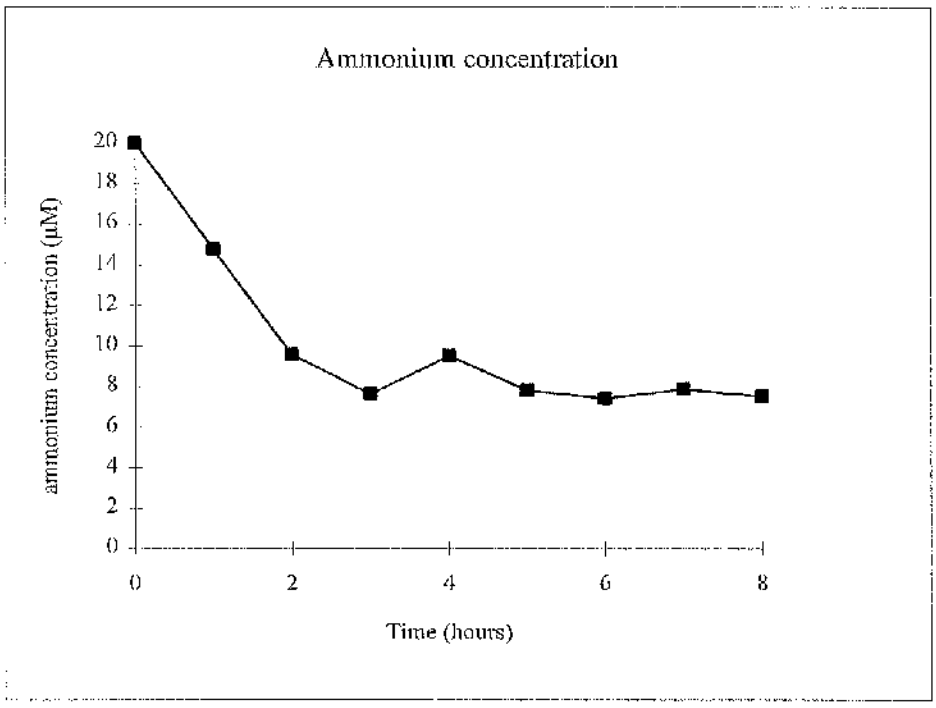


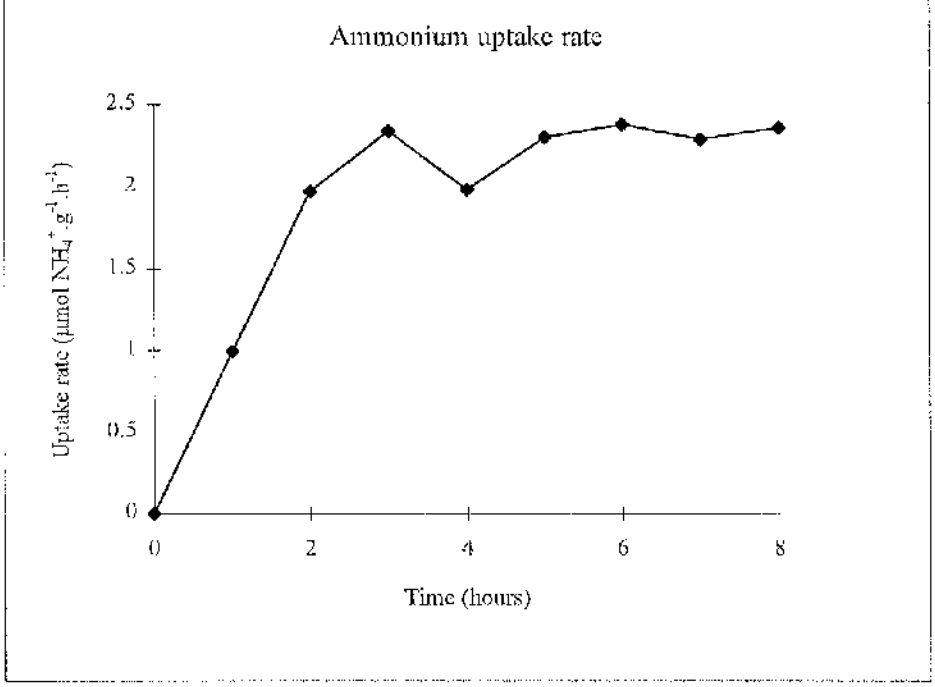
Only those animals that had attached to the surface of the chambers and fully 'flowered' were used. All experiments were carried out three days after feeding and during the animals' 12 hour light period. Once the anemones had settled, the seawater in the chambers was drained and replaced with filtered seawater enriched with ammonium sulphate. The inflows and air supplies to each chamber were then connected. The overflow from each chamber was collected and used to calculate the flow rate. During each experiment samples were taken from the outflow at hourly intervals for between 6 and 10 hours and stored in capped acid-washed glass vials at 4°C until analysis. Samples were also taken from the inflows to each chamber, the ammonium-enriched reservoir and from the outflow of a control chamber which did not contain an anemone. The ammonium concentration of duplicate seawater samples was assayed using the micro-assay described in section 2.4.2. The apparatus and associated tubing were thoroughly cleaned to prevent contamination with algae or bacteria which could produce detectable ammonium depletion.

Figure 2.6 illustrates both the ammonium concentration leaving a chamber containing a symbiotic anemone and the corresponding ammonium uptake rate. The ammonium concentration falls over the first three hours but then remains at a steady state for the next five hours. The difference between the inflow concentration and this steady state outflow concentration was used to calculate the steady state rate of ammonium uptake.

$$\text{Uptake rate} = \underset{(\mu mol.l^{-1})}{\Delta[NH_4^+]} \times \underset{(l.h^{-1})}{\text{flow rate}} \times \underset{(g^{-1})}{(1/\text{dry weight})} = \mu mol\ NH_4^+.g^{-1}.h^{-1}$$

Figure 2.6
Ammonium concentration of the outflow from a chamber containing a symbiotic anemone with a 20μM ammonium inflow concentration and the corresponding ammonium uptake rates.
Illumination of 300μmol photons.$m^{-2}.s^{-1}$.





2.6.1 Ammonium flux in *Anemonia viridis*

A series of experiments were carried out to see whether the flow-through ammonium depletion apparatus described above produced results which were comparable with those obtained by Davies (1988) who used a closed chamber depletion method. Uptake by symbiotic anemones was measured at ammonium concentrations of 20, 30 and 40μM. Ammonium excretion by aposymbiotic anemones was measured at 20μM.

2.6.2 Methods

The anemones used in these experiments had been maintained under photosaturating light of 300μmol photons.$m^{-2}.s^{-1}$ (Harland and Davies, 1994) and were unfed for a period of at least three weeks before use in experiments. They are thus comparable with the unfed animals of Davies (1988). The anemones were unfed to eliminate any post-prandial effects on nitrogen metabolism (Fitt, 1984; Wilkerson & Muscatine, 1984) which have been documented for respiration in *Anemonia viridis* (Tytler & Davies, 1986).

Ammonium uptake by symbiotic anemones in the light was measured using the flow-through apparatus under an illumination of 300μmol photons.$m^{-2}.s^{-1}$ (figure 2.4). This protocol is described in section 2.6. The rate of uptake from seawater supplemented with ammonium at either 20, 30 or 40μM was recorded. In two experiments, ammonium efflux by unfed aposymbiotic anemones was also measured. Aposymbiotic anemones were maintained under the same conditions as symbiotic anemones and were starved for a period of three weeks before use. These experiments were carried out in the presence of a 20μM ammonium inflow concentration.

2.6.3 Results

In all incubations conducted the concentration of ammonium leaving experimental chambers containing symbiotic anemones was lower than that leaving control chambers. In other words symbiotic anemones showed the capacity to remove ammonium from the seawater. Aposymbiotic individuals increased the concentration of ammonium assayed showing that these anemones released ammonium. After 2 to 3 hours, the ammonium concentration of the outflow was seen to reach a steady state. This is illustrated in figure 2.7 where positive rates indicate uptake and negative rates indicate release of ammonium.

The steady state mass specific ammonium uptake rates recorded for symbiotic anemones in the light are listed in table 2.1 and summarised in figure 2.8.

Table 2.1 Steady state ammonium uptake rates of symbiotic and excretion rates of aposymbiotic anemones from incubations carried out at 20, 30 and 40μM ammonium. Mean, expressed per g dry weight (±SD).

Incubation concentration (μM)	Anemone type	Mean steady state uptake rate ($\mu mol\ NH_4^+.g^{-1}.h^{-1}$)
20	Symbiotic (n=7)	1.487 (0.291)
20	Symbiotic (n=4)	2.013 (0.584)
20	Symbiotic (n=8)	2.246 (0.477)
20	Symbiotic (n=2)	2.174 (0.211)
20	Symbiotic (n=8)	2.187 (0.477)
30	Symbiotic (n=4)	3.795 (1.186)
30	Symbiotic (n=4)	3.771 (0.781)
40	Symbiotic (n=2)	3.847 (0.901)
40	Symbiotic (n=5)	4.696 (0.955)
20	Aposymbiotic (n=2)	-2.295 (1.325)
20	Aposymbiotic (n=1)	-2.028

The rates recorded are from independent experiments. Positive rates indicate uptake and negative rates indicate release of ammonium.

The ammonium uptake rate data collected from symbiotic anemones showed homogeneity of variance (Levene's test). Statistical difference between ammonium concentration treatments was assessed using one way analysis of variance followed by Tukey pairwise comparison. This analysis showed that the rate of ammonium uptake increased significantly between 20μM and both 30μM and 40μM ($p<0.05$) but the increase between 30μM and 40μM was not significant ($p>0.05$).

Figure 2.7
Mean rate of ammonium uptake by symbiotic (n=4) and release by aposymbiotic (n=2) anemones incubated with a 20μM ammonium inflow concentration and 300μmol photons.$m^{-2}.s^{-1}$ illumination (±SE).

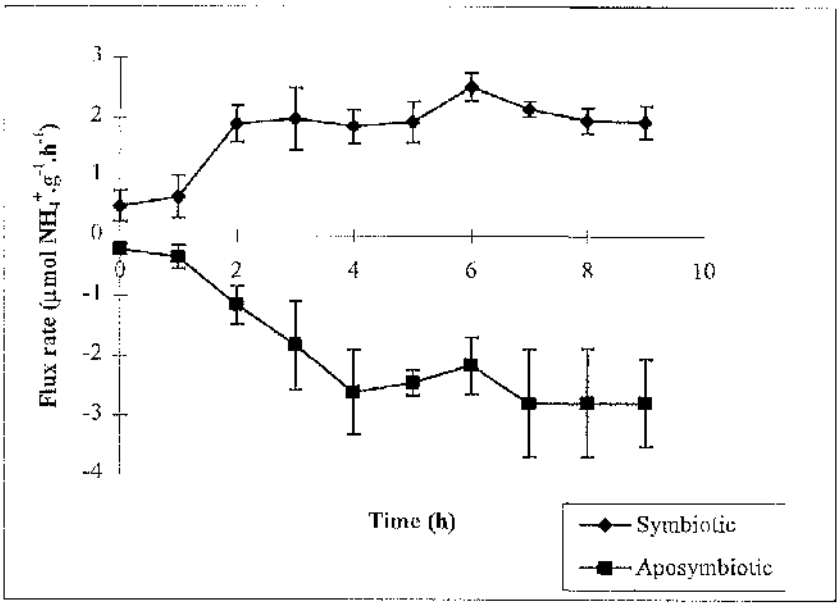
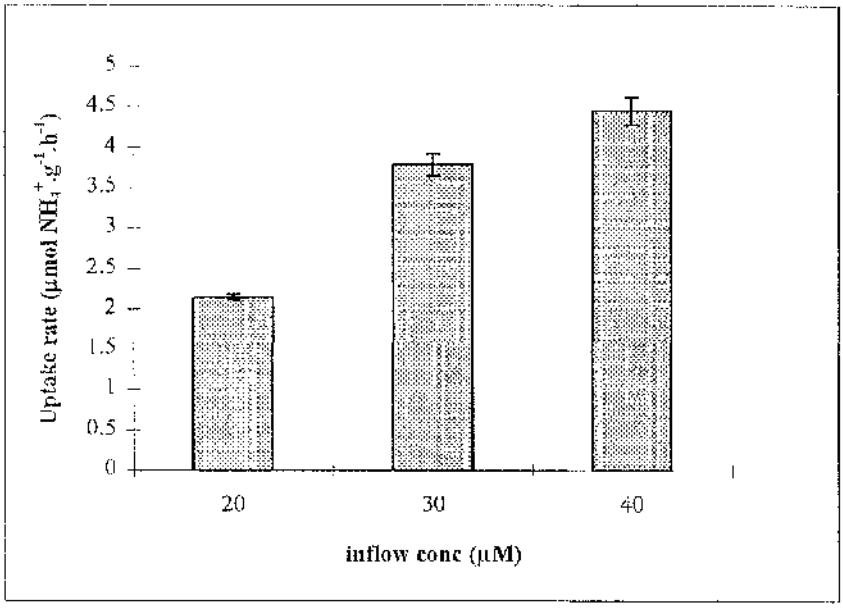


Figure 2.8
The effect of inflow concentration on the rate of ammonium uptake by symbiotic anemones with an illumination of 300μmol photons.$m^{-2}.s^{-1}$. The mean ammonium uptake rates of all experiments are shown (±SE) at 20μM (n=29), 30μM (n=8) and 40μM (n=7).



2.6.4 Discussion

In all experiments the concentration of ammonium in seawater leaving the incubation chambers containing symbiotic anemones in the light was lower than that entering (or leaving control chambers which did not contain anemones). The difference between inflow and outflow concentrations was used to calculate the rate of ammonium uptake by the anemones. The rates of ammonium uptake measured corresponded closely to those recorded by Davies (1988) and are compared in table 2.2.

Table 2.2 Comparison of the rates of ammonium uptake calculated during this study and that of Davies (1988). All experiments were conducted on unfed symbiotic *Anemonia viridis* under 300µmol photons.$m^{-2}.s^{-1}$ illumination.

Concentration of ammonium (µM)	**Uptake rate from this study (µmol $NH_4^+.g^{-1}.h^{-1}$)**	**Uptake rate from Davies (1988) (µmol $NH_4^+.g^{-1}.h^{-1}$)**
20	2.129 (0.515)	2.344
30	3.782 (0.979)	3.574
40	4.454 (1.005)	4.804

Uptake rates from this study are expressed as the mean of all the data collected (±SD). The uptake rates from the data of Davies (1988) have been calculated using the equation for the line describing the relationship between ammonium concentration and uptake rate.

When aposymbiotic anemones were incubated in flow-through chambers supplied with 20µM ammonium the concentration leaving the chambers was seen to increase. This increase was assumed to represent ammonium production by the anemone. Davies (1988) found that the rate of ammonium production by aposymbiotic anemones declined with increasing ammonium concentration of seawater. Using the equation of the line that describes the relationship between aposymbiotic nitrogen flux and ammonium concentration, the results of Davies (1988) predict that at 20µM there should be very little ammonium production (-0.058µmol $NH_4^+.g^{-1}.h^{-1}$). In contrast to this, the present study found the nitrogen flux of aposymbiotic anemones with a constant inflow of 20µM ammonium was -2.228µmol $NH_4^+.g^{-1}.h^{-1}$.

Davies (1988) interpreted the ambient concentration at which the net nitrogen flux was zero to be equal to the intracellular concentration of ammonium. On the basis that ammonium is lost by diffusion and that the rate of diffusion is directly proportional to the external concentration, Davies (1988) estimated the intracellular concentration of

ammonium in starved aposymbiotic anemones to be 20.72μM (±2.74). However, in the present study when aposymbiotic anemones were incubated with a 20μM inflow of ammonium, the concentration of ammonium leaving these chambers had increased suggesting that ammonium was produced and excreted into seawater at this external concentration. The reasons for this difference between these two sets of results are not clear. It seems unlikely that the intracellular ammonium concentrations could have differed between the studies since in both investigations aposymbiotic anemones were unfed for the same time period before experiment. It also seems unlikely that the different rates measured represent an artefact of the different methods used. Although Davies (1988) used a static system to record ammonium production, each incubation was limited to 30 minutes duration to minimise any metabolic inhibition caused by the build up of ammonium. The present study used a flow through system which will have removed ammonium as it was produced but at such a rate that a detectable increase in ammonium concentration could be measured.

2.7 Statistical analysis

All statistical analysis was carried out using Minitab statistical software on a personal computer.

Chapter 3

Nitrogen budget of symbiotic anemones

3.1 Introduction

Symbiotic cnidarians are able to deplete ambient ammonium when exposed to light (Kawaguti, 1953; Muscatine & D'Elia, 1978; Wilkerson & Trench, 1986; Davies, 1988) whereas aposymbiotic individuals excrete ammonium (Cates & McLaughlin, 1976; Szmant-Froelich & Pilson, 1977; Muscatine *et al.*, 1979; Wilkerson & Muscatine, 1984; Davies, 1988). Non-symbiotic ahermatypic corals also excrete ammonium (Muscatine & D'Elia, 1978; Burris, 1983). Some symbiotic corals show the capacity to take up nitrate (e.g. D'Elia & Webb, 1977; Franzisket, 1973, 1974; Webb and Wiebe, 1978; Wilkerson & Trench, 1986; Marubini, 1996; Marubini & Davies, 1996) but this has never been demonstrated in symbiotic anemones (Wilkerson & Muscatine, 1984; Davies, 1988). This is believed to be due to the inhibition of nitrate uptake and/or repression of the assimilatory enzyme, nitrate reductase (Syrett, 1981). Davies (1988) demonstrated that symbiotic *Anemonia viridis* took up ammonium and that there was a linear relationship between the weight-specific ammonium flux and the concentration of ammonium to which the anemones were exposed. Whether the ability of symbiotic cnidarians to take up ammonium could provide these organisms with a significant input of nitrogen to meet their metabolic requirements and allow growth, remains unclear.

Previous work on *Anemonia viridis* has suggested that this species could be autotrophic with respect to energy requirements when maintained without feeding under an illumination of 140μmol photons.m^{-2}.s^{-1} with a 12:12 photoperiod (Tytler, 1982; Tytler & Davies, 1986). However, when anemones were maintained without feeding under this light regime for 84 days they lost weight, albeit at a lower rate than anemones maintained in darkness (Tytler & Davies, 1986). These growth experiments were carried out in seawater at ambient nutrient concentrations (no data are available on the ammonium concentration but the seawater was not supplemented with any additional nutrient). These experiments were repeated by Davies (1988) using the same illumination. However, one group of anemones were maintained in seawater supplemented with ammonium to give a final concentration of approximately 20μM. This process was repeated daily to maintain an elevated level of ammonium in the seawater since uptake by the anemones reduced the ammonium concentration to 6μM after 24 hours. Under this ammonium regime the rate

of weight loss of symbiotic anemones was significantly reduced when compared with control animals. The failure to demonstrate growth in this study was thought to be due either to the concentration of ammonium being too low to allow a sufficiently high net nitrogen input to the symbiosis or to limitation by another nutrient such as phosphorus.

When algal-invertebrate symbioses are exposed to elevated levels of ammonium over several weeks, the zooxanthellae population density increases (Hoegh-Guldberg & Smith, 1989; Muscatine *et al.*, 1989; Dubinsky *et al.*, 1990; Stambler *et al.*, 1991; Hoegh-Guldberg, 1994; Muller-Parker *et al.*, 1994) as does the zooxanthellae mitotic index (Cook *et al.*, 1988; Fitt & Cook, 1989; McAuley & Cook, 1994; Beaver, 1996). When the coral *Stylophora pistillata* was subjected to 20μM ammonium treatment for 14 days the population density of zooxanthellae increased but there was no change in the protein content of the host (Muscatine *et al.*, 1989). In contrast, when the coral *Pocillopora damicornis* was subjected to 20μM ammonium treatment for 56 days both the population density of zooxanthellae (Hoegh-Guldberg, 1994) and the protein content of the host increased (Muller-Parker *et al.*, 1994). However, host protein did not increase when this coral was treated with 50μM ammonium (Muller-Parker *et al.*, 1994) and a second study on the same species did not find any effect of 20μM ammonium treatment on host protein content (Achituv *et al.*, 1994). Similarly, treatment with 20μM ammonium for 56 days did not alter the carbohydrate or lipid content of host tissue from *Pocillopora damicornis* (Achituv *et al.*, 1994). The response of the zooxanthellae population in *Anemonia viridis* to ammonium was investigated by Beaver (1996). When symbiotic anemones were maintained with 20μM ammonium, the mitotic index of zooxanthellae increased across a range of light intensities. However, when normalised to host protein, the population density of zooxanthellae did not increase significantly after 80 days treatment with 20μM ammonium.

Much past research into the ecophysiology of algal-invertebrate associations has concentrated on the carbon balance of zooxanthellae and host. This interest stemmed from the discovery that of the total inorganic carbon fixed by zooxanthellae only a small percentage is retained by the algae (Davies, 1984; Muscatine *et al.*, 1984; Tytler & Davies, 1986; Edmunds & Davies, 1986). The remainder is translocated to the host (Muscatine & Cernichari, 1969; Lewis & Smith, 1971; Trench, 1971a, b, c; Muscatine *et al.*, 1972; Trench, 1974) where this organic carbon supply can meet and sometimes

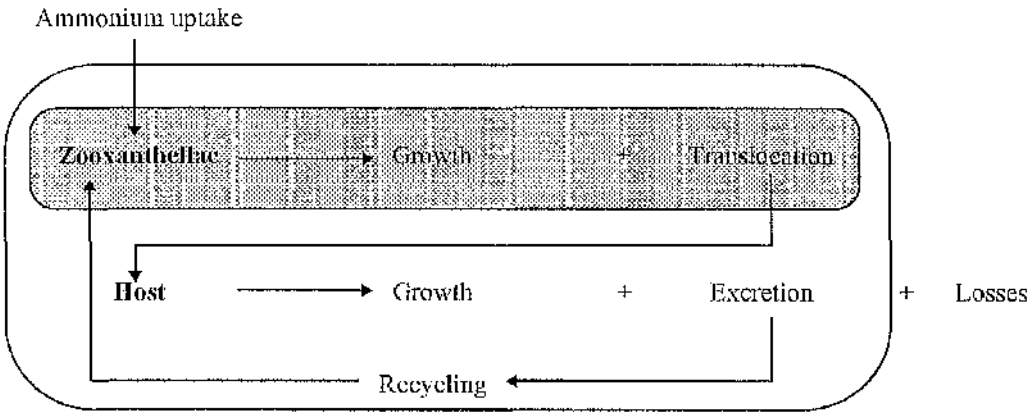
exceed the growth and respiratory demands of the animal host (Muscatine *et al.*, 1984; Davies, 1984; Edmunds & Davies, 1986). In order to arrive at such estimates, energy and carbon budgets of symbiotic associations have been constructed. These attempt to quantify the inputs of carbon from photosynthesis and from feeding to the overall carbon budget.

However, far less is known about the nitrogen budgets of symbiotic associations. Since nitrogen often limits growth in the marine environment, there is clearly a need to assess the importance of nitrogen supplied from both organic (feeding) and inorganic sources to the maintenance and growth of marine symbiotic associations. Nitrogen budgets have been estimated for only a few symbiotic associations namely the corals *Acropora palmata* (Bythell, 1988), *Stylophora pistillata* (Rahav *et al.*, 1989, whose data were adapted by Davies, 1992), the anemone *Anthopleura elegantissima* (Zamer & Shick, 1989) the giant clam *Tridacna gigas* (Hawkins and Klumpp, 1995) and green *Hydra* (McAuley, 1990). The nitrogen budgets for the two coral species were calculated on the basis of experiments carried out in seawater without supplementary ammonium. Bythell (1988) estimated that inorganic nutrient uptake from seawater could supply 30% of the total annual nitrogen demand of *Acropora palmata*. The balance was assumed to be supplied by feeding. The daily nitrogen budget for *Stylophora pistillata* calculated by Davies (1992) suggested that the growth data collated by Rahav *et al.* (1989) could be accounted for if the coral had access to an external nitrogen source. This value was derived to balance the budget and amounted to 20% of the input of nitrogen taken up and catabolic nitrogen recycled by the zooxanthellae. The nitrogen budget constructed for the giant clam *Tridacna gigas* by Hawkins and Klumpp (1995) measured the inputs of both inorganic and organic nitrogen which together accounted for about 70% of the total nitrogen requirements. McAuley (1990) compared the relative contributions of ammonium uptake and recycled ammonium to the nitrogen derived from digestion of a single brine shrimp by symbiotic green *Hydra* over a 24 hour period. Where digestion of a single brine shrimp was set to represent 100% of the nitrogen derived from feeding, uptake from 100μM ammonium was equivalent to 42% and recycled nitrogen to 21% of this brine shrimp derived nitrogen.

It was known that unfed symbiotic anemones in the light lost weight at a lower rate when supplied with ammonium than when maintained in control seawater (Davies, 1988). The aims of the experimental work described in this chapter were twofold. The first was to

see whether unfed symbiotic anemones would increase in weight if given a constant ammonium supply and the second was to produce a nitrogen budget based solely on ammonium uptake. The budget was derived using a simplified model of the nitrogen fluxes within this symbiosis and is illustrated in figure 3.1. In this model the only input of nitrogen is from ammonium taken up and assimilated by the zooxanthellae. Of this nitrogen, any that is not accounted for by zooxanthellae growth is assumed to be translocated to the host. Here the nitrogen is partitioned between host growth and a constant excretion term which, since symbiotic *Anemonia viridis* show no net loss of ammonium over 24 hours with irradiances above 190μmol photons.$m^{-2}.s^{-1}$ (Davies, 1988), is assumed to be recycled via the zooxanthellae.

Figure 3.1 Simplified model of the nitrogen fluxes in a symbiotic gastrodermal cell.



This model makes the following assumptions:

1. The only source of nitrogen available to the anemones was from the ammonium present in the seawater.
2. All the ammonium removed from the seawater was taken up by the anemones.
3. All the ammonium taken up by the anemones was assimilated by the zooxanthellae.
4. Any nitrogen not accounted for by zooxanthellae growth was translocated to the host with no losses from zooxanthellae expulsion.

In order to construct a nitrogen budget where the only input of nitrogen to the symbiosis was from ammonium added to the seawater, it was necessary to develop a system which allowed anemones to be maintained with a constant supply of ammonium. In addition

this system should allow the rates of ammonium uptake and excretion to be measured with minimal bacterial contamination. The development of this apparatus has been described in section 2.6. The results presented in section 2.6.4 show that this flow-through depletion methodology gave comparable ammonium uptake rates to those measured using the closed chamber depletion method of Davies (1988).

3.2 Methods

3.2.1 Effect of ammonium on the weight change of anemones

This experiment was intended to test the hypothesis that symbiotic anemones supplied with ammonium could increase in weight in the absence of holozoic feeding. Symbiotic anemones from standard experimental conditions (section 2.1) were buoyant weighed and transferred to two aquarium tanks. The anemones were maintained under an irradiance of 150μmol photons.$m^{-2}.s^{-1}$ which provided sufficient illumination to saturate photosynthesis in *Anemonia viridis* (Tytler, 1982) and was comparable to that used in the growth studies of Davies (1988) and Tytler and Davies (1986). Each tank contained ten animals. One tank was supplied with seawater containing 20μM ammonium, as described in section 2.5, while the second was supplied with seawater at ambient ammonium concentrations (<1μM). Both tanks were thoroughly cleaned twice a week to prevent algal growth. Each anemone was buoyant weighed at weekly intervals throughout the duration of the experiment and the ammonium concentrations in both tanks were monitored using the method described in section 2.4.1. The weight of each anemone was divided by its initial weight and multiplied by 100 to give the percentage of the initial buoyant weight. The animals were not fed during the experiment and any anemones which divided during the experiment were excluded from the analysis. It was intended to maintain these conditions for a period of 80 days. However, after 63 days a failure in the seawater supply to the control group ended this experiment. Since the ammonium-treated group was supplied from a seawater reservoir (see fig. 2.3) it was possible to continue this treatment for a total of 91 days.

3.2.2 Nitrogen Budget

To construct a nitrogen budget based on the model in figure 3.1, where the nitrogen source for symbiotic anemones was ammonium in the seawater, the following parameters were measured:

1. The rate of ammonium uptake in the light
2. The rate of ammonium excretion in the dark
3. The growth of zooxanthellae and host when subjected to a known input of ammonium

The amount of nitrogen translocated from zooxanthellae to host was then calculated from the difference between the amount of nitrogen taken up by the anemone and the amount of nitrogen accounted for by zooxanthellae growth. The growth of zooxanthellae and host

was assessed by measuring the change in total nitrogen of both fractions after subjecting symbiotic anemones to a measured input of ammonium. To measure growth in this way, it was necessary to analyse the total nitrogen content of zooxanthellae and host tissue from a group of control animals and compare these data with an equivalent analysis of experimental animals at the end of the incubation period with ammonium.

1. The rate of ammonium uptake

Before the experiment, control and experimental anemones were maintained for a period of four weeks without feeding under standard conditions (section 2.1) and acclimatised to an illumination of 300μmol photons.$m^{-2}.s^{-1}$ with a 12:12 daily photoperiod. Since the anemones were unfed no post-prandial effects on ammonium assimilation should occur (Davies, 1988). Experimental anemones (n=8) were buoyant weighed and transferred to individual incubation chambers within the apparatus described in section 2.6. These animals were then maintained under standard conditions with an experimental irradiance of 300μmol photons.$m^{-2}.s^{-1}$. Each chamber was supplied with a constant inflow of sterile (autoclaved) 0.3μm filtered seawater supplemented with 20μM ammonium. The anemones were subjected to this ammonium regime for 14 days during which time the system was regularly cleaned to prevent any algal growth. During this incubation the rate of ammonium uptake by each anemone was recorded as described in section 2.6. The rate of ammonium uptake at the end of the 12 hour dark period was also measured. Any anemones which divided during the experiment were excluded from the analysis. After 14 days the anemones were removed, buoyant weighed and separated into zooxanthellae and host fractions as described below.

2. The rate of ammonium excretion

The rate of excretion was determined by measuring the rate of ammonium produced by symbiotic anemones (n=8) in seawater at ambient ammonium concentrations (<1μM), after they had been maintained in the dark for 14 hours. The protocol used has been described in section 2.6.

3. The growth of zooxanthellae and host when subjected to a known input of ammonium

The total nitrogen content of zooxanthellae and host from control and experimental anemones was measured to assess the growth of both fractions in response to the measured input of nitrogen from ammonium uptake. Control anemones (n=8) were

buoyant weighed, sacrificed and each anemone was divided into two halves. Each half was blotted dry and wet weighed. One half was then freeze dried to determine its dry weight while the other half was homogenised in artificial seawater (section 2.3) containing 0.05% sodium dodecyl sulphate (SDS) (McAuley, 1986). Following homogenisation, the slurry of zooxanthellae and host material was allowed to stand for 20 minutes. Zooxanthellae and host tissues were then separated by repeated centrifugation and washing as described in section 2.3. The SDS washing technique of McAuley (1986) was used since preliminary experiments, and those of Beaver (1996), showed that this method counteracted the effects of mucus produced by the column and mesenteries when these tissues were homogenised. The zooxanthellae and host fractions from each anemone were then freeze dried and weighed. They were then stored in a desiccator until analysis. These dry weights were used to calculate the ratio of zooxanthellae to host biomass.

The total nitrogen content of the two fractions was analysed using the Kjeldahl procedure. This acid digestion converts all the nitrogen present in the sample to ammonium. The ammonium content of each sample was then assayed using the Nessler reaction. Both these methods have been extensively modified by individual researchers over years of use. The protocols followed here were a micro-Kjeldahl method adapted from the method of Lang (1958) and the Nessler reaction (pers. comm. D. Mousdale). The total nitrogen analysis procedure was assessed by digesting standards of ammonium sulphate and protein of known nitrogen content (bovine serum albumin, Sigma).

Reagents

1. Kjeldahl catalyst: 1% (w/v) selenium dioxide in 50:50 water:concentrated sulphuric acid (low in N).
2. 2N sodium hydroxide.
3. Nessler reagent: 4g of potassium iodide and 4g of mercuric iodide were dissolved in 25ml distilled water. This solution was then added to a stirred 750ml solution of distilled water and 3.5g gum acacia (Sigma). The volume was then made up to 1000ml and the reagent stored in a brown glass bottle at room temperature (stable for several weeks).
4. Standard: 4.73g ammonium sulphate in 1000ml distilled water (1mg N ml^{-1}).

Protocol

Samples containing between 25 and 100μg nitrogen were added to Pyrex tubes. Since no more than 100μl of liquid sample could be added to each tube, any samples which were more dilute were concentrated in the tubes by lyophilisation. Any liquid samples were made up to a volume of 100μl with distilled water. Each tube then had 250μl of Kjeldahl catalyst added and was transferred to a heating block at 350°C. After exactly 15 minutes the tubes were removed and allowed to cool to room temperature. To each tube 1.875ml of distilled water, 3ml 2N sodium hydroxide and 2ml Nessler reagent were added and the contents were immediately mixed using a vortex mixer. The absorbance of samples and standards was measured at 490nm (Philips PU8720 spectrophotometer). This assay was linear up to 100μg nitrogen as ammonia. This Kjeldahl procedure gave satisfactory estimates of the total nitrogen content of both ammonium sulphate and bovine serum albumin standards. These analyses were within ±1.5% of the known standard (data not shown).

The anemones from the experimental group were analysed using the same procedure.

3.3 Results

3.3.1 Effect of ammonium on the weight change of anemones

During this experiment four anemones divided (two from each treatment) and were excluded from the analysis leaving eight individuals from both the control and ammonium-treated groups. Over the first fourteen days there was no detectable weight change in either the ammonium-treated or control anemones. After 21 days and throughout the 91 day period of treatment with 20μM ammonium, these anemones showed an overall increase in buoyant weight. The buoyant weight of control anemones at ambient ammonium concentrations (<1μM) was recorded for 63 days after which a failure in the seawater supply ended the control treatment. Over this period these anemones showed an overall decrease in buoyant weight (figure 3.2). The rate of weight change for each treatment was calculated by fitting a regression line to the data. The regression for ammonium-treated anemones produced a positive slope which was significantly different from the negative slope corresponding to control anemones (ANCOVA proportion of initial buoyant weight by treatment, $F_{1,184}$=34.66, p<0.001). These regressions are summarised in table 3.1 which shows the difference between the two treatments as the percentage buoyant weight change per day.

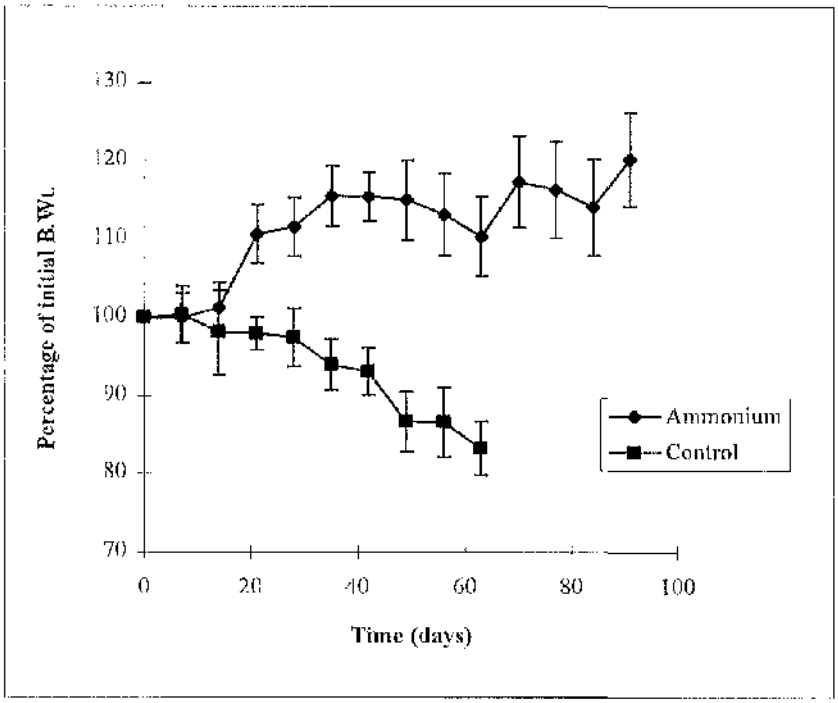
Table 3.1 Effect of ammonium treatment on the buoyant weight of unfed symbiotic anemones.

Treatment	% Buoyant weight change day^{-1}	R^2-adj.	p	Equation of line
Control	-0.263	24.6	<0.001	Prop.I.Bwt. = 1.03 - (0.00279d)
Ammonium	0.197	12.4	<0.001	Prop.I.Bwt. = 1.03 + (0.00185d)

Prop.I.Bwt., proportion of the initial buoyant weight; R^2-adj., coefficient of determination adjusted for the number of parameters in the model; p, significance of the regression.

Figure 3.2
Buoyant weight change of symbiotic anemones maintained in seawater supplemented with 20μM ammonium and in unsupplemented control seawater.

Anemones were maintained without feeding with an illumination of 150μmol photons.$m^{-2}.s^{-1}$
B.Wt, buoyant weight. n=8 for both treatments (±SE).



3.3.2 Nitrogen budget

Throughout the 14 day exposure the anemones took up ammonium with no net loss from the symbiosis during the night. The mean uptake rate was 2.2μmol $NH_4^+.g^{-1}.h^{-1}$ and the uptake rates recorded over the first two days of the incubation are illustrated in figure 3.3. The rate of excretion of symbiotic anemones after 14 hours darkness was -0.3μmol $NH_4^+.g^{-1}.h^{-1}$.

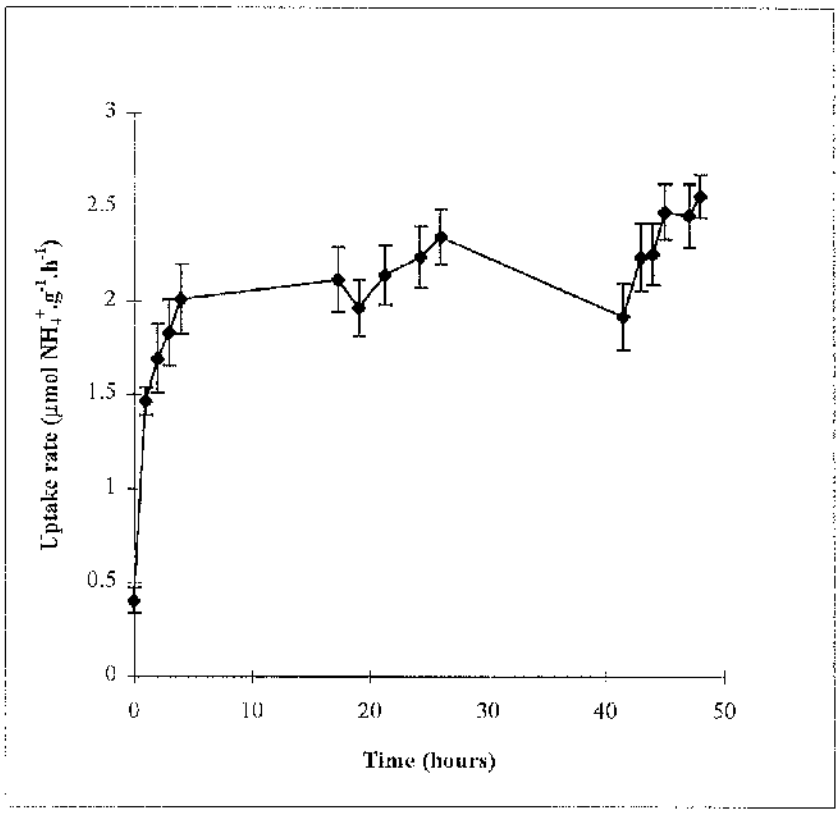
The proportion of the symbiosis that was zooxanthellae showed a slight but insignificant increase from 17.5% (SD=9.0) of total biomass in the control group to 18.2% (SD=4.7) in the ammonium treated experimental group (Two sample t-test, $p>0.05$). During the calculation of the nitrogen budget, zooxanthellae were taken to represent 18% of the anemone mass. The total nitrogen content of both zooxanthellae and host fractions, shown in table 3.2, increased significantly following exposure to the constant inflow of 20μM ammonium for 14 days. During the ammonium incubation, the weight change of the anemones was very variable but showed an overall weight loss of 5.36% (SD=4.55).

Table 3.2 The total nitrogen content of zooxanthellae and host fractions from symbiotic anemones before (control) and after (experimental) a 14 day exposure to a constant input of 20μM ammonium.

Fraction	Control	Experimental	Change	p
% N content of zooxanthellae	6.60 ± 1.10	9.50 ± 0.72	+2.9	<0.01
Mass zooxanthellae.g^{-1} total (mg)	180	170	-10	
Mass zooxanthellae N.g^{-1} total (mg)	12	16	+4	
% N content of host	10.73 ± 3.34	14.8 ± 3.4	+4.07	<0.05
Mass host.g^{-1} total (mg)	820	776	-44	
Mass host N.g^{-1} total (mg)	88	115	+27	

Results are the mean ±SD (n=8). Significance of the difference between control and experimental treatments was assessed using a two sample t-test. Masses are expressed per gram dry weight of anemone where 18% of the total mass is zooxanthellae for both experimental and control groups.

Figure 3.3
Mean rate of ammonium uptake (±SE) of symbiotic anemones over the first 48 hours of a 14 day exposure to a constant inflow of 20µM ammonium enriched seawater (n=8).



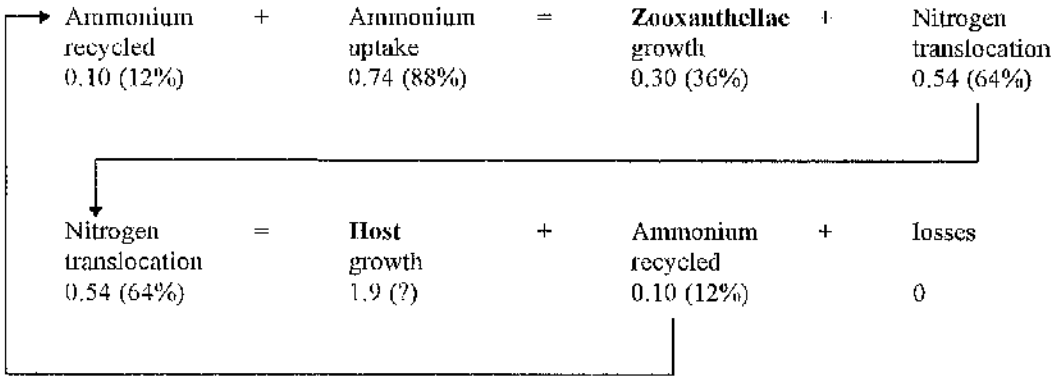
The parameters measured are listed below together with the corresponding nitrogen flux parameters (see figure 3.1) which the data were used to derive (flux units are shown normalised to dry weight over a 24 hour day):

Parameter measured	**Nitrogen flux in model**	**mg $N.g^{-1}.d^{-1}$**
Rate of ammonium uptake	Nitrogen input to the symbiosis	0.74
Rate of ammonium excretion in the dark	Nitrogen recycled in the light	0.10
Change in zooxanthellae total N	Zooxanthellae growth	0.30
Change in host total N	Host growth	1.90

The final parameter in the model of nitrogen fluxes was the nitrogen translocated from zooxanthellae to host. This was calculated on the assumption that any nitrogen not used in zooxanthellae growth was translocated to the host:

$\therefore$ (0.74+0.10) - 0.30 – 0.54 mg $N.g^{-1}.d^{-1}$

These data produce the following budget where the values represent mg $N.g^{-1}.d^{-1}$ and the figures in parentheses represent the percentage of the total input of ammonium to the zooxanthellae:



These data do not produce a balanced budget. The nitrogen acquired from ammonium uptake (0.74mg $N.g^{-1}.d^{-1}$) was assumed to represent the only input of nitrogen to the symbiosis. If this nitrogen, together with recycled nitrogen (0.10mg $N.g^{-1}.d^{-1}$), accounts for all the zooxanthellae growth (0.30mg $N.g^{-1}.d^{-1}$) and the remainder is translocated to the host (0.54mg $N.g^{-1}.d^{-1}$), then this translocation can only account for 28% of the measured host growth (1.9mg $N.g^{-1}.d^{-1}$).

3.4 Discussion

3.4.1 Effect of ammonium on the weight change of anemones

Over the first fourteen days of this experiment there was no difference in the weight change of control or ammonium-treated anemones. However, during the rest of the experiment control anemones in seawater at ambient ammonium concentrations (<1μM) lost weight whereas anemones in seawater at 20μM ammonium gained weight. This has since been confirmed by Beaver (1996) who also demonstrated that unfed symbiotic *Anemonia viridis* increased in weight by 11.4% over 80 days in 20μM ammonium-supplemented seawater and that almost 60% of this weight change could be accounted for by increased host protein. The assimilation of ammonium into host-derived high molecular weight material is investigated further in chapter 5. In the present study, unfed symbiotic anemones increased in weight by 15.8% over the same time period in 20μM ammonium-supplemented seawater. These two studies are the first to show that 20μM ammonium can support growth of an algal-invertebrate symbiosis. In contrast, symbiotic anemones under identical conditions but in seawater at ambient ammonium concentrations showed a weight loss amounting to 21% over an 80 day period.

These data compare well with previous data on the growth of *Anemonia viridis* summarised in table 3.3. The rate of weight change of starved anemones in normal seawater in this study lies within the reported range of -0.139% to -0.349% day^{-1}. The increase in weight of 0.197% day^{-1} of starved anemones with 20μM ammonium is close to that of 0.143% day^{-1} measured by Beaver (1996). Similar trends in weight change were described for symbiotic *Anthopleura elegantissima* which also lose weight when starved in the light but at a lower rate than aposymbionts or symbiotic anemones in darkness (Muscatine, 1961). *Anthopleura elegantissima* only gained in weight when fed in seawater at ambient ammonium concentrations (Sebens, 1980) and was found to deplete lipid reserves more rapidly when starved in the dark than when starved in the light (Fitt & Pardy, 1981).

Table 3.3 The rate of weight change of symbiotic *Anemonia viridis* under defined nitrogen availability, light regime and temperature. Negative rates of weight change correspond to a loss in weight and positive rates to a gain in weight over time.

Feeding regime	Ammonium concentration (μM)	Light regime (μmol photons.m^{-2}.s^{-1})	Temp (°C)	Weight change (% day^{-1})	Source
Starved	Ambient	Light (18:6)	15	[1]-0.214	Taylor (1969)
Starved	Ambient	140 (12:12)	10	-0.203	Tytler & Davies (1986)
Starved	Ambient <0.5	140 (12:12)	10	-0.139	Davies (1988)
Starved	Ambient <0.5 - 1.3	300 (12:12)	11	-0.349	Beaver (1996)
Starved	Ambient <1	150 (12:12)	15	-0.263	This study
Starved	5.64 (±4.33)	140 (12:12)	10	-0.027	Davies (1988)
Starved	20	300 (12:12)	11	0.143	Beaver (1996)
Starved	20	150 (12:12)	15	0.197	This study
Fed	Ambient	Light (18:6)	15	[1]0.086	Taylor (1969)
Fed	Ambient <0.5 - 1.3	300 (12:12)	11	0.569	Beaver (1996)

[1] The percentage weight change per day for these data was calculated from the percentage weight change after 140 days. There is no information on the exact level of illumination (Taylor, 1969). The light level is shown with the photoperiod in parentheses. Ambient refers to seawater which was not supplemented with ammonium. If available the concentration of ammonium is shown (±SD).

These results demonstrate that an ammonium supply to symbiotic anemones can allow growth. Clearly, a 20μM ammonium concentration is environmentally irrelevant to this organism which is found in coastal waters where the ammonium concentration is between <0.5 and 1.3μM (Davies, 1988). However, this experiment demonstrates an important principle; a symbiotic anemone supplied with an inorganic nitrogen supply can achieve net growth in the absence of holozoic feeding. The route by which ammonium is metabolised in symbiotic anemones can be interpreted in terms of nitrogen recycling and/or conservation, as discussed in section 1.3. In the context of this experiment it appears that nitrogen is taken up by either zooxanthellae or host (or both) resulting in net growth of the anemone. This is discussed further below.

3.4.2 Nitrogen budget

Over the two week exposure to ammonium, the weight change of anemones was very variable but showed an mean decrease of 5.36% (±4.55). During the long term growth study discussed above there was no significant change in weight over this time period which suggests that this apparent weight loss may not be an accurate reflection of the rate at which anemones change in weight. Significant weight change was only detected after four weeks in the long term experiment.

The budget calculated from the data presented in section 3.3.2 fails to balance. The measured input to the symbiosis from ammonium uptake was insufficient to account for the observed increases in both zooxanthellae and host total nitrogen. Ammonium uptake together with recycled nitrogen from host catabolism accounted for all the zooxanthellae growth but only 28% of the host growth. The recycled ammonium component of the budget model (figure 3.1) was calculated from the rate of ammonium excretion after symbiotic anemones had been maintained in the dark for 14 hours. It is possible that this value is an underestimate of the true amount of ammonium produced and recycled within the symbiosis. Several symbioses have been shown to be capable of ammonium uptake in darkness (Wilkerson & Muscatine, 1984; Davies, 1988) and this property has been explained by postulating that a store of carbon skeletons in the zooxanthellae allows ammonium uptake to continue in the absence of photosynthesis as in other microalgae (Syrett, 1981). If a portion of the excretory ammonium from host catabolism was still retained by the symbiosis after 14 hours darkness, then this figure will represent an underestimate of the true value of the recycled nitrogen parameter. The results presented in section 5.3.1.2 show that symbiotic *Anemonia viridis* still show assimilation of

[^{15}N]ammonium after 12 hours darkness. Thus it appears that the data used to estimate the recycled nitrogen parameter in this model do not represent the true magnitude of host ammonium production. Given this, the nitrogen budget has been recalculated by substituting the value of nitrogen recycled with the ammonium excretion rate recorded from aposymbiotic anemones under a constant inflow of 20μM ammonium (-2.228μmol $NH_4^+.g^{-1}.h^{-1}$, see section 2.6.3). This value corresponds closely to the rate of aposymbiotic *Anthopleura elegantissima* excretion of -2.14μmol $NH_4^+.g^{-1}.h^{-1}$ recorded by Zamer and Shick (1987). The aposymbiotic anemones in the present study had been maintained in darkness for several years and were almost entirely devoid of zooxanthellae (Beaver, 1996).

Using this value the following 24 hour nitrogen budget is obtained (all figures are in units of mg $N.g^{-1}.d^{-1}$ and the figures in parentheses represent the percentage of the total input of ammonium to the zooxanthellae):

Ammonium recycled 0.75 (50%)	+	Ammonium uptake 0.74 (50%)	=	**Zooxanthellae** growth 0.30 (21%)	+	Nitrogen translocation 1.19 (79%)
Nitrogen translocation 1.19 (79%)	=	**Host** growth 1.9 (?)	+	Ammonium recycled 0.75 (50%)	+	losses 0

The budget obtained from this recalculation still does not balance. Nitrogen translocation now accounts for more of the observed host growth (63%) but still falls short of accounting for the total nitrogen requirements and any unmeasured loss component which might be expected. Such losses could come from mucus production (Bythell, 1988) and zooxanthellae expulsion (Beaver, 1996).

Both these budgets show that the measured nitrogen input (ammonium uptake) to the symbiosis was insufficient to account for the measured growth (zooxanthellae and host change in total nitrogen). In other nitrogen budgets this shortfall has been assumed to be supplied by one of the other two sources of nitrogen available to a symbiotic association, namely holozoic feeding and the uptake of dissolved organic nitrogen (discussed in

sections 1.2.1 & 1.2.2). The annual nitrogen budget of *Acropora palmata* estimated by Bythell (1988) predicted that inorganic nitrogen from ambient seawater concentrations could supply 30% of the corals' total nitrogen requirements. The balance was not measured, but calculated by subtraction and assumed to be supplied by holozoic feeding. In the present study it was assumed that the only source of nitrogen available to the symbiosis was from the 20μM ammonium present in the seawater. Given this supplemented inorganic nitrogen supply, the latter budget presented here estimates that 68% of the total nitrogen requirements (zooxanthellae and host growth) are supplied from the ammonium in seawater.

The nitrogen budget calculated for the giant clam symbiosis *Tridacna gigas* by Hawkins and Klumpp (1995) also failed to account for the observed nitrogen demand. This budget showed that ingested particles together with dissolved inorganic nitrogen uptake as ammonium supplied about 70% of the total nitrogen requirements to maintain observed rates of growth and metabolism. The authors put forward three possible explanations of this shortfall: (1) that the rate of DIN uptake was underestimated either due to low light levels which reduced the rate of ammonium uptake (Wilkerson & Trench, 1986) or limited ammonium availability in the closed incubation systems used; (2) that the clam symbiosis could use nitrate as an additional supply of DIN (Wilkerson & Trench, 1986; Fitt *et al.*, 1993); (3) that dissolved organic matter uptake could have contributed to nitrogen intake as in non-symbiotic bivalves (Hawkins & Bayne, 1991).

In the present study the rate of ammonium uptake was measured using a flow-through system and the light regime was standardised so it is unlikely that the measured nitrogen input from ammonium is underestimated. It is more likely to represent an overestimation since bacteria and algae associated with the surface of the anemones will have taken up ammonium. It is also unlikely that the anemones were using nitrate as an additional nitrogen source since symbiotic anemones, including *Anemonia viridis*, are unable to take up nitrate (Wilkerson & Muscatine, 1984; Davies, 1988). Since there was no nitrogen input from holozoic feeding, the only other possible source would be from the uptake of dissolved organic nitrogen. As discussed in section 1.2.2 both symbiotic and non-symbiotic cnidarians are capable of taking up dissolved organic nitrogen from seawater (Stephens 1962; Shick, 1975; Schlichter, 1982; Ferrier, 1991; Wilkerson & Kremer, 1992). This source of nitrogen can make detectable contributions to nitrogen demand of non-symbiotic cnidarians such as *Aurelia aurita* (Shick, 1975) as well as symbiotic

species such as *Linuche unguiculata* (Wilkerson & Kremer, 1992). Ferrier (1991) showed that free amino acids were taken up at environmentally realistic concentrations by four species of scleractinian corals. In addition, a 2μM concentration of amino acids was predicted to satisfy 80% of the respiratory demand of the symbiotic *Heteroxenia fuscescens* (Schlichter, 1982).

Schlichter (1980) and Schlichter *et al.* (1987) reviewed the capacity of sea anemones to take up dissolved free amino acids (FAA) from seawater. This process has been shown to follow Michaelis-Menten kinetics with uptake affinities (K_t) which typically reflect the environmental concentration of FAA. For example, the K_ts for leucine and lysine were 1.9 and 2.6 x 10^{-6}M respectively in *Anemonia viridis* (Bajorat, 1979, cited by Schlichter, 1980). This is of a similar order as the concentration of amino acids in coastal seawater (ca. 10^{-7}M) (Shick, 1991). These amino acid uptake systems are specific for the classes of amino acid (Schlichter, 1978) and must work by active transport against the concentration gradient created by the high intracellular FAA concentration of sea anemones (reviewed by Shick, 1991; this study, section 4.3.1). Thus amino acid uptake by anemones is an energy-requiring process. It has been suggested that the capacity of marine invertebrates to take up dissolved FAA may reflect a nitrogen rather than an energy requirement (Shick, 1991; Ferrier, 1991). It is possible that the shortfall observed in the budget estimated here could be explained by the uptake of dissolved organic matter, principally amino acids, from the seawater.

The nitrogen budget calculated in this study is based on a simplified model of the nitrogen fluxes within this symbiosis (figure 3.1). The model relied on four central assumptions which will be summarised and reviewed here.

1. **The only source of nitrogen available to the anemones was assumed to be the ammonium present in the seawater.**

   As discussed above it appears that the ammonium input from the seawater supply was insufficient to account for the observed increases of zooxanthellae and host total nitrogen. This suggests that another source of nitrogen must have been available. In the absence of holozoic feeding, this nitrogen could have come from the uptake of dissolved organic material, in particular the uptake of dissolved free amino acids.

2. **All the ammonium removed from the seawater was assumed to be taken up by the anemones.**

The seawater used throughout this experiment had been filtered and sterilised to prevent bacterial and algal ammonium uptake. However, any organisms associated with the anemones could have been responsible for some of the ammonium uptake. Microscopic examination revealed the presence of ciliates and unicellular algae in association with anemone mucus. To minimise this source of error the experimental apparatus was kept clean and any excess mucus produced was removed. No account was made of this loss in the budget calculation. Surface sterilisation of anemones using a mixture of antibiotics was investigated but found to be deleterious to the animals.

3. **All the ammonium taken up by the anemones was assumed to be assimilated by the zooxanthellae.**

As suggested by Rees (1987) and Miller and Yellowlees (1989) and discussed in section 1.5, the host fraction may be capable of ammonium assimilation given a supply of carbon skeletons from zooxanthellae photosynthesis. This possibility that this pathway exists has not been considered in the present model. However, recalculating the budget using this pathway would not alter the fact that there seems to by insufficient nitrogen input to account for the growth.

4. **Any nitrogen not accounted for by zooxanthellae growth was assumed to be translocated to the host.**

If ammonium is assimilated by host tissue this assumption may not be strictly valid but the net flux of nitrogen into a symbiotic anemone will be the same whether assimilated by zooxanthellae or host tissue. In addition, this model made no measurement of nitrogen losses from the symbiosis via mucus production or zooxanthellae expulsion. The nitrogen budget of Bythell (1988) suggests that mucus production could represent a significant loss to the coral *Acropora palmata*. In this budget it was estimated that 50% of the total nitrogen requirement of the coral was lost as mucus and that this demand could not be met by inorganic nitrogen uptake at ambient dissolved inorganic nitrogen concentrations. In other words some of the nitrogen derived from feeding would be lost from *Acropora palmata* as mucus. Since *Anemonia viridis* continuously expels zooxanthellae (Taylor, 1969; Beaver, 1996),

symbiont loss may represent a further nitrogen demand to be accounted for in a comprehensive budget.

The results presented here showed that exposure to 20μM ammonium over 91 days supported anemone growth, whereas control anemones in seawater at ambient ammonium concentrations lost weight. However, when ammonium uptake was measured over a 14 day period it was insufficient to account for the measured changes in zooxanthellae and host total nitrogen suggesting that another source of dissolved nitrogen, such as free amino acids, could form an important part of the nitrogen budget of this symbiosis.

Chapter 4

Ammonium metabolism of symbiotic anemones

4.1 Introduction

The results described in the previous two chapters showed that symbiotic *Anemonia viridis* is capable of ammonium uptake from seawater which caused the total nitrogen content of both zooxanthellae and host to increase. A simple model of the nitrogen fluxes within the symbiosis was used to produce a nitrogen budget for the anemone. This budget suggested that ammonium taken up and recycled could account for 68% of the observed increase in total nitrogen but failed to account for all of it. A long term exposure to 20μM ammonium caused anemones to increase in weight. In other non-symbiotic marine invertebrates ammonium is a toxic metabolic product which is excreted. The conversion of inorganic nitrogen as ammonium to organic nitrogen will take place via the free amino acid pool, in particular via the amino acids glutamine and glutamate (Stryer, 1988). This free amino acid pool represents the available supply of nitrogen for protein synthesis and, since protein is constantly turned over, must be in a state of dynamic equilibrium with protein amino acids (Urich, 1990).

As discussed in section 3.1, ammonium retention and depletion are characteristic of symbiotic anemones. This has led many authors to conclude that the symbionts are the site of ammonium assimilation. There are two well described enzymatic routes by which symbiotic microalgae could assimilate ammonium; firstly via glutamate dehydrogenase (GDH) and secondly via the linked activity of glutamine synthetase (GS) and glutamine 2-oxoglutarate amidotransferase (GOGAT). GDH activity has been detected in zooxanthellae from hard corals (Crossland & Barnes, 1977; Dudler & Miller, 1988) and the sea anemone *Aiptasia pulchella* (Wilkerson & Muscatine, 1984). The amination reaction of GDH catalyses glutamate synthesis from ammonium and 2-oxoglutarate using as coenzyme either NADH or NADPH:

$$\text{2-oxoglutarate} + \text{NAD(P)H} + NH_4^+ \leftrightarrow \text{L-glutamate} + \text{NAD(P)}^+ + H_2O$$

Dudler and Miller (1988) characterised the induced activity of GDH enzymes in cultured zooxanthellae from the coral *Acropora formosa*. Following incubation in 200mM ammonium, these algal cells showed NADPH-specific activity predominantly in the

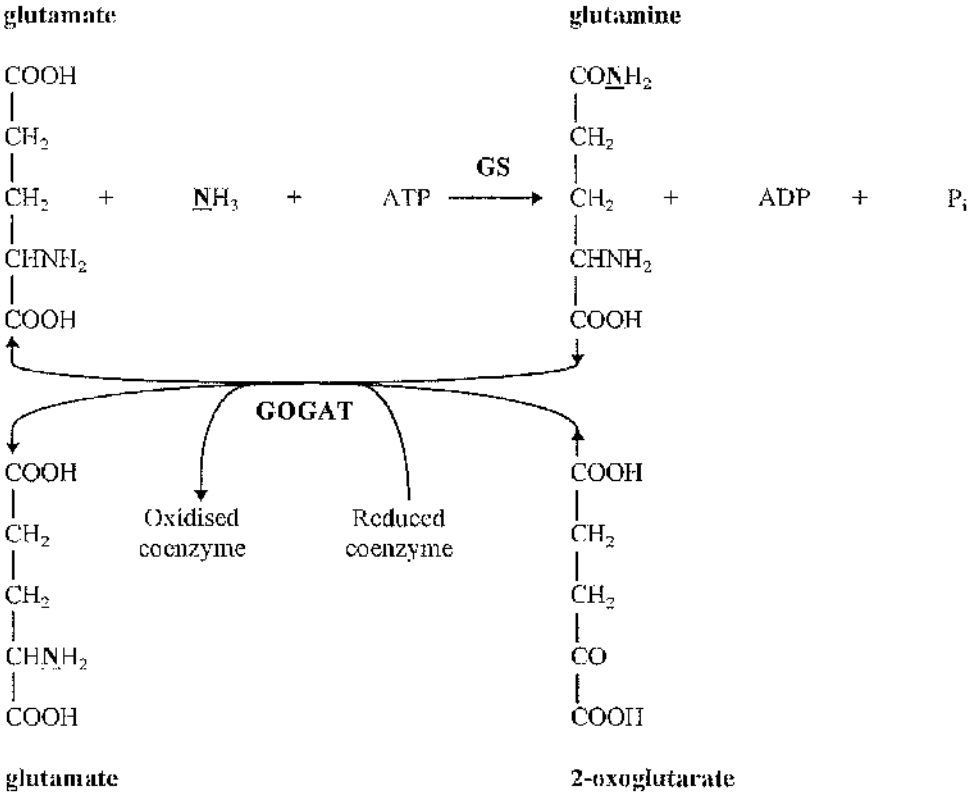
direction of glutamate synthesis (amination) and NADH-specific activity in both amination and deamination directions. Since NADPH-GDH was most active in the direction of glutamate synthesis and it had a high affinity for ammonium at low concentrations, these authors proposed that this enzyme could be involved in ammonium assimilation by zooxanthellae.

In higher plants the sole mechanism for the assimilation of ammonium is believed to be via the GS/GOGAT cycle (Miflin & Lea, 1977), a subject which has been reviewed by Lea *et al.* (1989). The GS/GOGAT cycle also appears to be a major route of ammonium assimilation in microalgae (Ahmed & Hellebust, 1988) and zooxanthellae (Summons & Osmond, 1981; Summons *et al.*, 1986). Prior to the discovery of glutamate synthase or GOGAT, it was believed that the GDH reaction was the route of ammonium assimilation. GOGAT catalyses the conversion of glutamine and 2-oxoglutarate to produce two molecules of glutamate. The cycle is completed if this reaction is coupled with glutamine synthetase (GS) which catalyses the reaction of glutamate with ammonium to produce glutamine. Thus for every GS/GOGAT cycle there is assimilation of one nitrogen from ammonium and the net production of one glutamate from 2-oxoglutarate. This cycle is illustrated in figure 4.1.

In higher plants there are two forms of GOGAT which have activity dependent either on NADH or ferredoxin (Fd) (Lea *et al.*, 1990). In zooxanthellae, while GS activity has been detected (Wilkerson & Muscatine, 1984; Anderson & Burris, 1987), GOGAT activity has not. The evidence for GS/GOGAT as the mechanism of ammonium assimilation in zooxanthellae is indirect. When zooxanthellae from the clam *Hippopus hippopus* were incubated with [^{15}N]ammonium, the greatest enrichment with ^{15}N was found in the amide group of glutamine. The lower enrichment of the amino groups of glutamine and glutamate pointed to a system in which nitrogen from the amide N of glutamine was transferred to the amino groups of glutamine and glutamate (Summons & Osmond, 1981). When *in vivo* experiments were carried out using ^{15}N to follow ammonium assimilation in a variety of coral and clam symbioses, a similar pattern of zooxanthellae amino acid enrichment with ^{15}N was seen. This indicates that *in vivo* the GS/GOGAT cycle is the major mechanism of ammonium assimilation in zooxanthellae (Summons *et al.*, 1986).

Figure 4.1

The GS/GOGAT cycle.
Ammonium is assimilated by glutamine synthetase (GS) to produce glutamine. The nitrogen from ammonium (N) forms the amide group of glutamine. The action of GOGAT completes the cycle by producing two molecules of glutamate from 2-oxoglutarate and glutamine. Isoenzymes of GOGAT are active with either NADH or reduced ferredoxin as coenzyme.



Further indirect evidence for this cycle comes from the effect of ammonium treatment on the levels of glutamine and glutamate in microalgae. Turpin and Harrison (1978) showed that adding ammonium to cultures of the dinoflagellate *Gymnodinium simplex* caused levels of glutamine to rise and levels of glutamate to fall suggesting that GS was actively assimilating ammonium. These authors proposed that GOGAT would complete the cycle to regenerate glutamate. The levels of these amino acids in freshly isolated zooxanthellae from the hydroid *Myrionema amboinense* changed in the same way following the addition of ammonium (McAuley & Cook, 1994). A final line of indirect evidence to suggest that the GS/GOGAT cycle is the major route of ammonium assimilation in zooxanthellae comes from the study by Rahav *et al.* (1989). This investigation showed that when the coral *Stylophora pistillata* was incubated with azaserine, a specific inhibitor of GOGAT, the coral excreted ammonium. If azaserine is assumed to inhibit only GOGAT, then this suggests that the GS/GOGAT cycle of zooxanthellae would have assimilated this ammonium *in vivo*. The only direct attempt to assay GOGAT activity in zooxanthellae was that by Wilkerson and Muscatine (1984). These authors failed to detect any NADH or NADPH-dependent GOGAT activity but did not investigate the possibility of a ferredoxin-dependent enzyme from zooxanthellae isolated from the anemone *Aiptasia pulchella*. To date, there are no published data on the GOGAT activity of zooxanthellae.

As discussed above, ammonium assimilation via GS will yield glutamine. When nitrogen is limiting, glutamine levels fall but glutamate levels are maintained. This means that the ratio of glutamine:glutamate (gln:glu) can provide a sensitive indicator of nitrogen status (Flynn, 1990) (section 1.8.6). This phenomenon has been observed in *Chlorella* cells cultured from the green *Hydra* symbiosis which, when transferred to a nitrogen-free culture medium, showed a decreased gln:glu ratio (McAuley, 1992). The same response was seen in zooxanthellae when the coral *Pocillopora damicornis* was maintained in ammonium-stripped seawater resulting in a reduction in the ratio of gln:glu (McAuley, 1994). However, Flynn (1990, 1991) defines physiological nitrogen-limitation as corresponding to a gln:glu ratio of less than 0.2. The gln:glu ratio of microalgal cells may increase when they are given an increased nitrogen supply but this does not imply nitrogen-limitation unless the ratio was originally less than 0.2 (McAuley, 1994).

Similarly, in host tissue there are two well characterised routes by which ammonium could be assimilated. These two reactions are again catalysed by the enzymes GDH and

GS (see reaction schemes above). GOGAT has not been detected in animal tissue (Fowden, 1981). In animal cells, the amination reaction of GDH is believed to provide a limited capacity to reassimilate some of the ammonium produced by deamination of amino acids and so synthesise glutamate (Fowden, 1981). It is generally accepted that NADPH-specific enzymes are most active in the synthesis of glutamate whereas NAD^+-specific enzymes are most active in its breakdown (Smith *et al.*, 1975). Although most animal GDH enzymes are active with either coenzyme (Urich, 1990), those from the Cnidaria show most activity with NADPH (Hoffmann *et al.*, 1978; Bishop *et al.*, 1978; Male & Storey, 1983; Catmull *et al.*, 1987; Rahav *et al.*, 1989). Since NADPH-GDH was believed to assimilate ammonium, the high activity of this enzyme in host tissue from symbiotic anemones and corals suggested that the host fraction had the capacity to assimilate ammonium (Miller & Yellowlees, 1989). This led Miller and Yellowlees (1989) to propose that ammonium assimilation could take place in the host fraction via NADPH-GDH (see figure 1, page 11). The 2-oxoglutarate needed for assimilation would be supplied by translocated photosynthate from the zooxanthellae. A further mechanism was proposed by Male and Storey (1983). These authors showed that GDH from host tissue of the anemone *Anthopleura xanthogrammica* could use glutamine as an alternative substrate to ammonium. This activity, coupled to ammonium assimilation via GS, would provide a means of incorporating ammonium to produce glutamine. If the glutamine produced by GS then provided a substrate for glutamine-linked GDH, then glutamate could be generated and provide a transamination substrate for the production of other amino acids. Functionally, this activity would be equivalent to GS/GOGAT (figure 4.1).

Whether the zooxanthellae or host tissue is the primary site of ammonium assimilation, the initial products of assimilation will be amino acids. There are no data available describing the free amino acid pool composition of host tissue from *Anemonia viridis* and only one analysis of the composition of zooxanthellae (McAuley, 1994). As a later investigation intended to follow the pathway of [^{15}N]ammonium assimilation into amino acids, it was necessary to analyse the free amino acid pools of both zooxanthellae and host to design subsequent experiments (chapter 5). Since treatment with 20μM ammonium increased the total nitrogen content of both zooxanthellae and host (section 3.3.2), its effect on the composition and concentration of free amino acids in both partners was examined and the gln:glu ratio of zooxanthellae was calculated.

The experimental work described in this chapter was intended to assay the activity of GDH in *Anemonia viridis* to see whether the host from this symbiosis contained similarly high levels of NADPH specific enzyme as other cnidarian symbioses. This assay was also carried out on zooxanthellae tissue. However, the investigations summarised above suggest that zooxanthellae assimilate ammonium via a different enzyme system, the GS/GOGAT cycle. Since GOGAT activity has not been demonstrated in zooxanthellae its activity in the symbionts of *Anemonia viridis* was investigated.

In this chapter the following questions were addressed:

1. What is the concentration and composition of the free amino acid pool from zooxanthellae and host and are these altered by maintaining symbiotic anemones in 20μM ammonium?
2. What is the activity of GDH in zooxanthellae and host?
3. Do zooxanthellae contain ferredoxin-dependent GOGAT?

4.2 Methods

4.2.1 Free amino acid analysis

The concentration of free amino acids and the composition of free amino acid pools were analysed from samples prepared according to the procedure described below. Following description of the analytical methods used, the method used to examine the effect of treatment with 20μM ammonium on the free amino acid pools will be given.

Tissue preparation

The free amino acid pool concentration and composition were determined from tentacle tissue of symbiotic anemones. Approximately 0.2g wet weight of tentacle from each anemone were homogenised per millilitre artificial seawater and the zooxanthellae and host fractions were separated (section 2.3). The zooxanthellae pellet produced was then sonicated (Microson Ultrasonic Cell Disruptor) in 2% (w/v) sulphosalicylic acid (SSA) to disrupt the cells and precipitate protein. The host supernatant was added to an equal volume of 4% SSA to produce the same final concentration. The extracts of both zooxanthellae and host were then left overnight at 4°C. After this, the precipitated protein was removed by centrifugation at 11,337g for 10 minutes and the supernatants, containing low molecular weight material including amino acids, were removed and frozen at -20°C until analysis.

4.2.1.1 Total free amino group concentration

The method used to assay free amino acid concentration was adapted from the original method of Moore and Stein (1948) (pers. comm. D. Mousdale). This method uses the ninhydrin reagent which not only reacts with amino acids but with other compounds having unsubstituted amino groups such as amines, amino-sugars and ammonia. Thus the colour reaction produced when a sample is analysed by this method will represent the concentration of all the ninhydrin-positive substances present in the sample.

Reagents

1. Solution of 2.15g citric acid and 4.35g sodium citrate in 125ml distilled water (pH 5.0). 0.2g stannous chloride were then dissolved in this solution with gentle heating (prepared on the day of use).
2. Solution of 0.5g ninhydrin in 12.5ml 2-methoxyethanol (prepared on the day of use).
3. 50:50 propan-1-ol:water.

4. 4% (w/v) 5-sulphosalicylic acid (SSA).

5. 1mM L-leucine standard.

Procedure

For tissue prepared as described above, a 25µl aliquot of SSA supernatant made up to 0.5ml gave results which were within the range of the assay. Equal volumes of the stannous chloride and ninhydrin reagents were mixed. A 1.5ml aliquot of this reagent was then added to 0.5ml aliquots of sample or standard and mixed. These tubes were then transferred to a boiling water bath for 5 minutes after which they were cooled to room temperature. Once at room temperature, 8ml of the propan-1-ol solution was added to each tube, the tubes were vortex mixed and allowed to stand for 30 minutes at room temperature. The absorbance of samples and standards were measured at 570nm against a reagent blank (Philips PU8720 spectrophotometer). This assay was linear up to 0.5µmole.

4.2.1.2 Free amino acid pool composition

Preliminary analysis of amino acid composition was carried out using thin layer chromatography (TLC). This provided information on the most abundant components of the free amino acid pool but failed to provide satisfactory separation of the amino acids and so further analysis was carried out using high performance liquid chromatography (HPLC).

Thin layer chromatography

Low molecular weight fractions of host material were prepared from tentacle tissue as described above but used 5% (w/v) trichloroacetic acid (TCA) rather than SSA to precipitate the high molecular weight fraction. After removing the precipitated protein by centrifugation at 11,337g for 10 minutes the supernatants were taken and extracted with an equal volume of diethyl ether to remove the TCA. The ether layer was then removed and the extraction was repeated three times. Following this, samples of the low molecular weight fraction and 0.1M amino acid standards (Sigma) were analysed by TLC using an ethanol:ammonium (70:30) solvent system (Stahl, 1965). Standards were prepared in a 10% (v/v) aqueous solution of propanol. The plates were then dried and the amino acids visualised by spraying the plates with a 0.2% (w/v) solution of ninhydrin in acetone.

High performance liquid chromatography

Extracts of both zooxanthellae and host from tentacle tissue were prepared as described above. These were then analysed by HPLC to determine the composition of amino acids and concentration of the individual amino acids making up the free amino acid pool. This analysis was carried out by Bioflux (Robertson Institute of Biotechnology, University of Glasgow) and used the *o*-phthalaldehyde derivitization method of Jarrett *et al.* (1985). This analysis allowed the concentrations of both glutamine and glutamate to be measured and these were used to calculate the gln:glu ratio of zooxanthellae (Flynn, 1990).

Effect of 20μM ammonium treatment on the free amino acid pool

Symbiotic anemones were maintained under standard conditions (section 2.1). One group of anemones were subjected to a constant input of 20μM ammonium-supplemented seawater using the apparatus described in section 2.5. A second group of anemones were maintained in control seawater at ambient (<1μM) ammonium concentrations. The anemones were kept under these conditions for 47 days during which time they were not fed. After 47 days, tentacle tissue was removed from five animals from the control and ammonium-treated groups. The zooxanthellae and host fractions were prepared for free amino acid analysis as described above (section 4.2.1). The concentration of total free amino groups was determined from five anemones of each group using the total free amino group method (section 4.2.1.1). The composition of the free amino acid pool was determined from two anemones of each group using HPLC (section 4.2.1.2).

4.2.2 Enzyme analysis

4.2.2.1 GDH assay (EC 1.4.1.2-4)

GDH activity of both zooxanthellae and host was assayed using tentacle tissue and according to the method of Catmull *et al.* (1987). Anemones were maintained under standard conditions (section 2.1) and tissue was taken four days after feeding.

Host tissue preparation

Approximately 100mg wet weight tentacle tissue was homogenised (Ultra-Turrax T25, Janke & Kunkel) per millilitre of buffer containing 5mM dithiotreitol (DTT) and 50mM N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid] (HEPES), pH 7.4. Zooxanthellae were removed by low speed centrifugation at 268g for 2 minutes and the supernatant was taken as the host cell extract. The cloudiness of this crude host extract

was reduced by a higher speed centrifugation at 11,337g for 5 minutes. This centrifugation was repeated three times. All tissue extracts and reagents were kept on ice. Initial experiments showed that the use of a motor-driven homogeniser did not reduce enzyme activity when compared to results obtained with a hand homogeniser (data not shown).

Zooxanthellae tissue preparation

Zooxanthellae were removed from tentacle tissue using the method described above. These algal cells were cleaned by repeated centrifugation and washing in artificial seawater (section 2.3) and resuspended in 2ml buffer containing 5mM DTT, 50mM HEPES, pH 7.4. The cells were broken by passing through a chilled French pressure cell three times at 82,737kPa (Aminco, SLM Instruments Inc.). The suspension was then centrifuged at 11,337g for 10 minutes to remove cell debris and the supernatant was taken as the tissue extract used in the enzyme assay.

Enzyme assay

GDH activity was measured by recording the oxidation of NAD(P)H as the reductive amination of 2-oxoglutarate occurred. The rate of coenzyme oxidation was recorded using a Philips PU8720 spectrophotometer at 340nm. This assay was carried out in a final volume of 1ml containing 50mM HEPES (pH 7.4), 100mM ammonium acetate, 0.2mM NADPH or NADH (Sigma) and tissue extract (80-160µg protein). The mixture was allowed to equilibrate to 25°C and the reaction initiated by adding 50µl 200mM 2-oxoglutarate. Glutamine-linked GDH activity was measured by replacing the 100mM ammonium acetate with 100mM glutamine (Male & Storey, 1983).

4.2.2.2 Fd-GOGAT assay (EC 1.4.7.1)

Ferredoxin-dependent GOGAT activity of zooxanthellae from tentacle tissue was measured using the method of Hecht *et al.* (1988) scaled down by a factor of 2. This activity was determined by measuring the production of glutamate during the reaction of 2-oxoglutarate and glutamine in the presence of the ferredoxin analogue, methyl viologen.

Zooxanthellae tissue preparation

In order to obtain enough zooxanthellae extract to assay enzyme activities, tentacle tissue was removed from three symbiotic anemones and wet weighed. The zooxanthellae and

host fractions were then separated by repeated centrifugation and washing in 0.3µm filtered seawater as described in section 2.3. The zooxanthellae were then resuspended in 2ml of extraction buffer containing 100mM KH_2PO_4/KOH pH 7.5, 0.5mM ethylene diaminetetraacetic acid (EDTA), 100mM KCl, 0.5% (v/v) Triton X100 and 0.1% (v/v) mercaptoethanol. The zooxanthellae suspension was then passed through a chilled French pressure cell three times at 82,737kPa (Aminco, SLM Instruments Inc.). Following this, 0.3g of AG 1-X2 ion exchange resin (200-400 mesh, acetate form) (Bio Rad) was added to the suspension to remove acidic amino acids present in the tissue extract. After 10 minutes on ice, the suspension was centrifuged at 11,337g for 10 minutes to pellet cell debris and resin. The supernatant was then removed and kept on ice until used for assay.

Enzyme assay

All reagents were prepared in buffer containing 500mM KH_2PO_4/KOH, pH 7.5. The reaction mixture contained 50µl 100mM glutamine, 50µl 100mM 2-oxoglutarate, 50µl 150mM methyl viologen (Sigma), 200µl of zooxanthellae extract, and was made up to a final volume of 0.75ml with buffer. The reaction mixtures were preincubated at 30°C for 10 minutes and the reaction initiated by adding 50µl of reductant (47mg $Na_2S_2O_4$ and 50mg $NaHCO_3$ in 1ml buffer). After 20 minutes incubation at 30°C the reaction was terminated by adding 0.5ml ethanol. Each reaction mixture was then transferred to an AG 1-X2 ion exchange column (200-400 mesh, acetate form, 6mm ∅, 40mm length). Glutamine was eluted from the columns with 7.5ml distilled water. Residual water was removed by centrifugation at 390g for 2 minutes. Glutamate was then eluted from the columns with 2.5ml 3M acetic acid. The centrifugation step was repeated to remove residual acetic acid. The concentration of glutamate in the acetic acid was determined using the total free amino groups method described before (section 4.2.1.1) and using glutamate standards prepared in 3M acetic acid. Negative controls were carried out in which one of the reagents (i.e. 2-oxoglutarate or glutamine or methyl viologen) were omitted. Any ninhydrin-positive colour development in these controls was subtracted from the experimental assays to take account of side reactions caused by glutaminase or transaminases present in the tissue extract.

To demonstrate that any activity detected was enzyme-mediated, a sample of the zooxanthellae tissue extract was placed in a boiling water bath for 5 minutes to denature all enzymes prior to assay. It is possible that apparent activity could be produced by

transaminase enzymes, other than GOGAT, present in the crude tissue extract. To control for this, the non-specific transaminase inhibitor aminooxyactetic acid (AOA) was used at a concentration of 1mM. A final control was made using the GOGAT inhibitor azaserine (AZS) at a concentration of 5mM. By eliminating GOGAT activity, AZS should eliminate all the activity detected by this assay whereas AOA should not.

Protein Estimation

Protein concentration of the extracts assayed was determined using the Coomassie Blue protein-dye binding procedure of Bradford (1976) with bovine serum albumin (Sigma) as the standard. Protein estimation based on the Lowry (1951) method was found to be unsuitable as the HEPES buffer produced a strong colour reaction in negative controls.

4.3 Results

4.3.1 Free amino acid analysis

4.3.1.1 Total free amino group concentration

Table 4.1 gives the concentration of free amino groups in zooxanthellae and host from symbiotic anemones maintained in ambient seawater or seawater supplemented with 20μM ammonium.

Table 4.1 Effect of ammonium treatment on the concentration of free amino groups in unfed symbiotic anemones. Mean ±SD (n=5). Concentrations have been normalised to wet weight of tentacle tissue.

Fraction	Seawater ammonium concentration (μM)	Free amino group concentration ($\mu mol.g^{-1}$)
Host	<1	100.4 (±21.4)
Host	20	113.3 (±27.1)
Zooxanthellae	<1	10.1 (±3.4)
Zooxanthellae	20	21.3 (±3.3)

Anemones were maintained for 47 days without feeding in seawater at ambient ammonium concentrations (<1μM) or in seawater supplemented with 20μM ammonium at an illumination of 150μmol photons.$m^{-2}.s^{-1}$.

When anemones were maintained in seawater supplemented with 20μM ammonium the concentration of total free amino groups in host tissue did not change (ANOVA p=0.427) whereas that in zooxanthellae increased by a factor of two (ANOVA + Tukey, p<0.001).

4.3.1.2 Free amino acid pool composition

Host tissue from symbiotic anemones was analysed by TLC. This analysis gave little resolution of the composition of the free amino acid pool but did show that there were two major components present in host extracts. These ran close to the standards of glycine and alanine in the ethanol/ammonium solvent system used. When host extracts were analysed using HPLC, the entire composition of the free amino acid pool was obtained. The free amino acid composition of host tissue is given in table 4.2 and zooxanthellae tissue in table 4.3. The analyses from anemones maintained in seawater supplemented with 20μM ammonium are also given in these tables.

Table 4.2 Free amino acid composition of host tissue from symbiotic anemones maintained in control seawater or seawater supplemented with 20μM ammonium.

	Control		Ammonium	
Amino Acid	**Concentration ($\mu mol.g^{-1}$)**	**% total**	**Concentration ($\mu mol.g^{-1}$)**	**% total**
Aspartate	1.920 (0.170)	2.137	1.417 (0.519)	1.263
Glutamate	3.466 (0.828)	3.830	1.994 (1.145)	1.747
Asparagine	0.360 (0.084)	0.398	0.247 (0.095)	0.220
Serine	1.920 (0.382)	2.126	1.755 (0.593)	1.568
Glutamine	0.450 (0.042)	0.501	0.367 (0.201)	0.323
Histidine*	0.390 (<0.001)	0.436	0.525 (0.063)	0.478
Glycine	46.298 (5.260)	51.466	66.351 (8.753)	60.329
Threonine*	0.810 (0.128)	0.899	0.727 (0.116)	0.660
Arginine	2.490 (0.762)	2.744	2.069 (0.742)	1.846
Alanine	Not detected	Not detected	0.097 (0.032)	0.087
Taurine	29.090 (0.976)	32.530	32.924 (5.029)	29.884
Tyrosine	Not detected	Not detected	Not detected	Not detected
γ-aminobutyrate	Not detected	Not detected	Not detected	Not detected
Methionine*	0.226 (0.022)	0.250	0.030 (<0.001)	0.028
Valine*	0.480 (0.042)	0.534	0.337 (0.159)	0.298
Tryptophan*	Not detected	Not detected	Not detected	Not detected
Phenylalanine*	0.060 (<0.001)	0.067	0.037 (0.011)	0.034
Isoleucine*	0.376 (0.064)	0.416	0.292 (0.138)	0.258
Leucine*	0.270 (0.170)	0.293	0.142 (0.095)	0.124
Ornithine	Not detected	Not detected	Not detected	Not detected
Lysine*	1.26 (0.722)	1.371	0.960 (0.382)	0.853
Total	89.866 (9.652)	100	110.275 (18.073)	100

Amino acid concentrations were normalised to wet weight of tentacle tissue. Mean ±SD (n=2).
*Essential amino acid.

Table 4.3 Free amino acid composition of zooxanthellae tissue from symbiotic anemones maintained in control seawater or seawater supplemented with 20μM ammonium.

	Control		Ammonium	
Amino Acid	**Concentration ($\mu mol.g^{-1}$)**	**% total**	**Concentration ($\mu mol.g^{-1}$)**	**% total**
Aspartate	0.848 (0.074)	8.260	1.717 (0.265)	5.416
Glutamate	1.845 (0.976)	16.433	5.459 (0.020)	17.200
Asparagine	0.090 (0.021)	0.852	0.300 (0.021)	0.945
Serine	0.825 (0.276)	7.653	2.347 (0.137)	7.398
Glutamine	1.658 (0.499)	15.482	5.856 (0.051)	18.453
Histidine*	0.428 (0.138)	3.975	0.892 (0.096)	2.810
Glycine	0.855 (0.509)	7.507	2.549 (0.042)	8.034
Threonine*	0.345 (0.106)	3.218	1.042 (0.053)	3.283
Arginine	1.485 (0.615)	13.550	2.662 (0.286)	8.393
Alanine	0.353 (0.138)	3.232	1.455 (0.531)	4.573
Taurine	0.435 (0.276)	3.787	2.130 (0.211)	6.714
Tyrosine	0.323 (0.138)	2.935	0.397 (0.096)	1.250
γ-aminobutyrate	Not detected	Not detected	0.045 (0.021)	0.141
Methionine*	0.008 (0.011)	0.095	0.277 (0.032)	0.874
Valine*	0.150 (0.042)	1.406	0.052 (0.011)	0.166
Tryptophan*	Not detected	Not detected	0.397 (0.032)	1.252
Phenylalanine*	0.105 (0.042)	0.960	0.270 (0.042)	0.850
Isoleucine*	0.083 (0.011)	0.798	0.202 (0.032)	0.639
Leucine*	0.150 (0.042)	1.406	0.352 (0.032)	1.110
Ornithine	0.338 (0.095)	3.164	0.480 (0.064)	1.511
Lysine*	0.570 (0.191)	5.287	2.857 (0.457)	8.992
Total	10.892 (4.180)	100	31.741 (0.423)	100

Amino acid concentrations were normalised to wet weight of tentacle tissue. Mean ±SD (n=2).
*Essential amino acid.

Figure 4.2

Free amino acid pool composition of host tissue from symbiotic anemones maintained without feeding in control seawater or seawater supplemented with 20µM ammonium under an illumination of 150µmol photons.$m^{-2}.s^{-1}$. Mean ±SE (n=2).

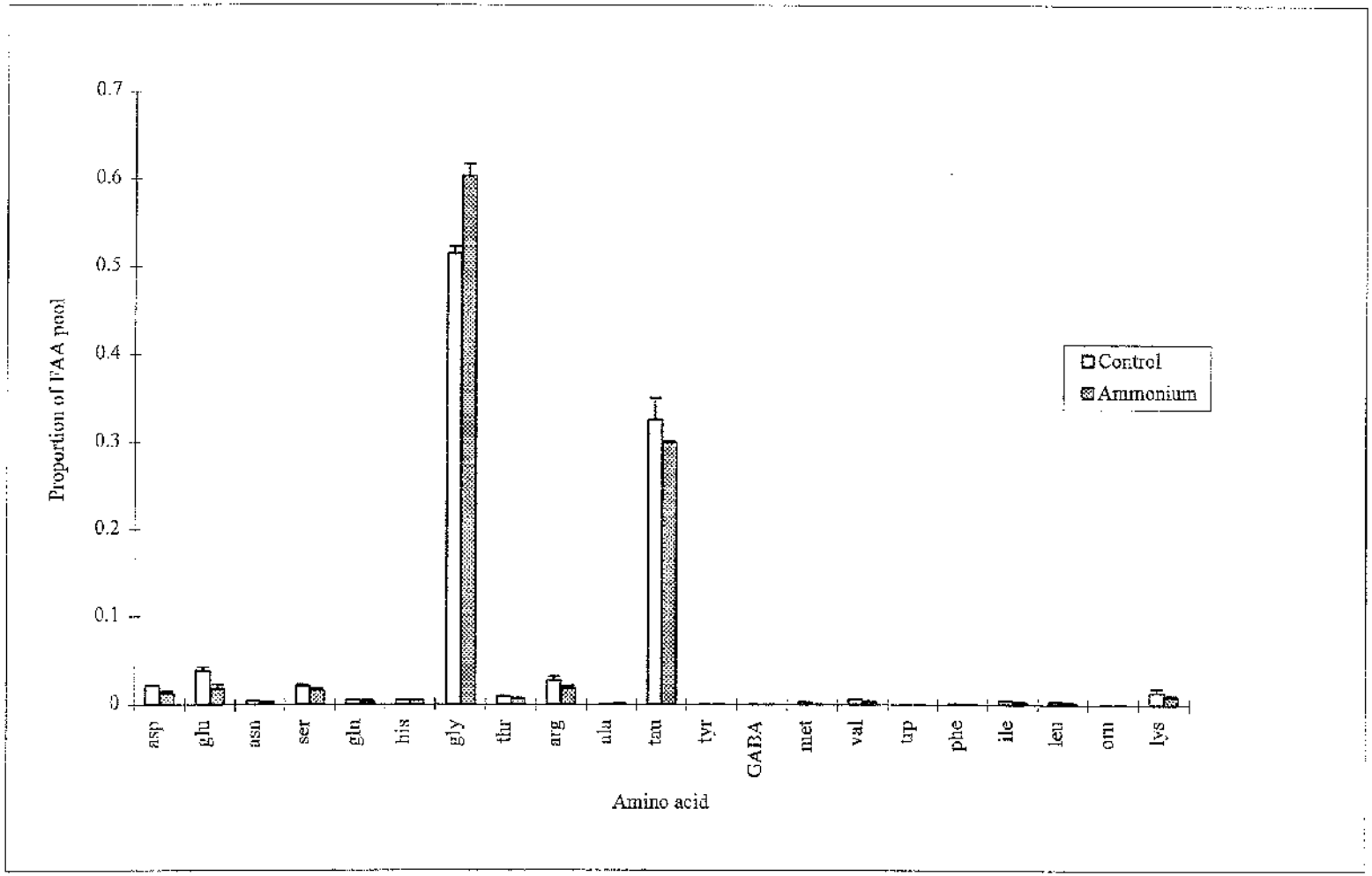
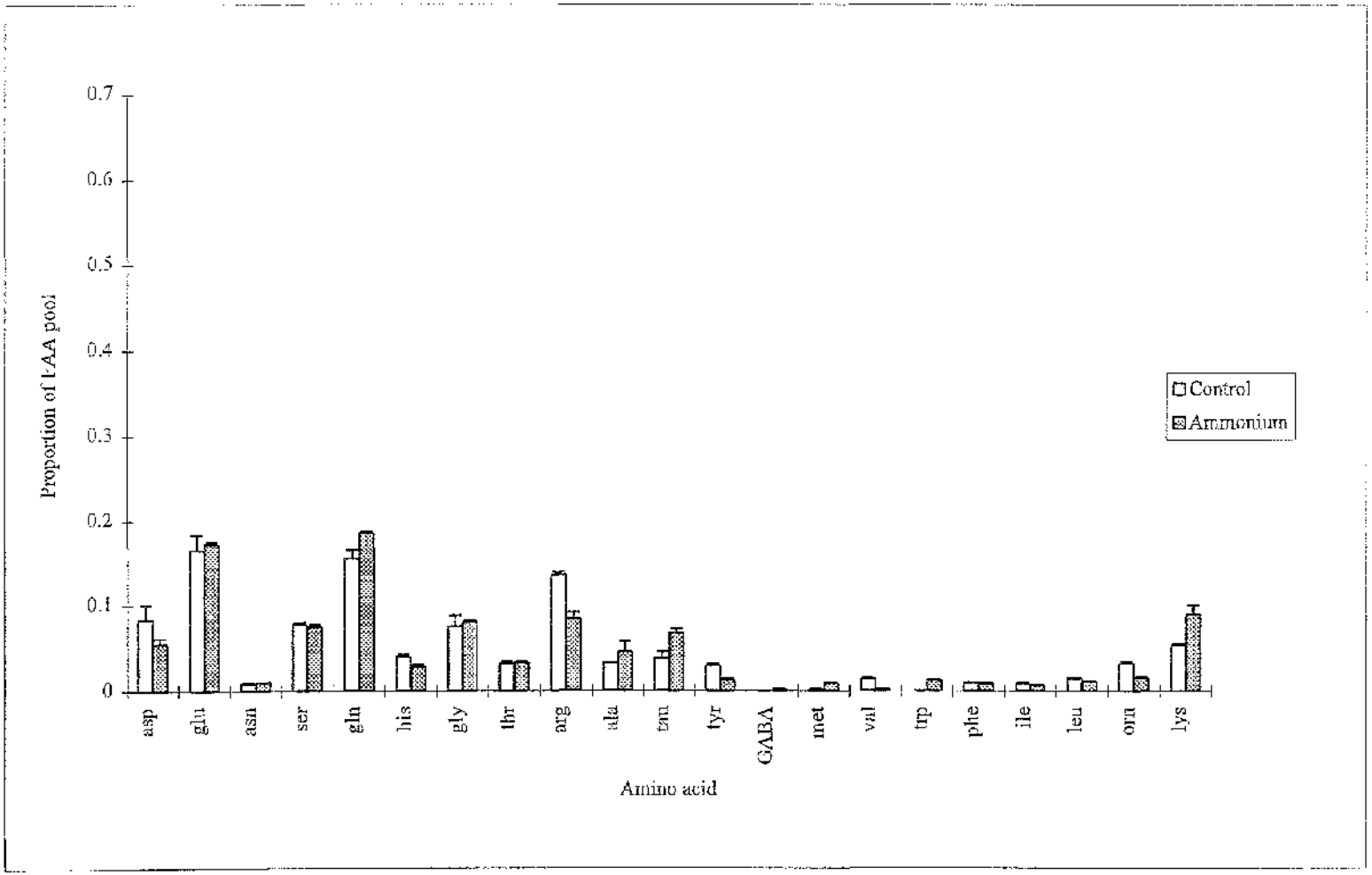


Figure 4.3

Free amino acid pool composition of zooxanthellae tissue from symbiotic anemones maintained without feeding in control seawater or seawater supplemented with 20μM ammonium under an illumination of 150μmol photons $m^{-2}.s^{-1}$. Mean ±SE (n=2).



The amino acid pool of host tissue (figure 4.2) was found to be dominated by two amino acids, glycine and the sulphonic amino acid taurine. This high glycine concentration confirmed the results of the TLC analysis although there was no evidence for high levels of alanine. Indeed in all but one of the analyses of host tissue, alanine was not detected. This could have been due to difficulty in resolving the alanine peak which was close to the much larger taurine peak on the HPLC system. Glycine and taurine accounted for approximately 85% of the total free pool in host tissue. In host tissue from anemones maintained with 20μM ammonium, glycine and taurine accounted for 90% of the free pool. In contrast, the free amino acid pool of zooxanthellae had a more even composition with no amino acids dominant (figure 4.3). In zooxanthellae, the most abundant amino acids were glutamate, glutamine and arginine which together accounted for 45% of the total free amino acid pool. The next most abundant amino acids were aspartate, serine and glycine which accounted for a further 24% of the total. The ratio of glutamine:glutamate was 0.961 (±0.238) in zooxanthellae from control anemones and 1.073 (±0.004) in zooxanthellae from anemones maintained with 20μM ammonium.

4.3.2 Enzyme analysis

4.3.2.1 GDH assay

The activity of GDH was expressed in units each equivalent to 1μmole coenzyme oxidised per minute. These units were then normalised to the protein content of the extract assayed. Host tissue was found to contain GDH. However, the assay of host extracts prepared from tentacle tissue was complicated by the presence of a substance with a peak of absorbance at 332nm, which was close to the 340nm wavelength used to assay NADPH/NADH. The compound responsible for this absorbance peak passed through a dialysis membrane indicating that it was of low molecular weight (~10,000g) and was not found in sonicated zooxanthellae tissue. This compound elevated initial absorbances and when the concentration of tissue extract was increased initial absorbance readings were too high to be measured. While limiting the concentration of tissue extract which could be used, this compound did not prevent the detection of GDH activity.

Both host and zooxanthellae extracts contained GDH which was more active in the presence of NADPH than NADH (table 4.4). GDH activity was also shown by both fractions when the ammonium substrate was replaced with glutamine. However, the activity recorded with the glutamine substrate was lower than that recorded with

ammonium in all treatments apart from NADH-linked activity in zooxanthellae. Here the glutamine-linked activity was greater than that seen with ammonium (figure 4.4).

Table 4.4 NADPH and NADH linked activity of GDH from host and zooxanthellae fractions with ammonium and glutamine substrates.

Fraction and coenzyme	GDH activity Ammonium	GDH activity Glutamine	Ratio of activity Ammonium:Glutamine
Host NADPH	23.8 (±15.9) n=6	4.17 n=1	5.7 : 1
Zooxanthellae NADPH	26.72 n=1	7.69 n=1	3.5 : 1
Host NADH	8.6 (±6.8) n=4	0.30 n=1	28.7 : 1
Zooxanthellae NADH	2.42 n=1	5.38 n=1	0.4 : 1

Enzyme activity is expressed as the mean number of milli units mg^{-1} protein (±SD).

4.3.2.2 Fd-GOGAT assay

The concentration of free amino groups detected in experimental assays were consistently higher than those found in controls where one of the assay reagents was omitted. Table 4.5 summarises the glutamate production measured as the total free amino group concentrations in the six experiments conducted.

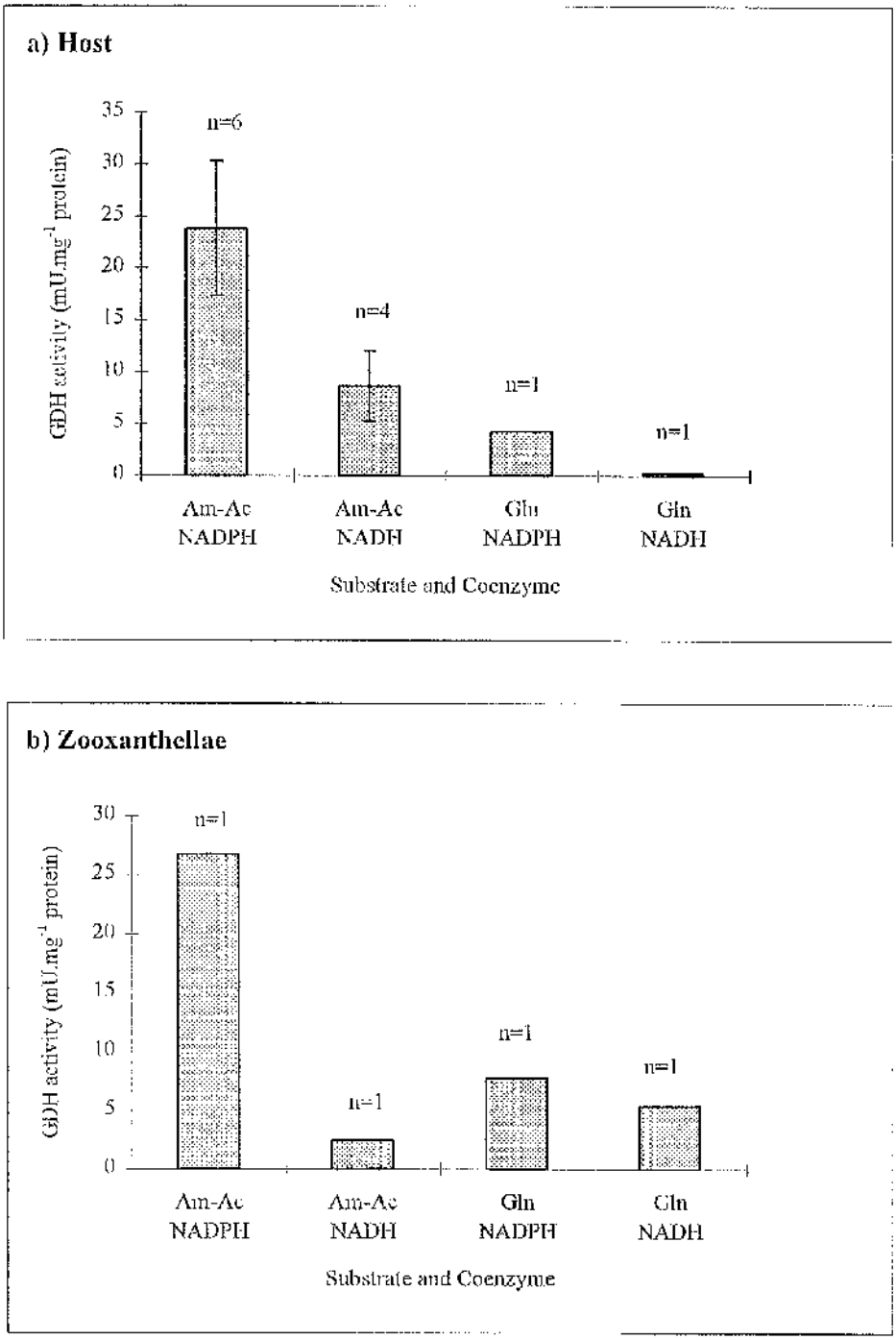
Table 4.5 Glutamate production as free amino group concentrations of control and experimental treatments recorded in 6 replicate GOGAT assays.

Assay	Glutamate, as free amino group concentration (mM)					
Experimental with all reagents	0.161	0.115	0.103	0.161	0.119	0.140
Control without methyl viologen	0.136	0.089	0.081	0.154	0.093	0.120
Control without 2-oxoglutarate	0.123	0.089	0.088	ND	ND	0.105
Control without glutamine	0.113	0.063	0.062	ND	ND	ND
Control without tissue extract	0.018	0.021	0.033	0.029	0.018	0.040
Control with boiled tissue extract	ND	ND	ND	0.097	ND	ND

ND, not determined.

Figure 4.4
Comparison of mean GDH activities (±SE) of host and zooxanthellae with the two coenzymes NADPH or NADH and the two substrates ammonium acetate (Am-Ac) or glutamine (Gln). n, number of animals.

One unit (U) of activity is equivalent to 1µmol coenzyme oxidised.min^{-1}.



It was assumed that any glutamate resulting from glutaminase or transaminase activity would be accounted for in the control containing the highest FAA concentration. GOGAT activity was therefore calculated from the difference between the experimental assay and the highest control. The enzyme activity was expressed in units where one unit equals 1μmol glutamate produced per minute. These units were then normalised to the protein content of the extract assayed (table 4.6).

Table 4.6 GOGAT activity of zooxanthellae extracts prepared from symbiotic anemones

Treatment	**GOGAT activity (mU)**
Experimental	2.85 (±1.59) n=6
Boiled extract	0.00 (-) n=1
5mM aminooxyacetic acid (AOA)	3.11 (±2.13) n=3
1mM azaserine (AZS)	0.00 (-) n=3

Mean activity is expressed as the number of milli units (mU) mg^{-1} protein (±SD).

The GOGAT activity detected was destroyed by boiling the zooxanthellae extract and was completely inhibited by treatment with AZS. Treatment with AOA, a non-specific transaminase inhibitor, did not have any effect on GOGAT activity suggesting that the activity recorded was not due to the presence of other transaminases in the extract.

4.4 Discussion

4.4.1 Free amino acid pools

When the free amino acid (FAA) pools of symbiotic anemones were analysed, the composition of the host was found to be dominated by just two amino acids, glycine and the sulphonic amino acid taurine. The FAA composition of the zooxanthellae differed from the host with glutamate, glutamine and arginine forming the dominant constituents. This compared well with the only other published FAA composition from zooxanthellae of *Anemonia viridis* (McAuley, 1994).

The FAA pools of anemones are frequently dominated by a few amino acids which play a significant part in osmoregulation. For example, taurine is the dominant constituent of the FAA pool of *Anthopleura xanthogrammica* (Male & Storey, 1983), *Bunodosoma cavernata* (Kasschau *et al.*, 1984), *Condylactis gigantea* (Herrera *et al.*, 1989), *Haliplanella lineata* (Shick, 1976) and *Metridium senile* (Deaton & Hoffman, 1988). Taurine is metabolically inert (Bishop *et al.*, 1983) and as a 'compatible solute' it will be less disruptive to enzyme function than would inorganic salts and basic amino acids (Hochachka & Somero, 1984). These factors may have selected for its osmoregulatory role in these species of sea anemone (Shick, 1991). In *Diadumene leucolena* the dominant free amino acid is glycine (Pierce & Minasian, 1974) which also forms a major component of the pool in *Haliplanella lineata* (Shick, 1976). These anemones are more likely to experience short-term fluctuations in salinity where there may have been selection for a flexible osmoregulatory system. Glycine and amino acids such as alanine and glutamate, which can also dominate these FAA pools, are readily metabolised by cnidarians and so could respond to short-term salinity change (Shick, 1991). In *Anemonia viridis* the high concentrations of taurine and glycine (figure 4.2) could reflect an osmoregulatory function.

The FAA concentration in animal cells shows considerable variation between species and between tissues within a species (Urich, 1990). However, in the Cnidaria the concentration of free amino acids is considerably higher than that in mammals. For example, while the concentration of FAA in mammalian brain is about 20μmol.g^{-1} (McIlwain & Bachelard, 1971), that in sea anemones can exceed 100μmol.g^{-1} (Shick, 1991). The concentration of free amino groups in host tissue (113μmol.g^{-1}) from

symbiotic anemones, compares well with the range of total FAA concentrations from sea anemones (32 to 114μmol.g^{-1}) summarised by Shick (1991).

The experimental work described in the previous chapter has shown that treatment with 20μM ammonium increases the total nitrogen content of both zooxanthellae and host. Treatment with 20μM ammonium appeared to increase the concentration of zooxanthellae FAA but did not affect that of the host. However, although the concentrations of glutamine and glutamate in the zooxanthellae increased, the resulting glutamine:glutamate ratio of 1.073 appears not to differ from the ratio of 0.961 in control anemones. The only other such ratio from *Anemonia viridis* was 1.44 from the zooxanthellae of anemones fed weekly in seawater containing 2-5μM ammonium (McAuley, 1994). All these ratios are greater than the 0.2 threshold below which microalgae are defined as nitrogen-limited (Flynn, 1990, 1991) suggesting that, by this criterion, zooxanthellae from *Anemonia viridis* may not be nitrogen-limited.

4.4.2 Enzymes of ammonium assimilation

The mechanism of ammonium assimilation in microalgal-invertebrate symbioses remains the subject of debate (Rees, 1987; Rees & Ellard, 1989; Miller & Yellowlees, 1989). Here two of the potential routes by which ammonium could be assimilated were examined. Miller and Yellowlees (1989) proposed that NADPH-GDH in the host could be the mechanism of ammonium assimilation. In the zooxanthellae, the linked activity of GS/GOGAT has been put forward as the assimilatory pathway (Summons & Osmond, 1981; Summons *et al.*, 1986).

The host tissue extracts prepared for GDH assay contained a low molecular weight factor which absorbed strongly in the UV region of the spectrum. It is possible that this interference was caused by mycosporine-like amino acids which are known to be present in both sea anemones and corals where they are believed to provide these animals with some protection from UV light (Dunlap & Chalker, 1986). Despite the presence of this UV absorbing factor, it was possible to detect GDH activity in the host tissue extracts. Host tissues from symbiotic anemones contained GDH which showed greater activity in the presence of NADPH than NADH. Most animal GDH is active with both coenzymes, though activity is usually greatest with NADH (Urich, 1990; Smith *et al.*, 1975). These results show that *Anemonia viridis*, in common with other Cnidaria, differs from most

animal systems in that GDH is more active with NADPH than NADH (Hoffmann *et al.*, 1978).

In mammalian, and most other animal systems, NADPH-GDH is most active in glutamate synthesis (amination) whereas NAD^+-GDH is most active in its breakdown (excretory deamination). NADPH-GDH has been found in the corals *Acropora formosa* (Catmull *et al.*, 1987), *Stylophora pistillata* (Rahav *et al.*, 1989) and the sea anemones *Metridium senile* (Bishop *et al.*, 1978) and *Anthopleura xanthogrammica* (Male & Storey, 1983). The activity of GDH from host tissue of *Anemonia viridis* is compared with equivalent values from other algal-invertebrate symbioses in table 4.7. The value determined during this study falls within the range of previously reported GDH activities. Dudler *et al.* (1987) presented evidence that the coral *Acropora latistella* contained two distinct GDH isoenzymes, a cytoplasmic NADPH-GDH and a mitochondrial NADH-GDH. These authors suggested that since the mitochondrial membrane is essentially impermeable to reduced coenzyme, conditions within the mitochondria would favour the deamination reaction and so provide 2-oxoglutarate as an energy-yielding substrate from glutamate. Whether or not a similar system operates in *Anemonia viridis* would require further investigation.

Table 4.7 Comparison of NADPH-GDH activities from host tissue of algal-invertebrate symbioses.

Species	NADPH-GDH activity (mU)	Source
Anemonia viridis	24	This study
Pocillopora damicornis	34	Yellowlees *et al.* (1994)
Anthopleura xanthogrammica	142	Male & Storey (1983)
Acropora latistella	7	Dudler *et al.* (1987)
Acropora formosa	180	Catmull *et al.* (1987)

One unit is equivalent to 1μmole coenzyme oxidised.min^{-1}.mg^{-1} protein.

Zooxanthellae tissue also contained GDH which showed greater activity in the presence of NADPH than NADH. Dudler and Miller (1988) found two GDH isoenzymes in zooxanthellae isolated from the coral *Acropora formosa*. The first showed specificity for NADPH and was most active in the direction of glutamate synthesis which was

interpreted as evidence for a role in ammonium assimilation. The second isoenzyme showed specificity for NADH and was active both in amination and deamination. It was suggested that the second enzyme carried out glutamate oxidation *in vivo*.

Both host and zooxanthellae GDH showed activity when glutamine replaced ammonium as the substrate for the amination reaction. This phenomenon was also reported from host tissue of the anemone *Anthopleura xanthogrammica* where the ratio of ammonium to glutamine activity was 11:1 at pH 7.0 and 2:1 at pH 8.0 (Male & Storey, 1983). The ratio of 6:1 determined during this study, at a pH of 7.4, compares well with these data. Glutamine-linked GDH activity is equivalent to that of GOGAT. Male and Storey suggested that this reaction may provide an alternative mechanism by which ammonium could be assimilated. Whether or not this represents a significant route by which ammonium assimilation could take place in the host is unknown. Male and Storey demonstrated that the ammonium and glutamine activities co-chromatographed throughout their purification procedure and that the activities could not be separated on the basis of molecular size by gel filtration. Therefore it appears that the glutamine-linked activity was not due to GOGAT contamination from zooxanthellae or bacteria; the molecular weight of GOGAT in the bacterium *Escherichia coli* was over twice that of GDH (Miller & Stadtman, 1972 cited by Male & Storey, 1983). However, glutamine-linked GDH activity was not detected in host tissue from the coral *Acropora formosa* (Catmull *et al.*, 1987). Whether or not glutamine-linked GDH activity coupled to GS could provide *Anemonia viridis* with an alternative route of ammonium assimilation awaits further investigation. GS appears to be the enzyme by which ammonium is assimilated by host tissue from the green *Hydra* symbiosis (Rees, 1987, 1989a). Its activity has recently been demonstrated in the host from the coral *Pocillopora damicornis* (Yellowlees *et al.*, 1994), the giant clam *Tridacna gigas* (Rees *et al.*, 1994) and the anemone *Aiptasia pallida* (Ferrier, 1996). Any further investigation of the ammonium assimilatory enzymes of *Anemonia viridis* should include an analysis of the activity of GS in the host.

This investigation has identified and quantified GOGAT activity in zooxanthellae for the first time. The only previous attempt to assay GOGAT activity in zooxanthellae was that of Wilkerson and Muscatine (1984). These authors failed to detect any NADH or NADPH-dependent GOGAT activity from zooxanthellae isolated from the anemone *Aiptasia pulchella* but did not investigate ferredoxin-dependent activity. Here GOGAT

activity has been detected using a ferredoxin analogue, methyl viologen. This suggests that GOGAT activity in zooxanthellae would be ferredoxin-linked *in vivo*. Since the activity assayed was unaffected by the non-specific transaminase inhibitor AOA, the enzyme activity recorded is unlikely to be due to the activity of transaminase enzymes which will have been present in the crude tissue extract prepared. On the other hand, the GOGAT inhibitor azaserine, completely eliminated all glutamate production suggesting that the activity seen was due to this enzyme.

To summarise, this investigation has shown that the free amino acid composition of zooxanthellae and host are markedly different. When anemones were maintained under 20μM ammonium the concentration of free amino groups increased in the zooxanthellae but appeared not to change in the host. There was no evidence that the ratio of glutamine:glutamate in zooxanthellae changed when anemones were maintained with 20μM ammonium for 47 days. GDH was detected in both zooxanthellae and host where it was most active with the coenzyme NADPH. In addition, GDH showed activity when glutamine replaced ammonium as the substrate. It is possible that this could provide an alternative route by which the host could assimilate ammonium. Zooxanthellae were shown to possess GOGAT active in the presence of a ferredoxin analogue. This suggests that *in vivo* zooxanthellae could assimilate ammonium via the activity of GS linked with ferredoxin-dependent GOGAT. While this provides some evidence for the existence of particular mechanisms of ammonium assimilation, it provides no information on whether or not these pathways operate *in vivo*. The following chapter describes an investigation carried out to follow the pathway of ammonium assimilation in symbiotic anemones.

Chapter 5

Pathway of ammonium assimilation in symbiotic anemones

5.1 Introduction

The experimental work described in chapter 3 investigated the quantitative importance of ammonium to symbiotic anemones in the light by constructing a nitrogen budget. This was followed by a preliminary study of the qualitative aspects of ammonium uptake by investigating the metabolism of ammonium in symbiotic anemones. In this chapter, some of the assumptions made by the nitrogen budget and the qualitative aspects of ammonium uptake will be further investigated by following the pathway of ammonium assimilation. In particular, the question of whether ammonium is assimilated by zooxanthellae or host tissue will be addressed (Miller & Yellowlees, 1989; Rees, 1989b). If ammonium is assimilated by the zooxanthellae, this leads on to the central issue of whether nitrogen is translocated to the host and if so in what form. Previous studies have provided indirect evidence that amino acids may form part of this translocate (Muscatine & Cernichiari, 1969; Trench, 1971b,c; Lewis and Smith, 1971; Muscatine *et al.*, 1972) but there is scant evidence for the transfer of 'high quality' nitrogen in the form of essential amino acids (but see Markell & Trench, 1993 and Falkowski *et al.*, 1993). If amino acids formed a significant part of the translocate from the zooxanthellae, then the host would not only gain a carbon input from the symbionts (Muscatine *et al.*, 1984; Davies, 1984; Edmunds & Davies, 1986) but a nitrogen input as well. This might explain the autotrophic growth demonstrated by symbiotic anemones supplied with 20μM ammonium in the light (section 3.3.1).

As discussed in previous chapters, symbiotic anthozoans are able to take up inorganic nitrogen as ammonium (and sometimes nitrate) in the light, whereas aposymbiotic individuals and non-symbiotic species show net excretion of inorganic nitrogen as ammonium. Clearly this phenomenon is somehow mediated by the zooxanthellae. Unlike certain coral species, symbiotic anemones, including *Anemonia viridis* (Davies, 1988), do not take up nitrate from seawater (Wilkerson & Muscatine, 1984; Davies, 1988). The mechanism of ammonium uptake in cnidarian-zooxanthellae symbiosis has been the subject of a debate over whether it is assimilated by the host or zooxanthellae (Miller and Yellowlees, 1989; Rees, 1989b). Thus although ammonium uptake is characteristic only of symbiotic individuals, the site of assimilation in cnidarian-zooxanthellae symbioses

remains unclear. One way of investigating whether zooxanthellae or host material assimilate ammonium is to follow this process with an isotopically-labelled tracer.

Isotope-based techniques allow investigation of the pathway of inorganic nitrogen uptake by providing a tracer which can be followed both during and after the uptake process. In addition, the use of isotope-based techniques can overcome several of the problems associated with investigations which rely on a depletion-based methodology, such as that used in chapter 3. Depletion-based methods rely on accurate determination of ammonium at low micromolar concentrations and take no account of ammonium depletion from adsorption onto surfaces (e.g. glass) or uptake by bacteria present in seawater. However, isotope enrichment of animal or plant tissue represents actual assimilation of inorganic nitrogen.

Previous studies which have investigated the pathway of ammonium assimilation using isotope-based tracer techniques can be divided into two categories: those using the radioactively labelled ammonium analogue ^{14}C-methylamine and those using ammonium enriched with the stable isotope ^{15}N as the tracer. Methylamine (alt. methylammonium) is metabolically inert and has been used to investigate the ammonium uptake kinetics of cultured zooxanthellae (Gunnerson *et al.*, 1988) and intact symbioses such as the coral *Madracis decactis* and the anemone *Aiptasia pallida* (D'Elia & Cook, 1988). However these investigations showed that ammonium competitively inhibited methylamine uptake. This would complicate the interpretation of uptake rates calculated from data based on methylamine assimilation in systems where the ratio of methylamine to ammonium was variable.

Problems of this nature can be overcome by using [^{15}N]ammonium, rather than its analogue. The degree of tissue enrichment after exposure to [^{15}N]ammonium can then be quantified by mass spectrometry. This approach was first used by Muscatine and D'Elia (1978) to investigate whether tissue from the coral *Pocillopora damicornis* was responsible for the disappearance of ^{15}N enriched ammonium from the incubating seawater. These authors found that the disappearance of ^{15}N label from the seawater was mirrored by the appearance of label in the coral tissues. This result was confirmed in a selection of corals by Burris (1983). There are relatively few other studies which have used ^{15}N as a tracer in this way. Muscatine *et al.* (1984) used data from the flux of [^{15}N]ammonium to calculate a nitrogen-specific zooxanthellae growth rate. Both this and

the growth rate calculated from measurement of mitotic index were low, and showed that zooxanthellae growth accounted for only a small fraction of the carbon fixed daily.

As discussed before, the principal site of ammonium assimilation in symbiotic anemones and corals is the subject of debate. There has been a general consensus that ammonium is assimilated by the zooxanthellae but it is possible that the host could also be responsible for some or all of the assimilation recorded. In the green *Hydra* symbiosis it has been suggested that carbon skeletons translocated to the host from the *Chlorella* symbionts provide the carbon substrate for host ammonium assimilation (Rees, 1987, 1989a,b). There is evidence that the host tissue from marine zooxanthellate symbioses contains enzyme systems which would be capable of assimilating ammonium and this has been put forward as evidence for the role of host tissue in assimilation (Miller & Yellowlees, 1989; Rees *et al.*, 1994; Ferrier *et al.*, 1996). However, the interpretation of whether or not host tissue is actively assimilating ammonium is complicated by the possibility that nitrogen-containing compounds appearing in the host could either have been assimilated directly or translocated from the zooxanthellae. It is equally possible that both processes are operating at the same time.

The work of Davies (1988) showed that nitrogen taken up by *Anemonia viridis* from seawater enriched with [^{15}N]ammonium appeared in the zooxanthellae over a 30 minute time course. No ^{15}N label was detected in the host fraction. However, these experiments relied on a density gradient centrifugation method to separate zooxanthellae from host material. It is not known whether the host fraction analysed represented the entire fraction or that portion of the gradient where host material accumulated. If the latter was the case, then it is possible that only a small proportion of the low molecular weight fraction of the host material would have been sampled. Since zooxanthellae were isolated as intact cells it is reasonable to assume that they would have retained the complete low molecular weight fraction together with high molecular weight biosynthetic products. Thus this study may have seriously underestimated the degree of labelling of the host fraction.

The only other studies to have investigated the site of inorganic nitrogen assimilation using ^{15}N have done so by following the division of label between zooxanthellae and host material (Wilkerson & Kremer, 1992; Hawkins & Klumpp, 1995). Wilkerson and Kremer (1992) examined the effect of both light and concentration on dissolved

inorganic nitrogen uptake by the symbiotic scyphozoan *Linuche unguiculata*. Zooxanthellae tissue was found to incorporate ammonium at a faster rate than host tissue. The detailed study of *Tridacna gigas* nutrition by Hawkins and Klumpp (1995) showed that zooxanthellae were responsible for assimilating [^{15}N]ammonium and that almost 100% of ^{15}N released from zooxanthellae was incorporated by host tissue. This conclusion was reached by finding that the ^{15}N lost from zooxanthellae over a 245 hour period following a 3.5 hour pulse with [^{15}N]ammonium could be accounted for by the ^{15}N appearing in the host fraction over that time period. In addition, these authors demonstrated that [^{15}N]ammonium uptake is modulated by nutritional state, photoperiod, ammonium concentration and a possible biological rhythmicity. Hoegh-Guldberg and Davidson (1996) used the indirect technique of $^{14}CO_2$ labelling followed by analysis of the N-containing labelled products to suggest that zooxanthellae of the symbiotic anemone *Aiptasia pulchella* were the site of ammonium assimilation. These studies argue that the zooxanthellae are the site of ammonium assimilation and therefore any label appearing in the host fraction will have been translocated from the zooxanthellae. However, such evidence does not prove this unequivocally since host tissue could be capable of direct assimilation of ammonium albeit at a lower rate than that shown by the zooxanthellae.

Despite the controversy which remains, the hypothesis that zooxanthellae are the major site of ammonium assimilation has been widely accepted and is an essential element of the depletion-diffusion model proposed to explain marine microalgal-invertebrate nutrient uptake (D'Elia, 1977; D'Elia *et al.*, 1983). The rate of ammonium uptake by cnidarian-zooxanthellae symbioses has been demonstrated in a number of studies to be positively correlated with the concentration of ammonium (e.g. Burris, 1983; Wilkerson & Muscatine, 1984; Wilkerson & Trench, 1986). This was also demonstrated for *Anemonia viridis* by both this study (section 2.6.3) and by Davies (1988) who used a depletion methodology to calculate the mass specific rate of ammonium uptake at a variety of concentrations. As discussed in section 1.4 these kinetics were interpreted as compatible with zooxanthellae-driven depletion of host cytoplasm followed by diffusion of ammonium from the incubating seawater, after the model proposed by D'Elia (1977) and D'Elia *et al.* (1983).

Ammonium assimilation by plant and microalgal cells requires a supply of fixed carbon provided by photosynthesis (Syrett, 1981). Since ammonium uptake is characteristic of

symbiotic individuals, the assimilation process would be expected to depend upon symbiont photosynthesis and would therefore require light. Gunnerson *et al.* (1988) demonstrated that methylamine uptake by cultured zooxanthellae was light-dependent and competitively inhibited by the presence of ammonium. D'Elia and Cook (1988) also demonstrated that methylamine uptake was light-dependent in intact symbiosis and was diminished by heterotrophic feeding. Davies (1988) showed that ammonium flux by *Anemonia viridis* was light-dependent and that it was possible to group ammonium flux rates on the basis of irradiance regime. Symbiotic anemones under high light took up ammonium at a faster rate than those under low light, whilst aposymbiotic anemones excreted ammonium at any light level.

There is evidence therefore that zooxanthellae may be the principal site of ammonium assimilation in *Anemonia viridis*. In chapter 4 the metabolism of ammonium in both zooxanthellae and host was discussed and the available data suggest that the initial products of ammonium assimilation will be the amino acids glutamine and glutamate. Ammonium assimilation by marine cnidarian symbioses is believed to be via the GS/GOGAT cycle of the zooxanthellae. This pathway is characteristic of plant cells (Syrett, 1981). GS/GOGAT has been demonstrated indirectly as the mechanism of ammonium assimilation in both isolated zooxanthellae from the giant clam *Hippopus hippopus* (Summons and Osmond, 1981) and from clam tissue slices (Summons *et al.*, 1986). These investigations analysed the products of ammonium assimilation in zooxanthellae using ^{15}N labelling and interpreted the pattern of amino acid enrichment. This showed that the amide group of glutamine became enriched with ^{15}N at a faster rate than the amino group, a pattern consistent with the transfer of labelled nitrogen via GS/GOGAT. The results presented in section 4.3.2.2 demonstrate that zooxanthellae from *Anemonia viridis* possess GOGAT. The initial products of ammonium assimilation in zooxanthellae of *Anemonia viridis* are not known. Ammonium assimilation by the host fraction could produce glutamine if catalysed by glutamine synthetase (Ferrier *et al.*, 1996) and/or glutamate if catalysed by glutamate dehydrogenase as proposed by Miller and Yellowlees (1989). The amino acid products of ammonium assimilation in host material have not been analysed in any cnidarian-zooxanthellae symbiosis.

As discussed in chapter 1, there is indirect evidence that nitrogen-containing compounds are present in the photosynthate translocated from zooxanthellae to host. However, the importance of this potential nitrogen source to the nutrition of the host is unknown.

Amino acids, principally alanine, were identified in the translocate of freshly isolated zooxanthellae after incubation with host homogenate (Trench, 1971c). Markell and Trench (1993) demonstrated the presence of essential amino acids in the glycoconjugate coat surrounding zooxanthellae after isolation, suggesting that these had been translocated through the algal amphiesma. It is usually the carbon skeleton (α-oxoacid) of an essential amino acid that cannot be synthesised and an animal may have some capacity to produce essential amino acids by transamination (Stryer, 1988). However, the essential amino acids lysine and threonine cannot be produced by transamination in animals and must therefore be present as such in the diet (Waterlow *et al.*, 1978). Thus labelling of lysine and threonine with ^{15}N in the host would imply translocation of these essential amino acids from the zooxanthellae and not host transamination of translocated α-oxoacids. Clearly this assumes that cnidarians have the same requirement for essential amino acids as vertebrate animals. Whether or not this is the case is unknown, although the recent investigation by FitzGerald and Szmant (1997) suggests that the Cnidaria may have the capacity to synthesise some of the amino acids which are regarded as essential in vertebrate animal taxa.

The experimental work described here addressed the following central questions:
What is the site of ammonium assimilation in symbiotic anemones?
What are the initial products of ammonium assimilation in symbiotic anemones and are these consistent with the GS/GOGAT cycle typical of microalgae?
Is there any evidence for a flux of nitrogen from zooxanthellae to host?

5.2 Methods

In this investigation, anemones were incubated with ^{15}N using the same protocol for a series of experiments. To avoid unnecessary repetition, preparative and analytical methods will be described in full here and the details of each experiment will be given in the subsequent sections. The techniques used to measure ^{15}N enrichment have been reviewed by Preston and Slater (1994).

5.2.1 Preparative and analytical methods

a) Incubation procedures for ^{15}N labelling

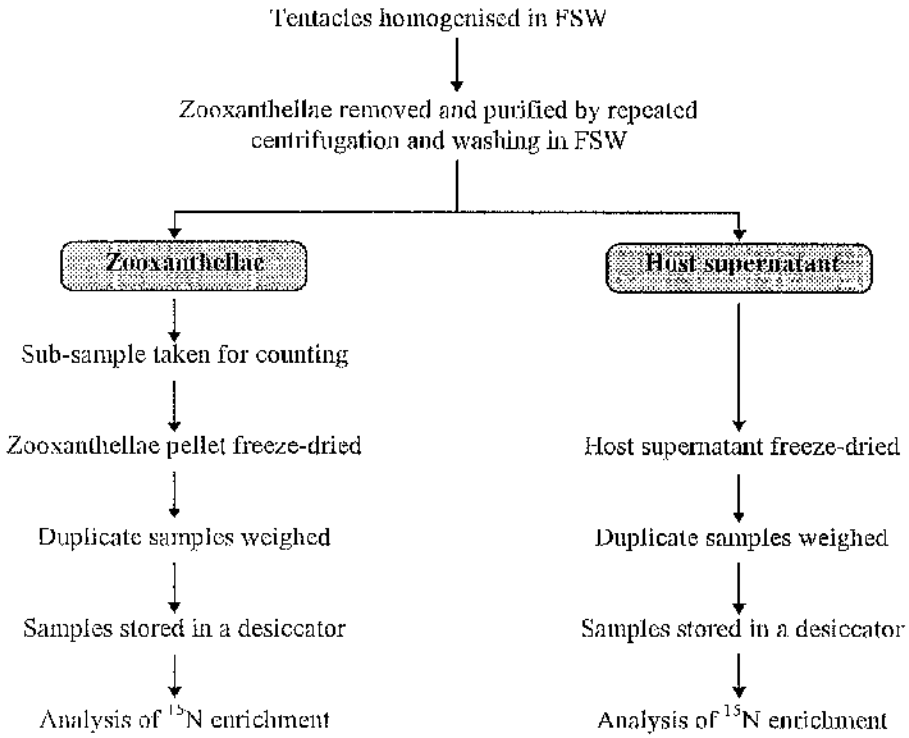
Anemones which had been maintained under standard conditions (section 2.1) were buoyant weighed (section 2.2) and anemones of a similar mass were transferred to 250ml volume glass beakers in a total volume (water plus anemone) of 200ml of 0.3µm-filtered seawater (FSW). The mean dry weight of the anemones used in these experiments was 357mg (SD=163, n=97). The animals were allowed at least one hour to attach to the base of the beaker, during which time they were maintained under the experimental irradiance at a temperature of 15°C. Only those animals that had attached to the glass and fully 'flowered' were used. All experiments were carried out three days after feeding and during the animals' 12 hour daylight period. Individual symbiotic anemones were incubated in FSW enriched with [^{15}N]ammonium. A concentrated stock of [^{15}N]ammonium, prepared in distilled water, was then added to each beaker to produce the final experimental concentration required. The incubation times, ammonium concentrations and procedures followed are described with the details of each experiment in the subsequent sections.

b) Separation of zooxanthellae and host fractions

Preliminary experiments showed that tentacle tissue from a single anemone provided enough algal material for analysis of total N enrichment when zooxanthellae and host were separated. However, after separation of zooxanthellae and host there was insufficient material from one anemone to analyse the total N enrichment of intact tentacle tissue. After incubation with ^{15}N, anemones were removed and rinsed in FSW to remove adhering labelled seawater. The tentacles were then removed, blotted dry and wet weighed before homogenisation in FSW. Zooxanthellae and host extracts were then

prepared using repeated centrifugations and washes in FSW as described in section 2.3. This protocol is summarised in figure 5.1.

Figure 5.1 Tissue preparation for analysis of ^{15}N enrichment by continuous flow-isotope ratio mass spectrometry (CF-IRMS).
Experimental details are given in the text.



c) Separation of high and low molecular weight material

Samples prepared for analysis of total ^{15}N enrichment by CF-IRMS were separated into high and low molecular weight material using trichloroacetic acid (TCA) precipitation. Samples of both zooxanthellae and host tissue were fractionated into high (TCA insoluble) and low (TCA soluble) molecular weight components by treatment with ice cold 5% (w/v) TCA for three hours. The samples were then centrifuged at 11,337g for 10 minutes and the supernatants and pellets separated. The supernatants were added to an equal volume of diethyl ether to extract the TCA. The ether-aqueous emulsion was vortex-mixed and then centrifuged at 350g to separate the solvent and aqueous layers. The aqueous layer was then taken and the ether extraction repeated three times. The low molecular weight TCA soluble fractions were concentrated by lyophilisation before drying in tin foil analysis cups (Elemental Microanalysis). The high molecular weight TCA insoluble fractions were lyophilised and then weighed into tin cups for analysis.

d) Analysis of total ^{15}N enrichment by continuous flow-isotope ratio mass spectrometry

Unless otherwise stated, duplicate samples (containing approximately 70μg N) of dried zooxanthellae and host material were weighed into tin foil analysis cups. The nitrogen within the samples was converted to N_2 gas by Dumas combustion and the mass/charge ratios (m/z 28, 29, 30) from each sample were then measured using continuous flow-isotope ratio mass spectrometry (CF-IRMS) (Europa Scientific, ANCA system) according to the procedure described by Preston and Owens (1983). The enrichment of the samples with ^{15}N was then derived and recorded as atom % excess (APE):

$R = m/z\ 28/29$

atom % $^{15}N = 100/(2R+1)$

atom % excess ^{15}N = (atom % $^{15}N_{sample}$) - (atom % $^{15}N_{standard}$)

The natural abundance of ^{15}N in zooxanthellae and host material was determined from three symbiotic anemones.

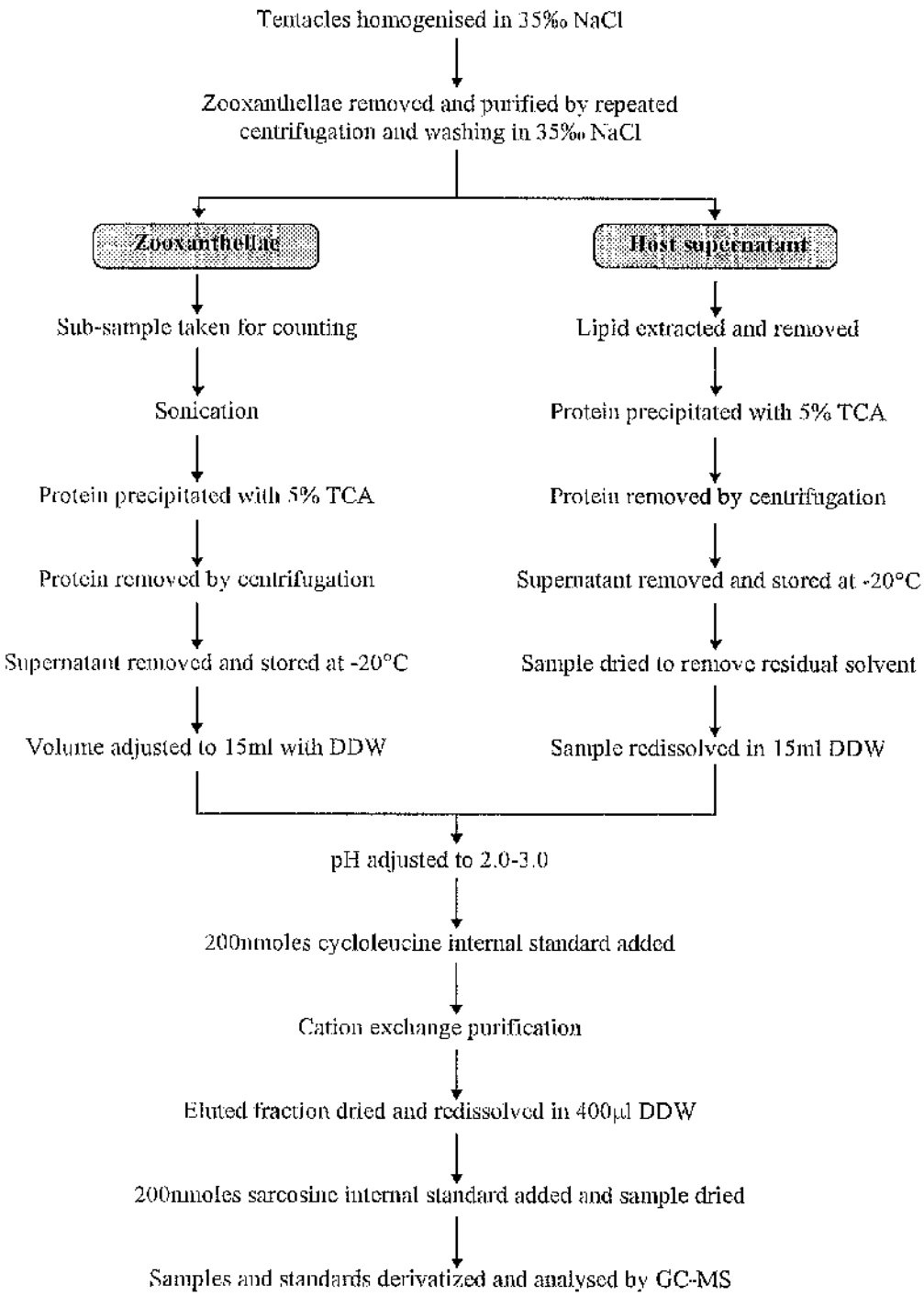
e) Analysis of amino acid ^{15}N enrichment by gas chromatography-mass spectrometry

The enrichment of the low molecular weight fraction was examined in more detail by analysing the ^{15}N assimilation into the free amino acid pools from both zooxanthellae and host. The enrichment analysis of free amino acids was carried out using gas chromatography-mass spectrometry (GC-MS).

After each incubation, the anemone was removed and rinsed in FSW. Tentacle tissue was then removed, blotted dry and wet weighed before homogenisation in a 35‰ NaCl solution in distilled water. Tentacle tissue was homogenised in a solution of NaCl since filtered seawater will have contained ions which could have interfered with the cation exchange process used to prepare samples of free amino acids for analysis by GC-MS. Zooxanthellae and host extracts were prepared using repeated centrifugations and washes in 35‰ NaCl using the method described in section 2.3.

Low molecular weight extracts for analysis by GC-MS were then obtained by the procedure summarised in figure 5.2. A 10ml volume of the host supernatant was vigorously mixed with 100ml chloroform:methanol (2:1) to extract total lipids before deproteinization. Following centrifugation at 350g for 10 minutes the aqueous layer was removed and added to an equal volume of ice cold 10% (w/v) TCA to produce a final concentration of 5% TCA. The samples were left on ice for a minimum of three hours.

Figure 5.2 Tissue preparation for analysis of ^{15}N enrichment of amino acids by gas chromatography-mass spectrometry (GC-MS).
Experimental details are given in the text.



The zooxanthellae were resuspended in a 2ml volume of 35‰ NaCl and an aliquot was removed for cell counting before the remainder was subjected to a brief pulse of sonication (Microson Ultrasonic Cell Disruptor) to break the cells. An equal volume of ice cold 10% (w/v) TCA was added to the zooxanthellae suspension and the samples were left on ice for a minimum of three hours. The precipitated protein was then removed from both zooxanthellae and host samples by centrifugation at 11,337g for 10 minutes. The supernatants were removed and frozen at -20°C.

Before sample purification by cation exchange, the samples were thawed and any residual solvent present in the host extracts was removed by drying in a vacuum centrifuge. The volumes of both extracts were then adjusted to 15ml with deionised distilled water (DDW) and the pH was brought to between 2.0 and 3.0 with 6M NaOH. 200nmol of cycloleucine were added as internal standard to each sample. The samples were then transferred to Dowex 50X8-200 (H^+) cation exchange columns (Sigma) which had been washed with 20ml DDW. A further 10ml DDW were passed through the column following the sample. The amino acid fraction was then eluted with 5ml of 2M NH_4OH followed by 5ml DDW. The amino acid fraction was then dried in a vacuum centrifuge and redissolved in 400µl DDW. Each sample was transferred to a 2ml derivatization vial and 200nmol of sarcosine internal standard were added before the samples were dried in a vacuum centrifuge. One step derivatization was carried out following the method of Chaves Das Neves and Vasconcelos (1987).

Preliminary experiments compared different derivatization conditions to produce silylated amino acid derivatives of glutamine and alanine (200nmol). Solvents (acetonitrile and dimethylformamide), temperatures (60, 100, 140°C) and heating times (30 and 60 minutes) were compared. Following this investigation *tert*-butyldimethylsilyl (TBDMS) amino acid derivatives were produced by adding 25µl dimethylformamide (DMF) and 25µl N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) to the dried samples or standards and heating at 140°C for 30 minutes in a sealed derivatization vial. Samples were then transferred to 200µl inserts for automatic injection (Fisons Instruments A200S) into the GC-MS. The gas chromatograph (Hewlett Packard 5890 Series II) was a high resolution bonded phase capillary column (30m x 0.25mm DB-5MS, 0.25µm film; J&W Scientific). Analysis was carried out with a temperature programme of 110°C held for 1 minute. The GC was interfaced with a VG Trio-1000

mass spectrometer (Fisons Instruments) which used an electron impact ion source operating at 150μA trap current and 45eV electron energy.

The samples were first analysed in scanning mode to demonstrate the presence of amino acids and other unknown compounds. The amino acids were separated by high resolution gas chromatography. The retention times and fragment ions recorded in this study are summarised in table 5.1. The ^{15}N enrichment of amino acids was then recorded in selected ion recording (SIR) mode.

The atom % excess enrichment of amino acids above natural abundance was calculated using the formula shown below. Thus E_{sample} represents the ^{15}N enrichment of the sample and $E_{standard}$ represents the natural abundance of ^{15}N. QM refers to the quantitation mass of the fragment ion recorded and QM+1 refers to a fragment ion enriched with ^{15}N.

$$E = \left(\frac{QM+1}{QM} \right)$$

$$E' = E_{sample} - E_{standard}$$

$$\text{Atom\%Excess} = \left(\frac{E'}{1+E'} \right) \times 100$$

However, as measured natural abundance is a function of peak area, then if there was a discrepancy between the peak areas of enriched sample and natural abundance standard the theoretical natural abundance of ^{15}N in each amino acid was used for this calculation (Caprioli, 1972).

Table 5.1 The retention times during gas chromatography and the fragment ions recorded during mass spectrometry of the amino acids analysed.

Amino acid	Retention Time (min)	Fragment Ion (Quantitation Mass)
Alanine	6.4	159/158
Glycine	6.7	219/218
Valine	7.6	187/186
Leucine	8.2	201/200
Isoleucine	8.4	201/200
Proline	8.8	185/184
Threonine	11.3	405/404
Phenylalanine	12.1	233/234
Aspartate	12.7	419/418
Glutamate	13.8	433/432
Glutamine	15.1	432/431, 433/431, 259/258
Tyrosine	16.8	467/466

5.2.2 Incorporation of ^{15}N into zooxanthellae and host fractions

These experiments were conducted to examine the effects of ammonium concentration and light level on the appearance of label in the zooxanthellae and host fractions of symbiotic anemones. All experimental treatments in this section were replicated with three anemones (n=3).

To investigate whether the zooxanthellae or host were the site of ammonium assimilation, anemones were incubated with ammonium sulphate (98% atom ^{15}N, Sigma) for 0.5, 10 and 30 minutes after which samples of zooxanthellae and host material were prepared as described before (figure 5.1). Anemones were incubated in FSW enriched to either 10 or 20μM [^{15}N]ammonium.

Incubations at 20μM [^{15}N]ammonium were carried out under three light levels; a 'high light' treatment of 300μmol photons.$m^{-2}.s^{-1}$, a 'low light' treatment of

150µmol photons.$m^{-2}.s^{-1}$ and a dark treatment. The experiment in darkness took place in the morning at the end of the 12 hour dark photoperiod. Incubations at 10µM [^{15}N]ammonium were carried out under the two light levels, 'high light' and 'low light' but not in darkness.

5.2.3 ^{15}N assimilation into high and low molecular weight material

Three anemones were incubated with 20µM [^{15}N]ammonium for 30 minutes under an illumination of 300µmol photons.$m^{-2}.s^{-1}$. Zooxanthellae and host fractions were then prepared (figure 5.1) and each fraction was treated with 5% (w/v) TCA to separate the high and low molecular weight material. The total nitrogen enrichment with ^{15}N of the high and low molecular weight material of both zooxanthellae and host were then recorded by CF-IRMS. Given the limited amount of TCA soluble material from the zooxanthellae, single samples were analysed for ^{15}N enrichment.

5.2.4 ^{15}N assimilation into free amino acids

Individual anemones were incubated for 30, 60 and 120 minutes with 20µM [^{15}N]ammonium under 300µmol photons.$m^{-2}.s^{-1}$ illumination. Experimental treatments were replicated with two anemones (n=2). After each incubation zooxanthellae and host fractions were prepared (figure 5.2) and the ^{15}N enrichment of the free amino acids of each fraction was analysed by GC-MS.

5.2.5 Nitrogen flux within the symbiosis

To investigate whether there was any evidence for total nitrogen transfer between zooxanthellae and host, anemones were incubated with a [^{15}N]ammonium pulse and then transferred to [^{14}N]ammonium which provided the chase substrate. Samples were then taken for analysis during the chase period. In addition, the total nitrogen enrichment of high and low molecular weight fractions of both zooxanthellae and host were analysed. This analysis was intended to provide some preliminary data on the flux of ^{15}N label from low molecular weight precursors to high molecular weight biosynthetic products.

Individual anemones were incubated under an irradiance of 300µmol photons.$m^{-2}.s^{-1}$ for a 30 minute pulse in either 10 or 20µM [^{15}N]ammonium enriched FSW. After this the [^{15}N]ammonium enriched seawater was poured off, the animals rinsed three times with FSW and a concentrated stock of [^{14}N]ammonium sulphate added to provide an

unlabelled chase substrate at the same concentration. Anemones were sacrificed at 0, 60 and 120 minutes into the chase and zooxanthellae and host extracts were prepared from tentacle tissue as described above (figure 5.1). Experimental treatments were replicated with three anemones (n=3). The total nitrogen enrichment of the zooxanthellae and host extracts prepared were then analysed by CF-IRMS. In addition, the zooxanthellae and host extracts prepared from anemones incubated in this pulse chase system with 20μM [^{15}N]ammonium were fractionated with TCA and the enrichment of the high and low molecular weight fractions were measured by CF-IRMS.

5.2.6 Flux of free amino acids within the symbiosis

A pulse-chase system was also used to investigate any amino acid flux between zooxanthellae and host. In these experiments, anemones were incubated for a 60 minute pulse in 20μM [^{15}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. This was intended to increase the initial amino acid enrichment from the 30 minute pulse labelling described before. Anemones were incubated under chase conditions with 20μM [^{14}N]ammonium for 120 and 300 minutes. Experimental treatments were replicated with two anemones (n=2). Tentacle tissue was taken and prepared for GC-MS analysis of amino acid enrichment with ^{15}N as described above (figure 5.2).

5.3 Results

5.3.1 Incorporation of ^{15}N into zooxanthellae and host fractions

The natural abundance of ^{15}N in the total nitrogen of zooxanthellae and host was measured from control anemones and used to calculate the enrichment of samples (atom % excess ^{15}N). The natural abundance in zooxanthellae material was 0.369 atom% ^{15}N (SD=0.0003, n=3) and that in host material was 0.370 atom% ^{15}N (SD=0.0012, n=3).

During each incubation with [^{15}N]ammonium, the zooxanthellae became enriched at a greater rate than the host (figure 5.3). Over the 30 minute time course of [^{15}N]ammonium assimilation both zooxanthellae and host fractions became enriched in a linear relationship with time. Tables 5.2a and 5.2b summarise the mean enrichments from each of the total N assimilation time course experiments carried out and analysed by CF-IRMS. Linear regression was used to fit regression lines by the least squares method and so calculate the rate of ^{15}N enrichment (atom % excess minute^{-1}). This unit is equivalent to the fractional synthetic rate of ammonium assimilation:

Fractional synthetic rate (% min^{-1}) =
(atom % excess change in product enrichment min^{-1} / atom % precursor enrichment) x 100

The precursor enrichment was taken as the enrichment of the added [^{15}N]ammonium (98 atom%). Since the concentration of ammonium in the seawater used in these experiments was below the limit of detection (<1μM) this ammonium was assumed not to have significantly reduced the precursor enrichment. The rates for zooxanthellae and host [^{15}N]ammonium assimilation are summarised in tables 5.3a and 5.3b and illustrated graphically in figures 5.4a and 5.4b.

Figure 5.3

Enrichment of zooxanthellae and host fractions from symbiotic anemones incubated with 20μM [^{15}N]ammonium at an illumination of 300μmol photons.m^{-2}.s^{-1}.

Means are shown (±SE) n=3. Error bars are not shown when smaller than data point symbol.

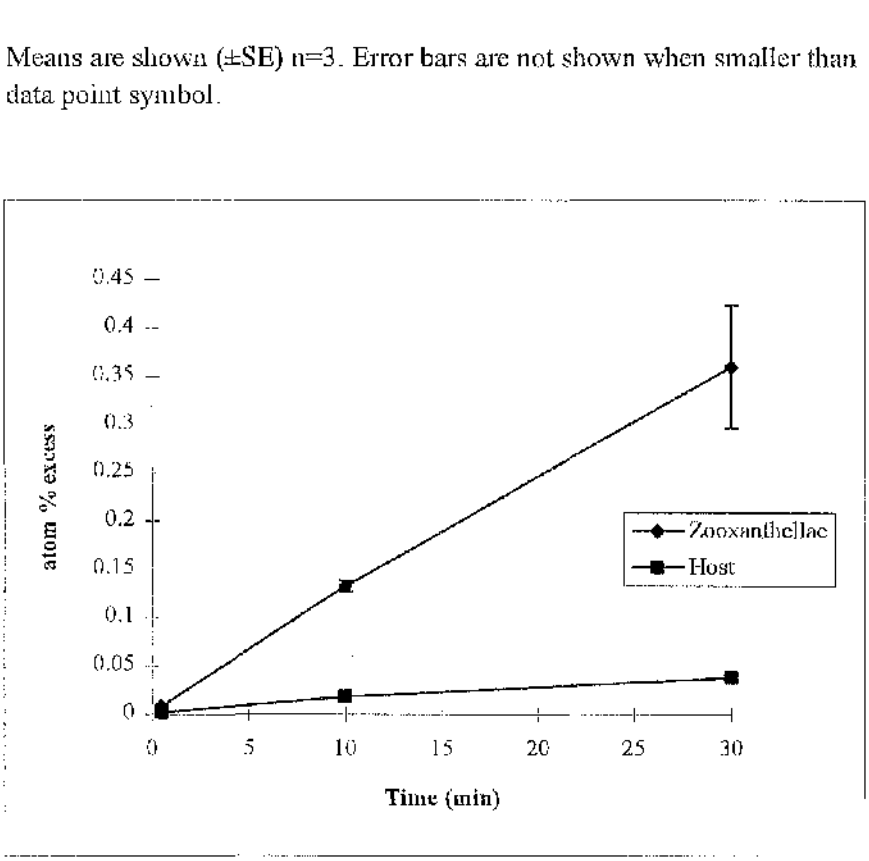
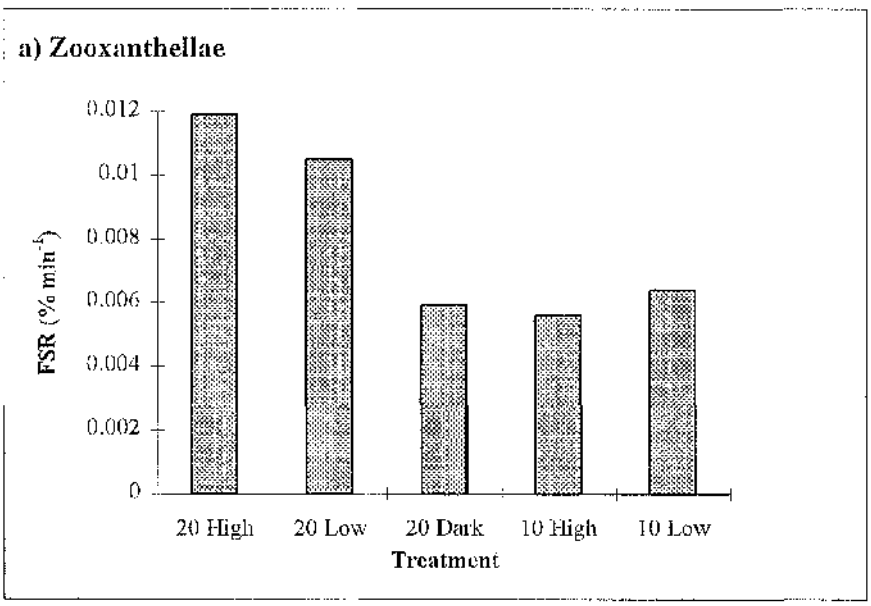
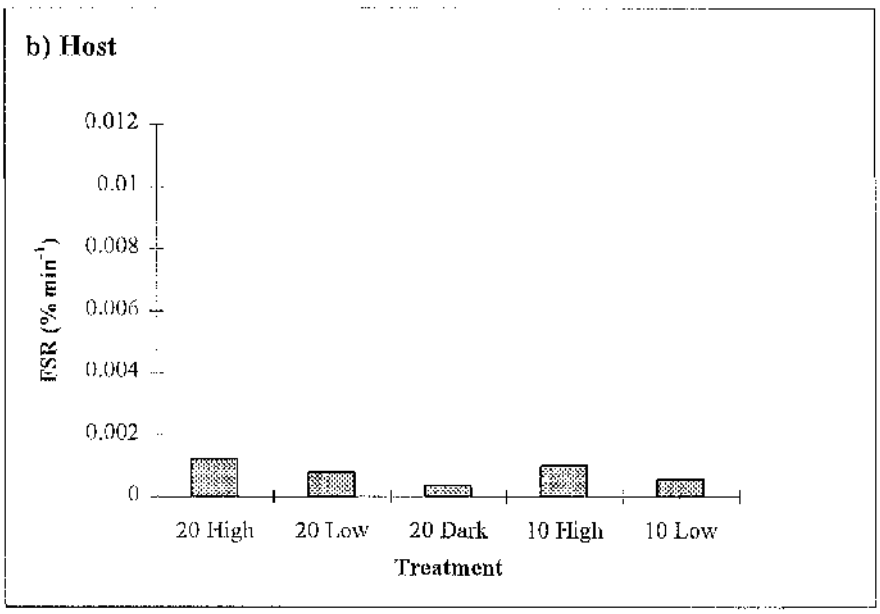


Figure 5.4

Rate of [^{15}N]ammonium incorporation into (a) zooxanthellae and (b) host total nitrogen.

Treatments 20 and 10 refer to the ammonium concentration (μM). FSR, fractional synthetic rate. The light level treatments were:
'High' light, 300μmol photons.m^{-2}.s^{-1}
'Low' light, 150μmol photons.m^{-2}.s^{-1}
'Dark', 0μmol photons.m^{-2}.s^{-1}





5.3.1.1 The effect of concentration on ammonium assimilation

Incubation with 20μM [^{15}N]ammonium produced a significant increase in the slope of the relationship between zooxanthellae enrichment and time when compared with treatment with 10μM [^{15}N]ammonium (fig. 5.4a). This was true under both high light (ANCOVA atom % excess by concentration $F_{1,17}$=14.29, p=0.002) and low light (ANCOVA $F_{1,17}$=10.60, p=0.006). However there was no significant effect of concentration treatment on the slope of the relationship between host enrichment and time (fig. 5.4b) under either high light (ANCOVA $F_{1,17}$=1.40, p=0.256) or low light (ANCOVA $F_{1,17}$=2.57, p=0.131).

5.3.1.2 The effect of light on ammonium assimilation

Incubation in the dark, with 20μM [^{15}N]ammonium, significantly reduced the slope of the relationship between zooxanthellae enrichment and time when compared with enrichments at both high light (ANCOVA atom % excess by light $F_{1,17}$=11.87, p=0.004) and low light (ANCOVA $F_{1,17}$=11.45, p=0.004). However, this relationship did not differ significantly between the high and low light treatments at either 20μM (ANCOVA $F_{1,17}$=0.67, p=0.426) or at 10μM [^{15}N]ammonium (ANCOVA $F_{1,17}$=1.76, p=0.205) (fig. 5.4a). The host fraction also showed that dark treatment significantly reduced the slope of the relationship between enrichment and time at 20μM [^{15}N]ammonium when compared with enrichments under both high light (ANCOVA $F_{1,17}$=24.88, p<0.001) and low light (ANCOVA $F_{1,17}$=6.5, p=0.023). In contrast to zooxanthellae, the slope of the relationship between host enrichment and time was significantly reduced by treatment with low light when compared with enrichment under high light at both 20μM (ANCOVA $F_{1,17}$=5.68, p=0.032) and 10μM [^{15}N]ammonium (ANCOVA $F_{1,17}$=18.13, p=0.001) (fig. 5.4b).

Table 5.2a ^{15}N enrichment of zooxanthellae from anemones exposed to a 30 minute time course with [^{15}N]ammonium. Mean atom % excess ^{15}N (±SD) n=3.

$^{15}NH_4^+$ concentration	Light level (μmol photons.m^{-2}.s^{-1})	^{15}N enrichment at 0.5 min	^{15}N enrichment at 10 min	^{15}N enrichment at 30 min
20μM	300	0.00808 (0.00294)	0.13226 (0.01002)	0.35951 (0.11023)
20μM	150	0.02419 (0.01371)	0.14871 (0.02835)	0.33146 (0.06592)
20μM	0	0.01024 (0.00639)	0.06877 (0.00922)	0.18312 (0.04336)
10μM	300	0.00731 (0.00132)	0.06014 (0.02750)	0.17086 (0.00581)
10μM	150	0.00998 (0.00284)	0.07178 (0.01646)	0.19689 (0.02388)

Table 5.2b ^{15}N enrichment of host tissue from anemones exposed to a 30 minute time course with [^{15}N]ammonium. Mean atom % excess ^{15}N (±SD) n=3.

$^{15}NH_4^+$ concentration	Light level (μmol photons.m^{-2}.s^{-1})	^{15}N enrichment at 0.5 min	^{15}N enrichment at 10 min	^{15}N enrichment at 30 min
20μM	300	0.00162 (0.00128)	0.01852 (0.00149)	0.03787 (0.00653)
20μM	150	0.00106 (0.00199)	0.01135 (0.00135)	0.02446 (0.00771)
20μM	0	0	0.00871 (0.00510)	0.01070 (0.00305)
10μM	300	0.00012 (0.00030)	0.00807 (0.00309)	0.02940 (0.00555)
10μM	150	0	0.00424 (0.00235)	0.01613 (0.00150)

Table 5.3a Rate of [^{15}N]ammonium enrichment of zooxanthellae at different incubation concentrations and light levels.

$^{15}NH_4^+$ concentration	Light level (μmol photons.m^{-2}.s^{-1})	FSR (% min^{-1})	R^2-adj.	p	Equation of line
20μM	300	0.01210	88.4	<0.001	APE= 0.0056+(0.0119t)
20μM	150	0.01071	92.5	<0.001	APE= 0.0242+(0.0105t)
20μM	0	0.00603	92.4	<0.001	APE= 0.0069+(0.0059t)
10μM	300	0.00569	69.4	<0.001	APE= 0.0037+(0.0056t)
10μM	150	0.00651	96.8	<0.001	APE= 0.0064+(0.0064t)

Table 5.3b Rate of [^{15}N]ammonium enrichment of host tissue at different incubation concentrations and light levels.

$^{15}NH_4^+$ concentration	Light level (μmol photons.m^{-2}.s^{-1})	FSR (% min^{-1})	R^2-adj.	p	Equation of line
20μM	300	0.00123	92.8	<0.001	APE= 0.0026+(0.0012t)
20μM	150	0.00081	85.1	<0.001	APE= 0.0015+(0.0008t)
20μM	0	0.00036	56.0	<0.001	APE= 0.0014+(0.0004t)
10μM	300	0.00102	94.3	<0.001	APE= -0.0008+(0.0010t)
10μM	150	0.00057	95.0	<0.001	APE= -0.0007+(0.0006t)

FSR, fractional synthetic rate; APE, atom % excess; t, time (minutes); R^2-adj., coefficient of determination adjusted for the number of parameters in the model; p, significance of the regression.

5.3.2 ^{15}N assimilation into high and low molecular weight material

The rate of ^{15}N enrichment of high and low molecular weight material from both zooxanthellae and host is illustrated in figure 5.5. The low molecular weight zooxanthellae material became enriched at by far the greatest rate (0.05% min^{-1}), with minor incorporation into the host (0.003% min^{-1}). Enrichment of high molecular weight material from both zooxanthellae (0.002% min^{-1}) and host (0.0003% min^{-1}) was also detected. These data are listed in table 5.9 under '^{15}N enrichment after 30 min pulse' which corresponds to a 30 minute incubation with 20μM [^{15}N]ammonium.

5.3.3 ^{15}N assimilation into free amino acids

Only the tissue extracts prepared from zooxanthellae were analysed successfully. Amino acid peaks from host tissue extracts were either very small relative to standards or were undetectable. Figure 5.6a illustrates a gas chromatography trace for a typical zooxanthellae sample. When this is compared with figure 5.6b, an equivalent trace from a host-derived extract, it is possible to see a large difference in peak area between zooxanthellae and host. The small size of the cycloleucine internal standard in the host sample (fig. 5.6b) demonstrates that this difference is not due solely to a lack of host-derived amino acids in the sample. A difference in peak area of this magnitude between sample and standard will greatly increase the error associated with the enrichment calculation making any such result largely meaningless (Slater *et al.*, 1995).

The failure to detect amino acids in host material could be due to either loss of sample during cation exchange purification or failure to derivatize prior to analysis. Since it was not possible to detect the sarcosine internal standard in the host analyses, it appeared that the derivatization step was inhibited in these samples. Clearly this inhibition was produced by a factor present in host material that was not removed by lipid extraction, TCA precipitation or cation exchange purification. In an attempt to remove any low molecular weight contaminant greater than 50kDa, the host extracts were redissolved in DDW and passed through 50kDa ultrafilters (Amicon). These samples were then dried and derivatized as described. However, this treatment also failed to produce detectable amino acid peaks. It was noted that the major difference between host and zooxanthellae samples was the presence of mucus in the host. It is possible that the presence of this mucus was the cause of the failure to detect amino acids in host-derived samples, and that the sample preparation described was inadequate to remove this factor.

Figure 5.5

Rate of [^{15}N]ammonium incorporation into zooxanthellae and host low and high molecular weight material when symbiotic anemones were incubated with 20μM [^{15}N]ammonium at 300μmol photons.m^{-2}.s^{-1} illumination.

Fractions refer to the low molecular weight (LMW) and high molecular weight (HMW) TCA soluble and insoluble fractions respectively. FSR, fractional synthetic rate.

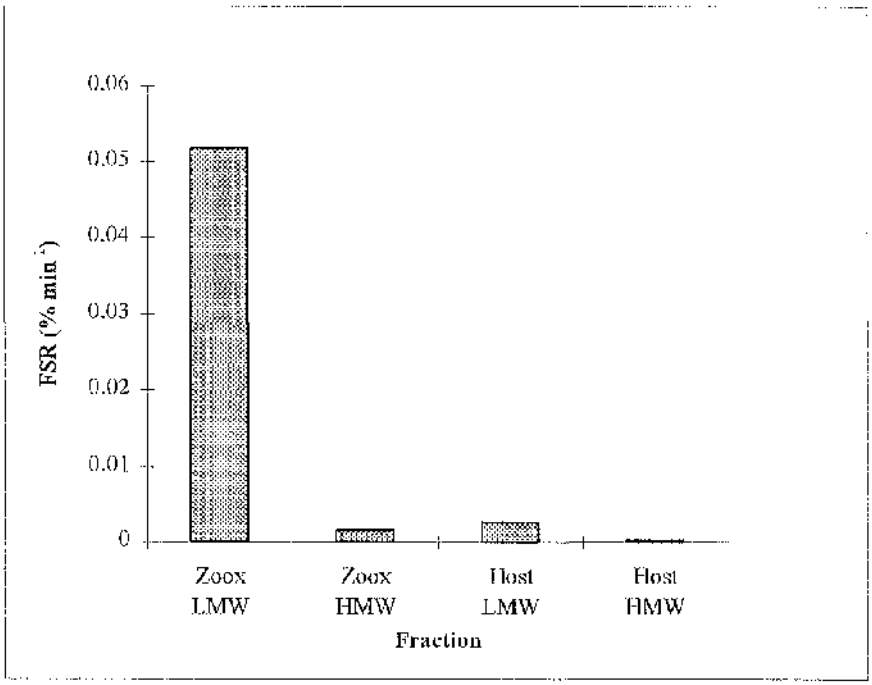
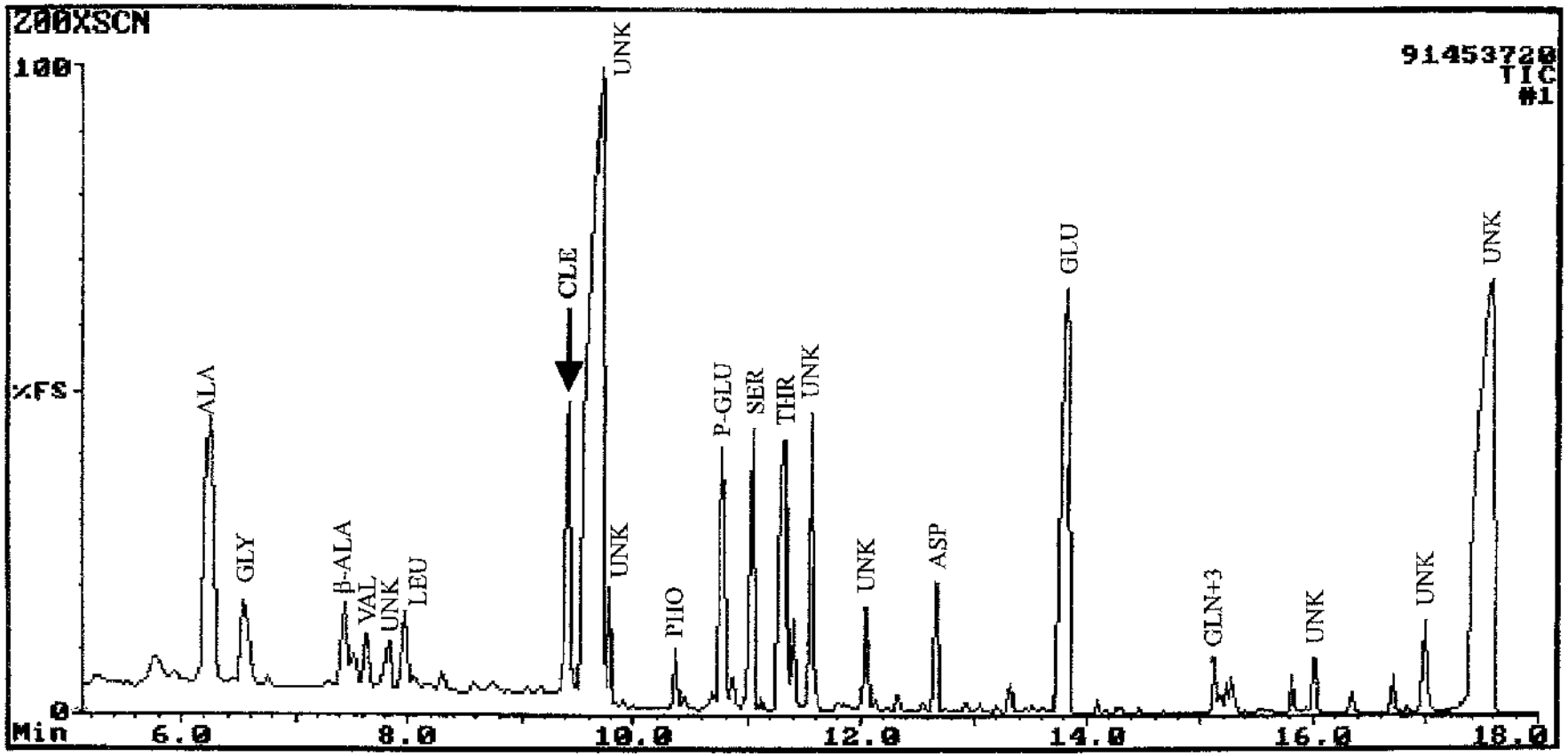


Figure 5.6a

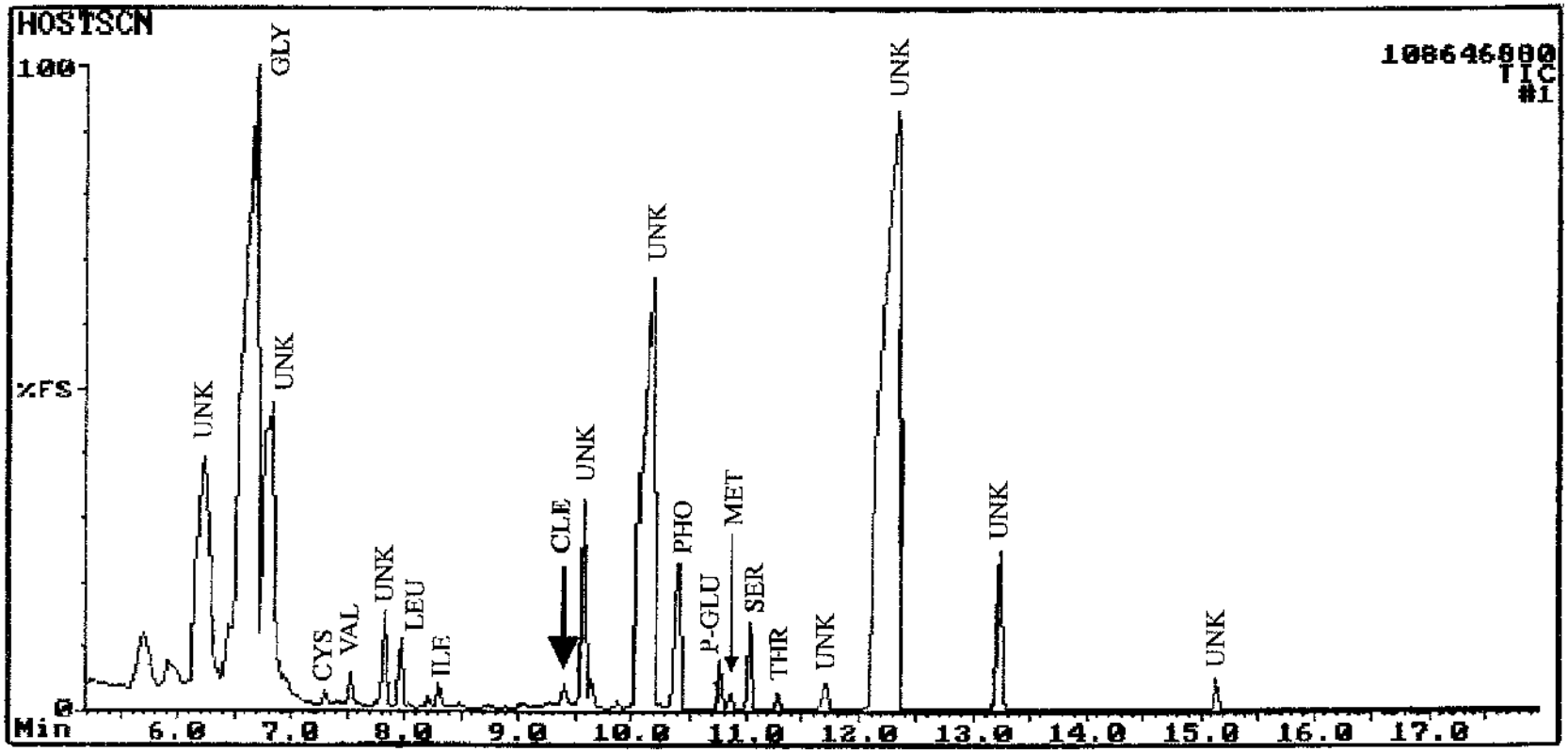
Total ion chromatogram from a typical zooxanthellae extract prepared for analysis by GC-MS. Arrow indicates the cycloleucince internal standard. Amino acid peaks are shown in order of retention time on the GC column.



ALA, alanine
GLY, glycine
β-ALA, β-alanine
VAL, valine
UNK, unknown
LEU, leucine
CLE, cycloleucine
PHO, phosphate
P-GLU, pyroglutamate
SER, serine
THR, threonine
ASP, aspartate
GLU, glutamate
GLN+3, glutamine+3 TBDMS

Figure 5.6b

Total ion chromatogram from a typical host exctract prepared for analysis by GC-MS. Arrow indicates the cycloleucine internal standard. Amino acid peaks are shown in order of retention time on the GC column.



UNK, unknown	VAL, valine	**CLE**, cycloleucine	MET, methionine
GLY, glycine	LEU, leucine	PHO, phosphate,	SER, serine
CYS, cysteine	ILE, isoleucine	P-GLU, pyroglutamate	THR, threonine

Table 5.4 Summary of amino acid enrichment from zooxanthellae from symbiotic anemones incubated for a 120 minute time course with 20μM [^{15}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination.
Mean atom % excess ^{15}N (±SD) n=2. (* indicates missing data)

Amino Acid	^{15}N enrichment at 30 min	^{15}N enrichment at 60 min	^{15}N enrichment at 120 min
Alanine	6.240 (*)	11.138 (0.917)	16.402 (3.326)
Glycine	0	1.839 (0.791)	4.284 (1.694)
Valine	0.875 (*)	3.107 (1.291)	3.962 (2.137)
Leucine	2.702 (*)	6.279 (5.392)	*
Threonine	0	1.238 (0.400)	5.280 (2.494)
Phenylalanine	1.413 (*)	1.860 (0.320)	2.430 (0.795)
Aspartate	13.872 (*)	20.591 (1.836)	27.426 (4.555)
Glutamate	15.463 (*)	22.403 (0.270)	27.136 (4.799)
Tyrosine	1.483 (*)	0.844 (*)	2.277 (*)

As illustrated in figure 5.6a it was possible to detect amino acids in samples from zooxanthellae and ^{15}N enrichment patterns were successfully analysed in the amino acids alanine, glycine, valine, leucine, threonine, phenylalanine, aspartate, glutamate and tyrosine. These data are summarised in table 5.4 and illustrated graphically in figures 5.7a-5.7i. These figures represent histograms of the atom % excess ^{15}N enrichment as well as plots of the same data as a percentage of the initial enrichment. The initial enrichment has been taken as the first time point where enrichment with ^{15}N was detectable. This was the first time point in all cases except for glycine (fig. 5.7d) and threonine (fig. 5.7h).

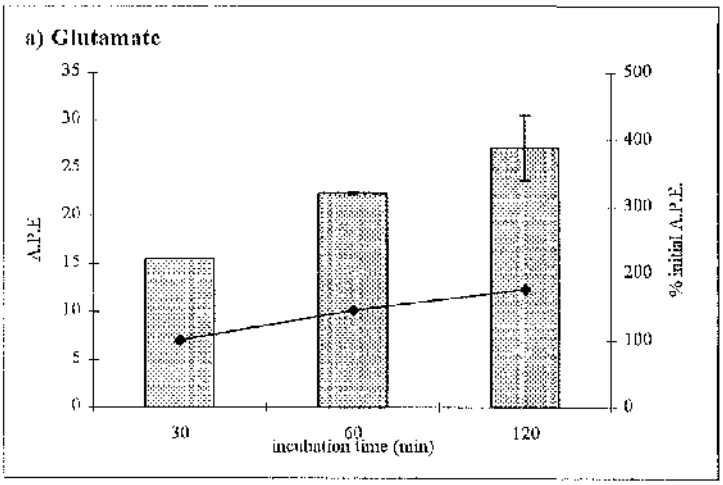
Glutamine produced small peak areas, suggesting failure at the derivitization step. However, in one preliminary experiment a sufficiently large peak was achieved to give reliable enrichment data. These data are shown in table 5.5. In this table, the ^{15}N enrichment from the amide group represents a derived value calculated on the assumption that the enrichment of the amide or amino (432/431) mass fragment was the average of the amide and amino (259/258) mass fragments. Since it was not possible to repeat the glutamine analysis no statistical comparisons have been made using these data.

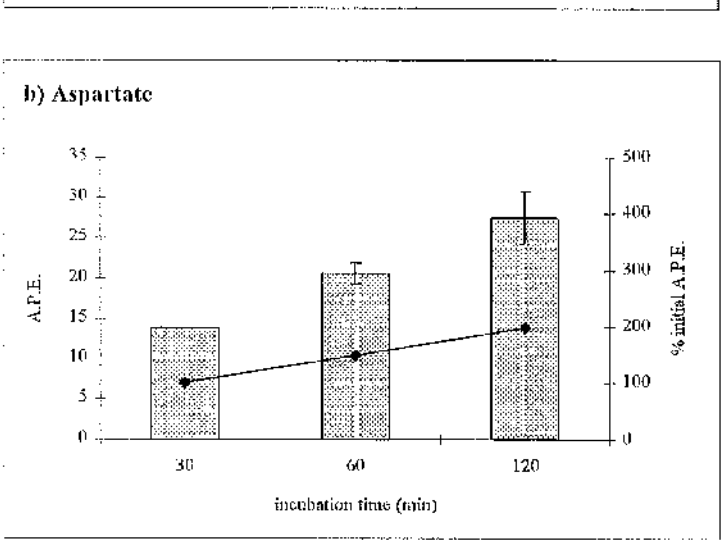
Table 5.5 Enrichment of glutamine and glutamate from zooxanthellae following a 30 minute incubation with 20μM [^{15}N]ammonium. Anemones were incubated under an irradiance of 300μmol photons.$m^{-2}.s^{-1}$.
Mean mole % excess of replicate analyses (n) (±SD).

Amino acid and nitrogen group	**Retention time (min)**	**Mass fragment (QM)**	**^{15}N enrichment (mole % ^{15}N excess)**	**n**
Glutamate (amino)	13.8	433/432	6.432 (2.040)	6
Glutamine (amide or amino)	15.1	432/431	7.442 (2.416)	4
Glutamine (amide and amino)	15.1	433/431	6.459 (1.299)	4
Glutamine (amino)	15.1	259/258	2.275 (2.062)	3
Glutamine (amide)	-	-	12.609	-

Figure 5.7 (a-c)

Enrichment in atom % excess (A.P.E.) of the amino acids glutamate, aspartate and alanine from zooxanthellae. Anemones were incubated with 20μM [^{15}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2).
Lines represent the mean percentage of the initial enrichment.





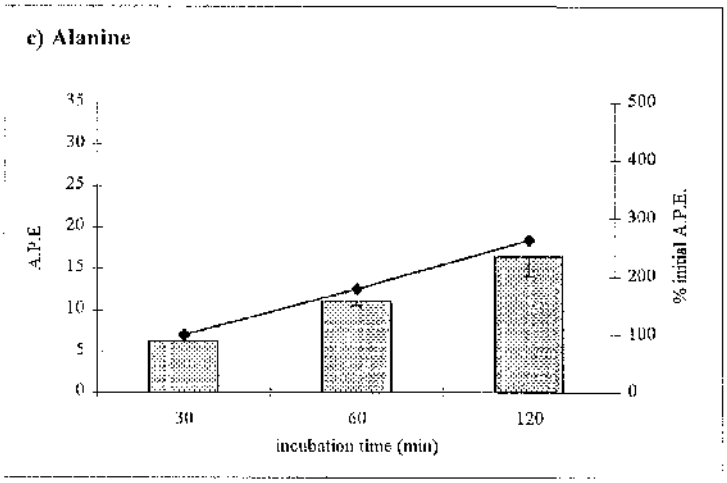
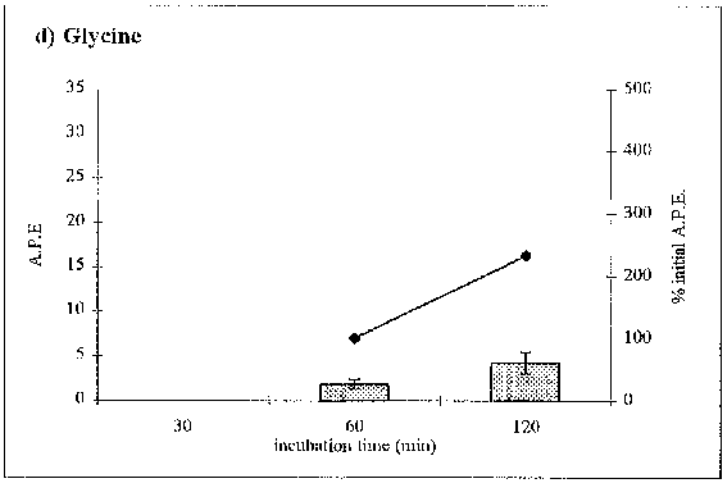
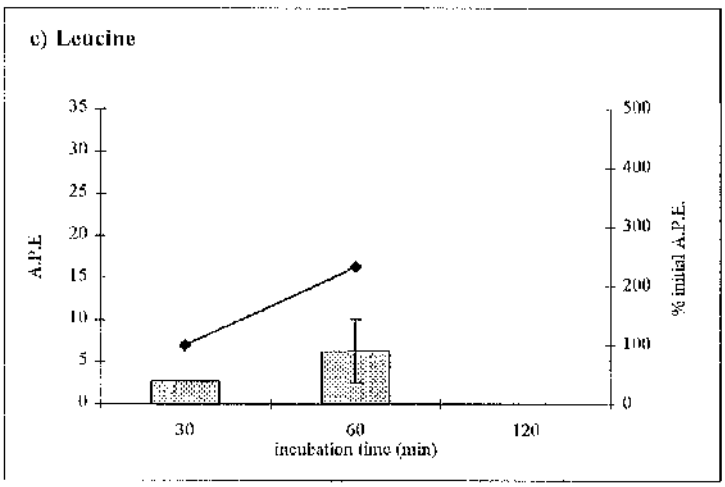


Figure 5.7 (d-f)

Enrichment in atom % excess (A.P.E.) of the amino acids glycine, leucine and valine from zooxanthellae. Anemones were incubated with 20µM [^{15}N]ammonium under 300µmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2).
Lines represent the mean percentage of the initial enrichment.





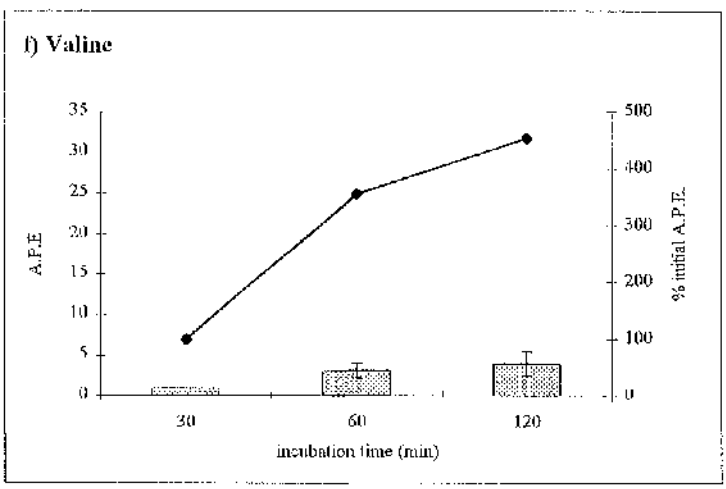
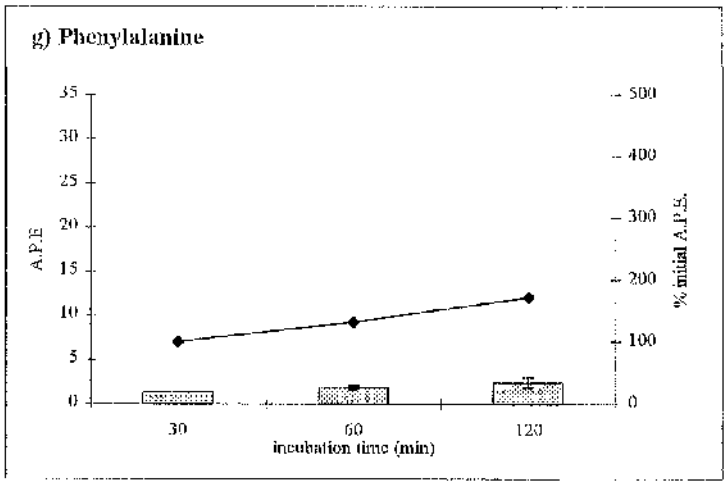
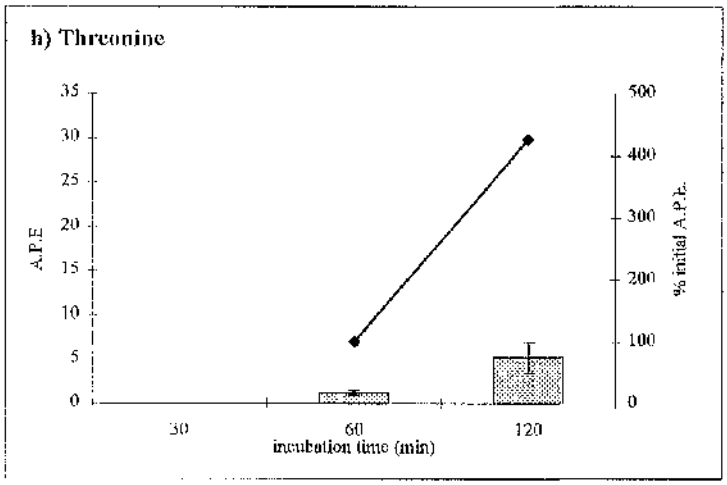
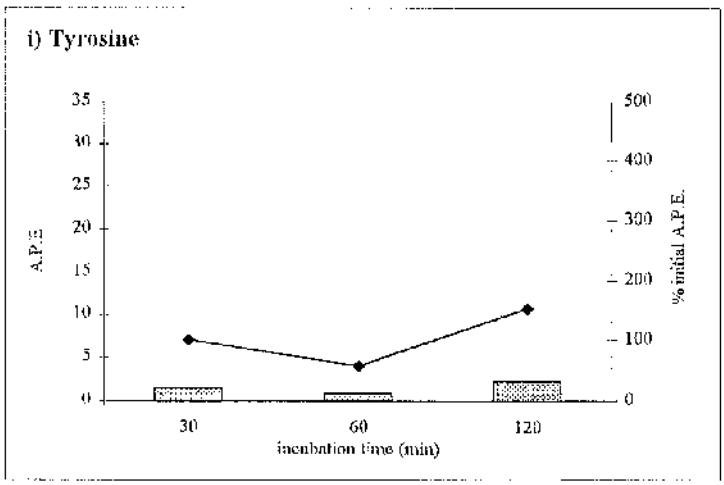


Figure 5.7 (g-i)

Enrichment in atom % excess (A.P.E.) of the amino acids phenylalanine, threonine and tyrosine from zooxanthellae. Anemones were incubated with 20μM [^{15}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2).
Lines represent the mean percentage of the initial enrichment.







Rates of amino acid enrichment were calculated using linear regression to fit a least squares model to the data. Where the data were non-linear and formed a hyperbolic relationship, the reciprocal of both dependent (enrichment) and independent (time) variables were taken before fitting a line using linear regression. The reciprocal of the gradient of this line was taken as the rate of enrichment (atom % excess min^{-1}) and the corresponding fractional synthetic rate was calculated. The rates of amino acid enrichment are listed in table 5.6.

Table 5.6 Rate of zooxanthellae amino acid enrichment calculated from time course data.

Amino Acid	FSR (% min^{-1})	R^2-adj.	p	equation of line
Glutamate	0.9357	88.1	0.012	1/APE = 0.0276+(1.09(1/t))
Aspartate	0.7286	91.4	0.007	1/APE = 0.0253+(1.40(1/t))
Alanine	0.2602	95.1	0.003	1/APE = 0.0277+(3.92(1/t))
Glycine	0.0400	76.0	0.015	APE = -0.507−(0.0392t)
Valine	0.0301	75.5	0.036	1/APE = -0.077+(33.9(1/t))
Phenylalanine	0.0936	50.3	0.110	1/APE = 0.352+(10.9(1/t))
Threonine	0.0641	72.8	0.042	APE = -2.33+(0.0628t)

FSR, fractional synthetic rate; APE, atom % excess; t, time (minutes); R^2-adj., coefficient of determination adjusted for the number of parameters in the model; p, significance of the regression.

Figure 5.8 illustrates this data set using the predictive equations in table 5.6. From these data and concentrations of each amino acid determined by HPLC (see section 4.3.1.2), it is possible to calculate the rate of amino acid synthesis normalised to wet weight of tentacle tissue:

Since, atom% excess min^{-1} = number of ^{15}N atoms incorporated/100 N atoms/min

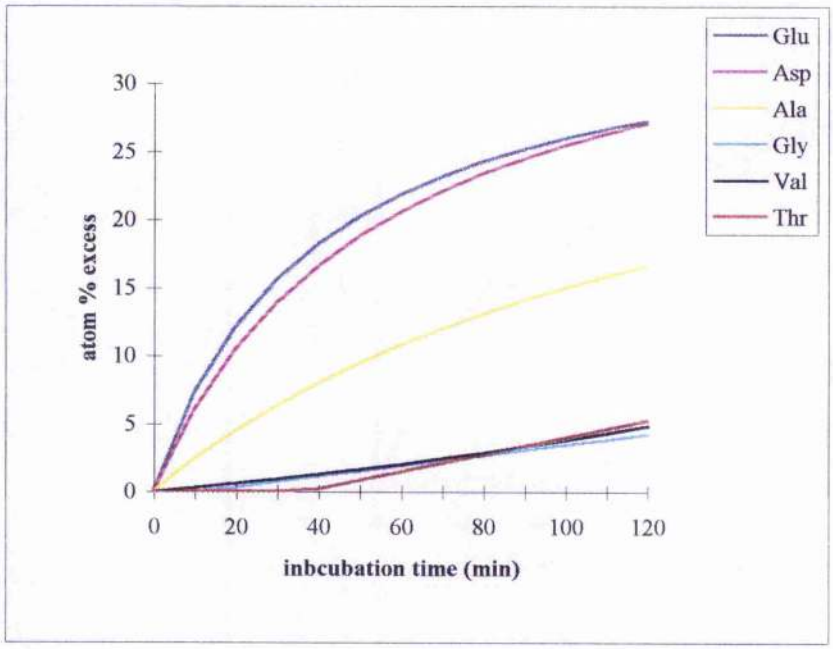
$\therefore$ (atom% excess min^{-1} x $\mu gN.mg^{-1}$ tentacle) / 100 = μgN incorporated.mg^{-1} tentacle.min^{-1}

$\therefore$ (μgN incorporated mg^{-1} min^{-1}) / 14 = μg-atom N incorporated.mg^{-1} tentacle.min^{-1}

Figure 5.8

Enrichment of zooxanthellae amino acids with ^{15}N following incubation with 20μM [^{15}N]ammonium.

The graphs represent the rate at which ^{15}N enrichment increases in each amino acid when symbiotic anemones are supplied with [^{15}N]ammonium at 300μmol photons.m^{-2}.s^{-1} illumination. The equation for each line was derived by linear regression (see table 5.6). The lines plotted here are all from significant regression analyses ($p<0.05$).



The rates of [^{15}N]ammonium assimilation into these amino acids have been compared with the total low molecular weight (TCA soluble) enrichment measured by CF-IRMS. This comparison is summarised in table 5.7. Taking the rate of total zooxanthellae low molecular weight enrichment following 30 minutes incubation with ^{15}N from table 5.9 (data at 0 chase time), it is possible to calculate the rate of ^{15}N assimilation by the above method. This value was taken to represent 100% of the low molecular weight zooxanthellae N incorporated. The sum of the amino acid enrichments measured in this analysis account for 64.5% of the total low molecular weight N assimilation by zooxanthellae.

Table 5.7 Rate of ^{15}N assimilation from ammonium into amino acids and total low molecular weight (LMW) material from zooxanthellae. Means (±SD) n=2. See text for details.

Amino Acid	Rate of assimilation ng-atom N.mg^{-1}.min^{-1}	Percentage of total low molecular weight assimilation
Glutamate	17.267 (9.132)	28.788
Aspartate	6.176 (0.541)	10.297
Alanine	0.917 (0.359)	1.530
Glycine	0.342 (0.204)	0.570
Threonine	0.221 (0.068)	0.369
Phenylalanine	0.098 (0.040)	0.164
Valine	0.045 (0.013)	0.075
Glutamine[1]	13.627 (4.098)	22.720
Total	38.694 (14.454)	64.513
Total LMW	59.979 (18.623)	100

[1]The data from glutamine is a derived value calculated from the data in table 5.5. These data are from a separate, preliminary experiment.

5.3.4 Nitrogen flux within the symbiosis

Following a 30 minute pulse with [^{15}N]ammonium, the total nitrogen enrichments of zooxanthellae and host fractions were recorded over 60 and 120 minutes chase in [^{14}N]ammonium. These data are summarised in table 5.8. The total nitrogen enrichment of zooxanthellae did not change significantly over the 120 minutes chase in unlabelled ammonium at either 10μM (ANOVA F_2=0.16, p>0.05) or 20μM (ANOVA F_2=2.70, p>0.05). This pattern was mirrored in the host fraction where there was also no significant change in total nitrogen enrichment over the chase time period at either 10μM (ANOVA F_2=1.66, p>0.05) or 20μM (ANOVA F_2=1.2, p>0.05).

Table 5.8 Enrichment of (a) zooxanthellae and (b) host fractions from anemones during a chase period in [^{14}N]ammonium following a 30 minute pulse in [^{15}N]ammonium under an illumination of 300μmol photons.m^{-2}.s^{-1}. Mean atom % excess ^{15}N (±SD) n=3.

a) Zooxanthellae

$^{15}NH_4^+$ concentration	^{15}N enrichment after 30 min pulse	^{15}N enrichment at 60 min chase	^{15}N enrichment at 120 min chase
20μM	0.24564 (0.03718)	0.19725 (0.04363)	0.18259 (0.01845)
10μM	0.16888 (0.04568)	0.16204 (0.04031)	0.15089 (0.03013)

b) Host

$^{15}NH_4^+$ concentration	^{15}N enrichment after 30 min pulse	^{15}N enrichment at 60 min chase	^{15}N enrichment at 120 min chase
20μM	0.03207 (0.00308)	0.02986 (0.00150)	0.03291 (0.00262)
10μM	0.01732 (0.00203)	0.02049 (0.00307)	0.01953 (0.00086)

Tissue samples from the experiment carried out at 20μM ammonium were separated into high and low molecular weight components using TCA and the total nitrogen enrichments analysed by CF-IRMS. These data are given in table 5.9. Over the chase time of 120 minutes, there was an apparent trend for the enrichment of low molecular weight material to decrease and for high molecular weight material to increase. However,

in zooxanthellae this trend was not significant for either the high (ANOVA F_2=2.83, p>0.05) or low (ANOVA F_2=1.58, p>0.05) molecular weight material. In the host fraction, there was a significant increase in the enrichment of high molecular weight material (ANOVA + Tukey p<0.001) and a significant decline in the enrichment of low molecular weight material but only between the zero time point and both the 60 and 120 minutes time points (ANOVA + Tukey p<0.05), suggesting that nitrogen from LMW material was transferred to HMW (i.e. protein) during the chase.

Table 5.9 Enrichment of high and low molecular weight (MW) fractions of zooxanthellae and host tissues incubated under 300µmol photons.m^{-2}.s^{-1} illumination for a 30 minute pulse with 20µM [^{15}N]ammonium followed by a chase in 20µM [^{14}N]ammonium. Mean atom % excess (=SD) n=3.

Tissue	TCA fraction	^{15}N enrichment after 30 min pulse	^{15}N enrichment at 60 min chase	^{15}N enrichment at 120 min chase
Zooxanthellae	Low MW	1.55498 (0.29826)	1.26947 (0.21797)	1.26528 (0.14266)
	High MW	0.04665 (0.00892)	0.04676 (0.00840)	0.05865 (0.00099)
Host	Low MW	0.07817 (0.01762)	0.05090 (0.00385)	0.05058 (0.00422)
	High MW	0.00767 (0.00175)	0.01537 (0.00047)	0.01936 (0.00102)

5.3.5 Flux of free amino acids within the symbiosis

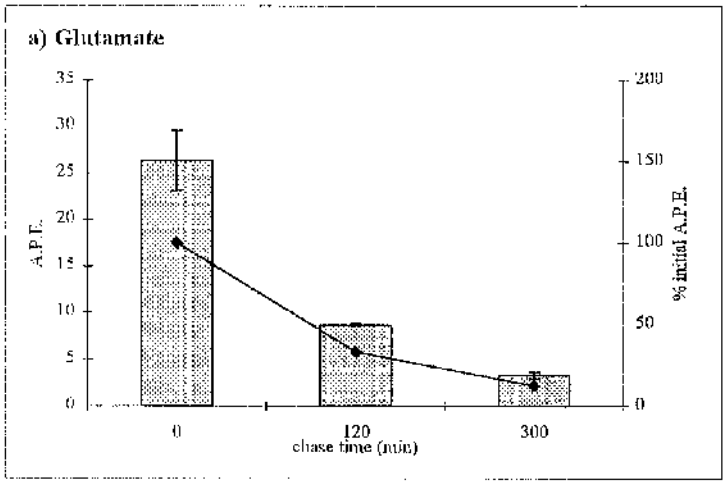
The enrichment of amino acids was only detected in samples from zooxanthellae and these data are summarised in table 5.10 and illustrated by the histograms in figures 5.9a-5.9i. During the 300 minute chase in [^{14}N]ammonium following the 60 minute pulse with 20µM [^{15}N]ammonium, the enrichment of glutamate, aspartate, alanine, glycine and valine declined. The enrichment of leucine remained constant whereas that of threonine and phenylalanine increased (see figures 5.9a-5.9h) but not to the initial enrichment of glutamate, aspartate and alanine.

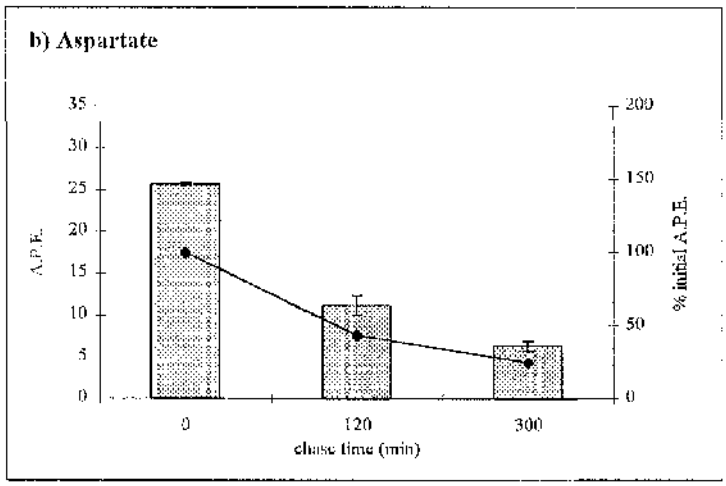
Table 5.10 Summary of amino acid enrichment of zooxanthellae from symbiotic anemones during a chase period in [^{14}N]ammonium following a 60 minute pulse with 20µM [^{15}N]ammonium under an illumination of 300µmol photons.m^{-2}.s^{-1}.
Mean atom % excess ^{15}N (±SD) n=2 (* indicates missing data).

Amino Acid	^{15}N enrichment after 60 min pulse	^{15}N enrichment after 120 min chase	^{15}N enrichment after 300 min chase
Alanine	24.228 (3.779)	11.519 (2.723)	6.508 (3.183)
Glycine	5.265 (1.475)	3.920 (1.526)	2.220 (1.573)
Valine	16.454 (1.684)	7.431 (2.348)	5.560 (1.112)
Leucine	14.795 (*)	12.687 (9.608)	14.304 (3.847)
Threonine	5.583 (0.232)	6.904 (0.320)	7.661 (0.320)
Phenylalanine	2.009 (*)	2.363 (1.172)	3.576 (0.244)
Aspartate	25.667 (0.231)	11.188 (1.642)	6.330 (0.861)
Glutamate	26.302 (4.547)	8.684 (0.230)	3.288 (0.452)
Tyrosine	*	1.769 (*)	*

Figure 5.9 (a-c)

Enrichment in atom % excess (A.P.E.) of the amino acids glutamate, aspartate and alanine from zooxanthellae. Anemones were incubated for a 1h pulse with 20μM [^{15}N]ammonium followed by a chase in [^{14}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2). Lines represent the mean percentage of the initial enrichment.





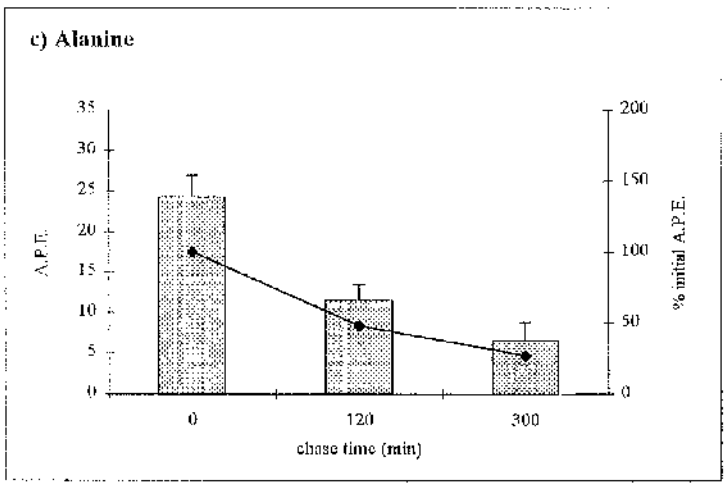
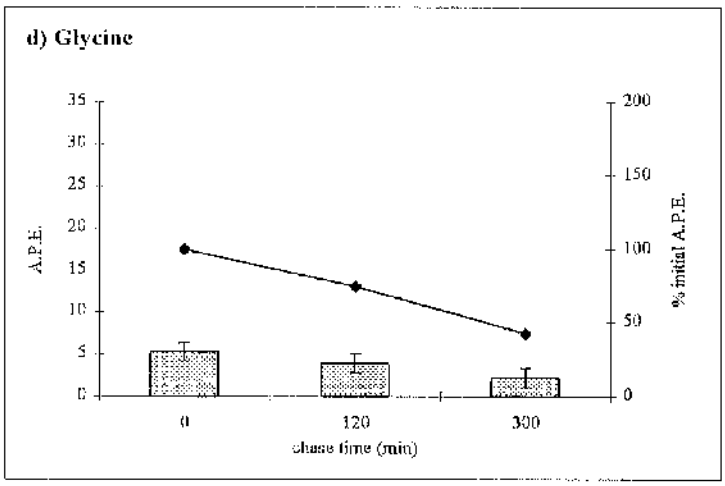
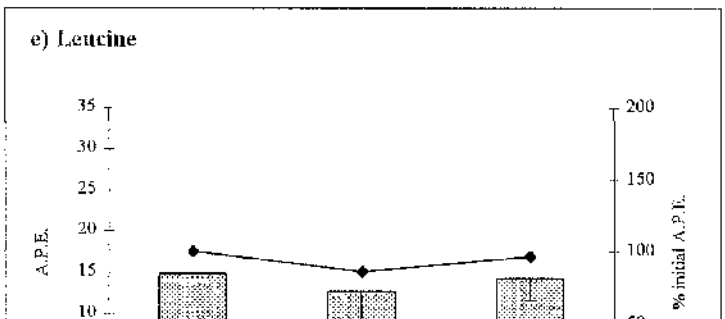


Figure 5.9 (d-f)

Enrichment in atom % excess (A.P.E.) of the amino acids glycine, leucine and valine from zooxanthellae. Anemones were incubated for a 1h pulse with 20μM [^{15}N]ammonium followed by a chase in [^{14}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2). Lines represent the mean percentage of the initial enrichment.





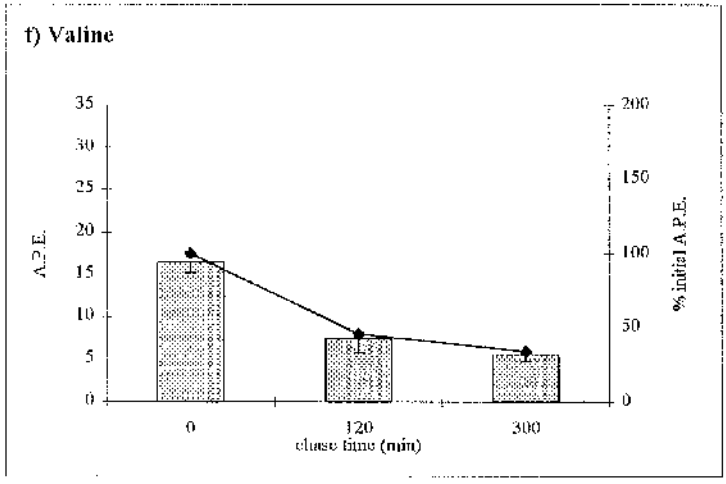
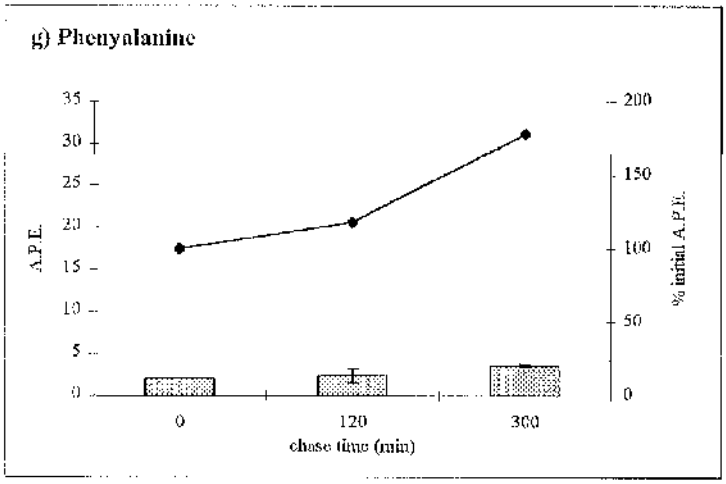
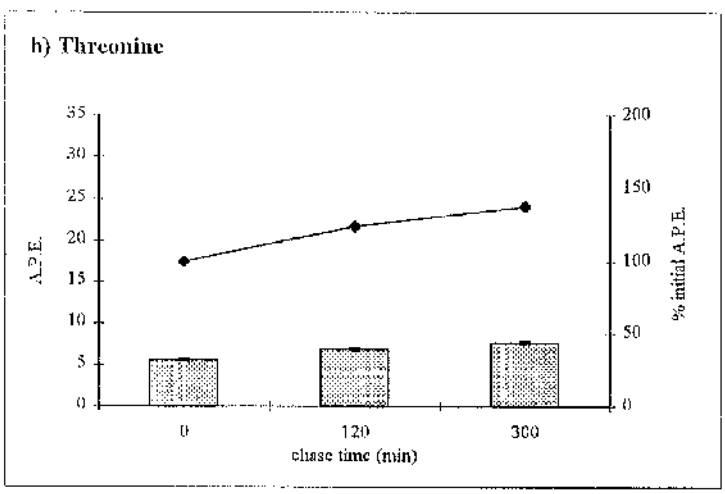
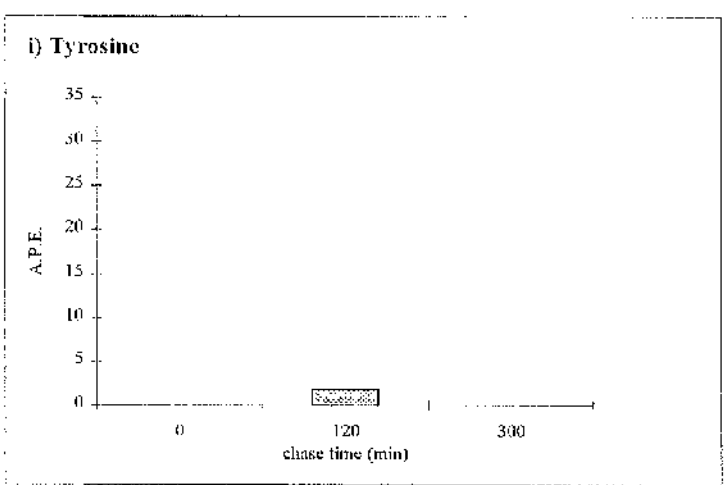


Figure 5.9 (g-i)

Enrichment in atom % excess (A.P.E.) of the amino acids phenylalanine, threonine and tyrosine from zooxanthellae. Anemones were incubated for a 1h pulse with 20μM [^{15}N]ammonium followed by a chase in [^{14}N]ammonium under 300μmol photons.m^{-2}.s^{-1} illumination. Mean enrichment ±SE (n=2). Lines represent the mean percentage of the initial enrichment.







The rate of enrichment change during the chase in [^{14}N]ammonium was calculated using linear regression to fit a least squares model to the data. Where the data were non-linear, the natural log of the dependent (enrichment) variable was taken before fitting a line using linear regression. This analysis is summarised in table 5.11 and illustrated graphically, using the equations for each of the lines, in figure 5.10.

Table 5.11 Rate of zooxanthellae amino acid enrichment change calculated from pulse-chase data. Negative rates indicate declining enrichment.

Amino Acid	FSR ((log) % min^{-1})	R^2-adj.	p	equation of line
Glutamate	-0.00694	95.8	<0.001	log APE = 3.16-(0.00680t)
Aspartate	-0.00465	91.5	0.002	log APE = 3.14-(0.00456t)
Alanine	-0.00457	77.8	0.013	log APE = 3.10-(0.00448t)
Glycine[1]	-0.01031	46.3	0.082	APE = 5.22-(0.01011t)
Valine	-0.00354	70.2	0.023	log APE = 2.65-(0.00347t)
Phenylalanine[1]	0.00572	44.7	0.132	APE = 1.83+(0.00561t)
Threonine[1]	0.00685	84.7	0.006	APE = 5.78+(0.00671t)

FSR, fractional synthetic rate; APE, atom % excess; t, time (minutes); R^2-adj., coefficient of determination adjusted for the number of parameters in the model; p, significance of the regression.
[1]Data for the amino acids glycine, phenylalanine and threonine were not log-transformed before analysis. Therefore the units for the corresponding rates are % min^{-1}.

The half life ($T_{½}$) of each amino acid with declining enrichments during the chase was calculated by using the same linear regression analysis to fit a line to the relationship between the natural log of the percentage initial enrichment and time (see table 5.12). Here the initial enrichment is taken as 100% and the half life is the time corresponding to 50% enrichment.

Figure 5.10

Enrichment of zooxanthellae amino acids with ^{15}N during a chase in 20μM [^{14}N]ammonium following a 60 minute pulse with 20μM [^{15}N]ammonium.

The graphs represent the rate at which ^{15}N enrichment changes in each amino acid when symbiotic anemones are transferred from [^{15}N]ammonium to [^{14}N]ammonium at 300μmol photons.m^{-2}.s^{-1} illumination. The equation for each line was derived by linear regression (see table 5.11). The lines plotted here are all from significant regression analyses ($p<0.05$).

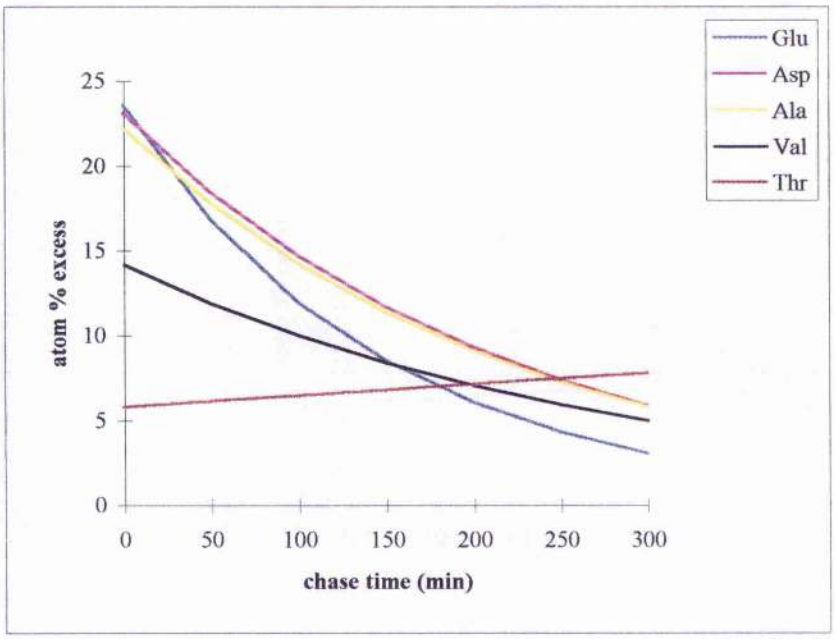


Table 5.12 Half lives of zooxanthellae amino acids calculated by linear regression.

Amino Acid	$T_{½}$ (min)	R^2-adj.	p	equation of line
Glutamate	78.96	95.6	0.003	log % I.E. = 4.43-(0.00656t)
Aspartate	120.74	88.3	0.011	log % I.E. = 4.43-(0.00429t)
Alanine	129.76	70.1	0.049	log % I.E. = 4.47-(0.00430t)
Glycine	256.61	44.4	0.133	% I.E. = 98.5-(0.1890t)
Valine	288.51	58.1	0.083	log % I.E. = 4.36-(0.00311t)

APE, atom % excess; t, time (minutes); R^2-adj., coefficient of determination adjusted for the number of parameters in the model; p, significance of the regression; % I.E., the percentage initial enrichment. Data for glycine were not log transformed before analysis.

Using the relationship, $T_{½} = \ln(2)k$, it is possible to calculate the rate constant k which, together with the concentration of each amino acid (section 4.3.1.2), gives the rate of flux of amino acid (table 5.13).

Table 5.13 Rate of amino acid flux in zooxanthellae

Amino Acid	k	Concentration ($nmol.mg^{-1}$ tentacle)	Rate ($nmol.mg^{-1}$ min^{-1})
Glutamate	0.008778	1845.34 (975.95)	16.199 (8.567)
Aspartate	0.005741	847.66 (74.24)	4.866 (0.426)
Alanine	0.005342	352.57 (137.91)	1.883 (0.737))
Glycine	0.003250	855.16 (509.20)	2.779 (1.655)
Valine	0.002403	150.03 (42.43)	0.360 (0.102)

5.4 Discussion

5.4.1 Incorporation of ^{15}N into zooxanthellae and host fractions

In all the experiments carried out, the zooxanthellae became enriched with ^{15}N from [^{15}N]ammonium at a greater rate than the host. That the host fraction showed any enrichment over a 30 minute time course is in contrast with the results of Davies (1988), who only detected zooxanthellae enrichment following incubation of *Anemonia viridis* for 30 minutes in seawater supplemented with 20μM [^{15}N]ammonium. Davies (1988) separated zooxanthellae from host using the density gradient centrifugation method of Tytler and Davies (1983). It is possible that by using this method the host fraction, in particular the low molecular weight material, was not completely sampled. The present study has demonstrated that the low molecular weight fractions are the most enriched with ^{15}N, so loss of this fraction by Davies (1988) would have given apparently low or non-existent enrichments. This represents the first demonstration of ^{15}N enrichment from [^{15}N]ammonium of host tissue from the *Anemonia viridis* symbiosis.

The pattern of enrichment seen shows that the zooxanthellae are the main site of ammonium assimilation in symbiotic anemones. Zooxanthellae became enriched at between 5.6 and 16.9 times the rate of host tissue. This pattern has also been found in other zooxanthellar symbioses when they have been investigated using tracers with both ^{15}N (Wilkerson & Kremer, 1992; Hawkins & Klumpp, 1995) and ^{14}C (Hoegh-Guldberg & Davidson, 1996). Wilkerson and Kremer (1992) found that zooxanthellae from *Limulus unguiculata* became enriched at between 1.25 and 2 times the rate of host tissue. However, these results cannot eliminate a role for direct ammonium assimilation by the host. The symbiotic sea anemone *Aiptasia pallida* is known to possess glutamine synthetase (GS) in both zooxanthellae and host fractions. Ferrier *et al.* (1996) found that the level of GS activity in the host fraction of this anemone was positively correlated with zooxanthellae density and was highest under conditions of low nutrient availability. Such results suggest that the host tissues could play a role in ammonium assimilation in the *Aiptasia pallida* symbiosis. It is possible that a similar pathway could exist in the *Anemonia viridis* symbiosis and be responsible for some or all of the ammonium assimilation seen in host tissue.

The rate of ^{15}N assimilation by zooxanthellae when anemones were incubated at 20μM [^{15}N]ammonium was approximately twice that of anemones incubated at 10μM

[^{15}N]ammonium. This was true under both the high and low light levels used. However, the rate of ammonium enrichment in the host fraction did not increase significantly with the [^{15}N]ammonium concentration under either of the light levels. Davies (1988) investigated the kinetics of this relationship for both the intact symbiosis and isolated zooxanthellae and found that there was a linear relationship between the concentration of ammonium and the rate of depletion by the intact symbiosis, with no evidence of saturation up to 30μM ammonium. Similarly, the results presented in section 2.6.3 show that ammonium uptake by symbiotic anemones is concentration-dependent. However, the kinetics of ammonium uptake by freshly isolated zooxanthellae from *Anemonia viridis* differed from those of the intact symbiosis; isolated zooxanthellae took up ammonium at a faster rate and showed uptake saturation (Davies, 1988). These results were interpreted as being consistent with the depletion-diffusion model of ammonium uptake outlined by D'Elia (1977) and D'Elia *et al.* (1983), where diffusion of ammonium into the symbiotic cell is driven by the concentration gradient created by zooxanthellae ammonium assimilation.

The rate of ^{15}N enrichment of zooxanthellae from anemones maintained in the dark after 12 hours dark pre-incubation was significantly lower than that for anemones incubated under either light level. There was no significant difference in zooxanthellae enrichment between the two light levels at either ammonium concentration. Dark treatment also reduced the rate of host enrichment in comparison with the two light levels. Unlike the zooxanthellae, the rate ^{15}N enrichment of the host fraction was significantly greater under the higher light treatment than under the lower light treatment at both ammonium concentrations. This is the first demonstration of increased host enrichment with ^{15}N from [^{15}N]ammonium when an algal-invertebrate symbiosis is incubated under increased illumination.

Ammonium uptake in the dark, albeit at reduced rates when compared with uptake in the light, has been demonstrated by a number of other studies (Muscatine & D'Elia, 1978; Wilkerson & Muscatine, 1984; Wilkerson & Trench, 1986; Davies, 1988). The length of time that ammonium assimilation continues in darkness is proportional to the duration of previous light exposure (Muscatine & D'Elia, 1978). This phenomenon is characteristic of nitrogen-limited algal cells, where a supply of reduced carbon remains to assimilate nitrogen in the absence of photosynthesis (Syrett, 1981).

The fact that no difference in zooxanthellae enrichment was evident between the two light treatments could be explained in the context of the light groups defined by Davies (1988). In that study, ammonium flux in symbiotic *Anemonia viridis* could be divided into two groups: individuals incubated under either 100-300μmol photons.$m^{-2}.s^{-1}$ ('high light') or 0-50μmol photons.$m^{-2}.s^{-1}$ ('low light'). In the present study both the light treatments used (150 and 300μmol photons.$m^{-2}.s^{-1}$) correspond to the 'high light' group defined by Davies (1988). It it is unlikely that the rate of photosynthesis differs significantly between these light levels, since above 120μmol photons.$m^{-2}.s^{-1}$ the relationship between the rate of photosynthesis and irradiance in *Anemonia viridis* becomes curvilinear and is saturated at around 160μmol photons.$m^{-2}.s^{-1}$ (Tytler, 1982).

The increased ^{15}N enrichment of the host fraction under the higher of the two light levels could be interpreted in terms of either the recycling or conservation hypotheses. However, it is still necessary to explain why host enrichment increased under the higher light level, whereas zooxanthellae enrichment was unchanged. If nitrogen was assimilated by the host using carbon skeletons from zooxanthellae translocated photosynthate (Rees, 1987; Rees & Ellard, 1989), one would expect the rate of host assimilation to depend directly on the rate of photosynthesis. As discussed above, the rate of zooxanthellae photosynthesis will have been similar at the two light levels. Therefore it seems unlikely that host enrichment is simply determined by the rate of photosynthesis. A possible explanation is that under higher light, although the overall photosynthetic rate of zooxanthellae does not differ, the composition of photosynthate translocated to the host does. In other words, under higher light more nitrogen-containing compounds are translocated to the host. Muscatine *et al.* (1984) showed that light and shade-adapted colonies of the coral *Stylophora pistillata* had different C:N uptake ratios. The different C:N uptake ratios found in *Stylophora pistillata* colonies would be reflected in different C:N ratios of the translocate from zooxanthellae to host. Therefore, the zooxanthellae could produce translocated photosynthate which would have a C:N ratio dependent upon habitat irradiance (Muscatine *et al.*, 1984). In the present study it was intended to analyse the products of ammonium assimilation in the host fraction to see whether or not their composition varied with irradiance. Unfortunately, as discussed in section 5.3.3, it was not possible to analyse amino acids from host-derived samples.

5.4.2 ^{15}N assimilation into high and low molecular weight material

The TCA soluble or low molecular weight (LMW) compounds were most highly enriched following a 30 minute incubation with [^{15}N]ammonium. When both zooxanthellae and host extracts were fractionated with TCA, the enrichment was highest in LMW zooxanthellae material followed by LMW host material. The high molecular weight (HMW) material from zooxanthellae was the next most enriched class of compounds followed by HMW material from the host which had the least enrichment.

5.4.3 ^{15}N assimilation into free amino acids

The analysis of free amino acid enrichment following incubation with [^{15}N]ammonium was only successful in samples prepared from zooxanthellae. The exact reason for the failure of samples prepared from the host to derivatize prior to GC-MS analysis is not known but appears to be due to some factor present in the host extracts which was not removed by lipid extraction, TCA precipitation, cation exchange purification or 50kDa ultrafiltration. This factor may be associated with the mucus which was present in host but not zooxanthellae material. Therefore there are no data available on either the assimilation of [^{15}N]ammonium into amino acids by the host fraction, or the translocation of ^{15}N enriched amino acids from zooxanthellae to host. In zooxanthellae, the most highly enriched amino acids were glutamate, aspartate and alanine. Unfortunately glutamine failed to derivatize reliably in all but one preliminary experiment. However, in this preliminary experiment the enrichment of glutamine was greatest in the amide position which is consistent with the GS/GOGAT cycle as the mechanism of ammonium assimilation (Summons & Osmond, 1981).

The amino acids analysed represented 65% of the total LMW zooxanthellae enrichment. Of this, glutamate accounted for 29%, glutamine 23% aspartate 10% and alanine 2%, with the other amino acids detected accounting for just over 1% of the total amino acid enrichment. In other words, over the 120 minutes of this time course investigation, approximately 60% of the total enrichment of LMW material can be accounted for by the three amino acids glutamate, glutamine and aspartate. The remaining low molecular weight enrichment could consist of ^{15}N assimilated into other amino acids, peptides and nucleotides.

Among the amino acids from zooxanthellae enriched with ^{15}N were a number of essential amino acids. The free amino acid pool of zooxanthellae from *Anemonia viridis* was

investigated in this study using HPLC (section 4.3.1.2). Table 5.14 lists the amino acids that were identified by HPLC and summarises whether or not they showed any evidence of enrichment.

Table 5.14 Summary of zooxanthellae amino acids and their enrichment with ^{15}N from [^{15}N]ammonium.

Amino acid	Evidence for enrichment
Aspartate	Yes
Glutamate	Yes
Asparagine	Not detected
Serine	?
Glutamine	Yes
Histidine*	?
Glycine	Yes
Threonine*	Yes
Arginine	?
Alanine	Yes
Taurine	Not determined
Tyrosine	Yes
Methionine*	?
Valine*	Yes
Phenylalanine*	Yes
Isoleucine	?
Leucine*	Yes
Ornithine	Not detected
Proline	?
Lysine*	?

*essential amino acid; ? some evidence for enrichment

All those amino acids which were detected have either been listed as definitely being enriched or as showing some evidence of enrichment. The latter are listed with a question mark and refer to amino acids where the enrichment calculation was either unreliable (Slater *et al.*, 1995) or the data set was too incomplete to allow further analysis. The present study identified reliable enrichment from a total of nine amino acids including three of the essential amino acids (threonine, valine and phenylalanine). There are data to suggest enrichment of a further three essential amino acids (histidine, methionine and

lysine). Threonine and phenylalanine showed increasing enrichment during the chase in [^{14}N]ammonium following incubation in [^{15}N]ammonium, suggesting that ^{15}N was transferred to these amino acids during the chase. Threonine, together with lysine, cannot be synthesised by transamination in animal cells (Waterlow *et al.*, 1978). This represents the first step in any translocation of essential amino acids from zooxanthellae to host and is the first such evidence from the *Anemonia viridis* symbiosis.

5.4.4 Nitrogen flux within the symbiosis

When symbiotic anemones were incubated with a 30 minute pulse of [^{15}N]ammonium the total nitrogen enrichment of zooxanthellae did not change over 120 minutes chase in [^{14}N]ammonium at either 10 or 20μM. The pattern was repeated in the host fraction. However, when these samples were fractionated into high and low molecular weight components there was a trend towards decreasing enrichment in LMW material and increasing enrichment in HMW material. This trend was not statistically significant in zooxanthellae material but was significant in host material. The increasing enrichment of HMW host material demonstrates that nitrogen from an inorganic source was appearing in host-derived HMW material (i.e. protein) over a 150 minute time period.

5.4.5 Flux of free amino acids within the symbiosis

When the same experimental pulse-chase design was used to follow the flux of ^{15}N through amino acids, glutamate was found to turn over most rapidly, followed by aspartate, alanine, glycine and valine. ^{15}N enrichment of the essential amino acids threonine and phenylalanine increased over the 300 minute chase period. Since no host material was analysed successfully it is impossible to say whether or not the two essential amino acids lysine and threonine, which cannot be synthesised by animal cell transamination, became enriched in the host fraction. Although high molecular weight host material incorporated ^{15}N, it is not possible to say whether the nitrogen was originally assimilated by the zooxanthellae and translocated to the host, or was assimilated directly by the host.

These results do not provide any evidence for or against a transfer of nitrogen from zooxanthellae to host. Over the time period used in this investigation (120 minute chase time), there was no trend of decreasing zooxanthellae enrichment in parallel with increasing host enrichment. This suggests that if this transfer of nitrogen is taking place in *Anemonia viridis* it is doing so over a longer time scale. It is of interest to note that the

evidence for zooxanthellae nitrogen translocation to the host in *Tridacna gigas* came from experiments which had a time course of 245 hours (Hawkins & Klumpp, 1995).

5.4.6 Summary

The experimental work described in this chapter has confirmed that *Anemonia viridis* can take up [^{15}N]ammonium and that both zooxanthellae and host material become enriched with ^{15}N. This is the first demonstration of host ^{15}N enrichment from *Anemonia viridis*. Whether the zooxanthellae are the only site of assimilation remains open to debate. However, the zooxanthellae did become enriched at between 5.6 and 16.8 times the rate of the host suggesting that the symbionts assimilated ammonium more actively than the host or the rate of nitrogen translocation from zooxanthellae to host was considerably less than the rate of ammonium assimilation by the zooxanthellae. The initial products of ammonium assimilation were of low molecular weight. Low molecular weight zooxanthellae material became enriched at 20 times the rate of low molecular weight host material. Similarly, the rate of enrichment of zooxanthellae high molecular weight material was greater, by 6 times, than that of host high molecular weight material. However, there was a significant increase over time in the enrichment of host high molecular material originating from [^{15}N]ammonium. This provides evidence that host protein is synthesised using nitrogen supplied to the symbiosis as ammonium in the seawater.

The rate of total nitrogen enrichment of zooxanthellae was doubled when the concentration of [^{15}N]ammonium in the seawater was doubled. This mirrors the positive relationship between ammonium concentration and uptake rate described in section 2.6.3 and by Davies (1988). However, any difference in host enrichment with ammonium concentration was not significant. Ammonium assimilation in both zooxanthellae and host was enhanced by light and although reduced, was not eliminated by 12 hours darkness. The only significant difference in total nitrogen assimilation between the two light levels used was seen in the host fraction. This is the first direct evidence from any microalgal-invertebrate symbiosis that the hosts' access to nitrogen from an inorganic supply in the seawater is determined by light level.

[^{15}N]ammonium assimilation into amino acids from zooxanthellae was measured and accounted for 65% of the total nitrogen enrichment of zooxanthellae low molecular weight material. 96% of the amino acid enrichment measured was accounted for by

glutamate, glutamine and aspartate. Of these amino acids, the flux of nitrogen through glutamate was the most rapid (no data are available for glutamine). Although glutamine analysis failed in most experiments, preliminary data showed that the pattern of enrichment was consistent with the GS/GOGAT cycle as the mechanism of ammonium assimilation. The activity of GOGAT from zooxanthellae from *Anemonia viridis* was recorded in section 4.3.2.2 and the pattern of glutamine enrichment provides further evidence that this enzyme system mediates zooxanthellae ammonium assimilation. Following assimilation into glutamine and glutamate, nitrogen from ammonium was shown to appear in essential amino acids from zooxanthellae. This represents the first step in any essential amino acid translocation from zooxanthellae to host.

Chapter 6

Discussion

The concept of nitrogen cycling has been central to the interpretation of microalgal-invertebrate symbioses as potentially autotrophic. While it is well established that symbiotic anemones and corals can be self sufficient with respect to carbon (Muscatine *et al.*, 1984; Davies, 1984; Edmunds & Davies, 1986), the contribution that zooxanthellae make to the nitrogen requirements of these organisms remains unclear. Early investigations revealed that these symbiotic associations retained excretory nitrogen and could take up inorganic nitrogen from micromolar concentrations in seawater (Kawaguti, 1953; Muscatine & D'Elia, 1978; Wilkerson & Trench, 1986; Davies, 1988). This immediately suggested that symbiotic animals may have access to a nitrogen source which was unavailable to non-symbiotic animals.

The initial aim of the research described here was to see whether access to an inorganic nitrogen supply would allow unfed symbiotic anemones to grow. Over a sixty day period, unfed anemones in seawater with a low concentration of ammonium lost weight, whereas anemones in seawater containing 20μM ammonium increased in weight. This provided a preliminary indication that ammonium uptake could support growth, so a 24 hour nitrogen budget for symbiotic anemones in 20μM ammonium was constructed. The budget showed that although the uptake of nitrogen from this ammonium supply could account for most of the observed growth, it failed to account for all of it. This implied that either the growth component measured was inaccurate or that a second, unmeasured source of nitrogen was available to the anemones. Since cnidarians, including *Anemonia viridis*, are capable of taking up free amino acids from seawater (Stephens 1962; Shick, 1975; Schlichter, 1978; Schlichter, 1982; Ferrier, 1991; Wilkerson & Kremer, 1992), it was suggested that this may have provided the anemones with a second source of nitrogen. Further nitrogen budget analysis should measure any amino acid uptake from seawater. This could be carried out using HPLC methodology of the sort described by Ferrier (1991). Unfortunately, it was not possible to conduct this analysis during the present study.

The growth component of the nitrogen budget relied on comparing two groups of anemones, one of which had been subjected to a known input of ammonium while the

second had not. Change in total nitrogen content between these two groups was used to calculate the rate of nitrogen-specific growth. Following exposure to ammonium, this method detected an increase in the total nitrogen content of both zooxanthellae and host. The increase in total nitrogen was presumably a reflection of protein production, since protein accounts for most nitrogen-containing biomass. Beaver (1996) showed that when symbiotic *Anemonia viridis* were maintained in seawater supplemented with 20μM ammonium, 60% of their weight increase was protein biomass. This also implies that the rate of protein synthesis may be increased by ammonium uptake. Further investigation into the effect of ammonium on the growth of this symbiosis could address this question by measuring the rate of protein synthesis in both zooxanthellae and host. An initial analysis of ammonium incorporation into protein was carried out in the present investigation by following the assimilation of [^{15}N]ammonium into high molecular weight material.

Further investigation is needed to produce a nitrogen budget for *Anemonia viridis* in the field. Davies (1988) measured both light levels and ammonium concentrations in the field (Loch Sween) and used these data, together with his analysis of nitrogen flux by anemones in the laboratory, to predict whether this symbiosis would show a net uptake of ammonium-nitrogen. This study suggested that, under field conditions, the anemones would suffer a net loss of nitrogen making holozoic feeding necessary for growth. The contribution holozoic feeding makes to the nitrogen requirements of *Anemonia viridis* in the field is not known, although the prey items taken by anemones were recorded by Möller (1978). In order to produce a meaningful nitrogen budget which included holozoic feeding it would be necessary to calculate both the nitrogen content of prey and the assimilation efficiency following prey ingestion. Such a calculation was carried out by Hawkins and Bayne (1985) who fed *Mytilus edulis* with ^{15}N labelled algae, measured its assimilation and calculated a nitrogen budget. A similar approach could be used to quantify the nitrogen input from feeding by *Anemonia viridis* if a suitable food source labelled with ^{15}N was available.

An extension of this technique could be used to see whether symbiotic anemones conserve protein. It has been suggested that the carbon-rich translocate from the zooxanthellae allows the host to divert amino acids from gluconeogenesis, reduce its rate of deamination and so conserve protein (Rees, 1987; Rees & Ellard, 1989; Szmant *et al.*, 1990). Kreeger *et al.* (1996) supplied *Mytilus edulis* with ^{15}N-labelled protein, which had

been extracted from microalgae grown with [^{15}N]nitrate and packaged in microcapsules. Following this, ^{15}N ingestion, incorporation and excretion were measured and ^{15}N loss via defecation was estimated indirectly from the difference between ingestion and the sum of incorporation and excretion. This analysis showed that *Mytilus edulis* retained amino-N from ingested protein which suggested that mussels catabolised protein to produce amino-N for amino acid synthesis rather than for energy production. It may be possible to supply symbiotic anemones with a similar ^{15}N enriched protein supply. Since the host receives a carbon-rich translocate from the zooxanthellae, it could be predicted that amino-N would be more conserved by symbiotic than by aposymbiotic anemones.

Following the investigation of the nitrogen budget of *Anemonia viridis* when supplied with 20μM ammonium, this study turned to the mechanism by which nitrogen cycling may take place. Although nitrogen cycling in microalgal invertebrate symbioses has been recognised for many years, the mechanism is still debated (Miller & Yellowlees, 1989; Rees, 1989b) and the role of recycling is particularly controversial (Douglas, 1987; Rees, 1989b; Douglas, 1994). For this reason, it was decided to examine both the hypotheses of nitrogen recycling and conservation. Nitrogen recycling takes place if excretory nitrogen from the host is taken up by its symbionts and converted to an organic form which can then be translocated from the symbionts and used by the host. The main criticism of nitrogen recycling in cnidarian symbioses is the lack of evidence for a substantial transfer of nitrogen from zooxanthellae to host (Douglas, 1987; Rees, 1989b; Douglas, 1994). Alanine is the only amino acid to have been consistently reported in the photosynthate released by freshly isolated zooxanthellae (Muscatine & Cernichiari, 1969; Trench 1971b, c; Muscatine *et al.*, 1972) and zooxanthellae *in hospite* (Lewis & Smith, 1971). There is very little evidence for the transfer of essential amino acids, although Markell and Trench (1993) did provide evidence that cultured zooxanthellae released water soluble proteins (termed glycoconjugates) which *in hospite* could provide the host with a supply of essential amino acids. The hypothesis of nitrogen conservation does not rely on there being a flux of nitrogen from symbiont to host. Host nitrogen is conserved in the presence of carbon translocation from the symbionts which allows the host both to reduce its rate of deamination and to reassimilate any catabolically produced ammonium (Rees, 1987; Miller & Yellowlees, 1989; Rees & Ellard, 1989; Rees, 1989b; Szmant *et al.*, 1990).

In their review of inorganic nitrogen uptake by symbiotic marine cnidarians, Miller and Yellowlees (1989) proposed that the host, not the zooxanthellae, was the site of ammonium assimilation. The main line of evidence used in support of this hypothesis was the fact that the host typically contains high levels of NADPH-GDH which, together with translocated carbon skeletons from the zooxanthellae, would allow the host to assimilate ammonium. *Anemonia viridis* also contained GDH which showed greater activity, in the direction of ammonium assimilation, with the coenzyme NADPH. However, it is another enzyme, GS, which appears to assimilate ammonium in host tissue from green *Hydra* (Rees, 1987, 1989a). Recently GS has also been identified in the host from a number of marine microalgal-invertebrate symbioses (Rees *et al.*, 1994; Yellowlees *et al.*, 1994; Ferrier *et al.*, 1996).

Whether GS or GDH could assimilate ammonium in the host is likely to depend on the affinity these enzymes have for ammonium at physiological concentrations. The intracellular ammonium concentration in host tissue of *Anemonia viridis* has not been measured but an indirect estimate was made by Davies (1988). This estimate of about 20μM for starved aposymbiotic anemones was within the range of 5-50μM suggested by Crossland & Barnes (1977) and Wilkerson & Muscatine (1984). GS has high affinity for ammonium (Stewart *et al.*, 1980) whereas NADPH-GDH has lower affinity (Smith *et al.*, 1975; Bishop *et al.*, 1978; Stewart *et al.*, 1980; Male & Storey, 1983). This implies that at the low intracellular ammonium concentrations predicted by the diffusion-depletion hypothesis (D'Elia, 1977; D'Elia *et al.*, 1983; D'Elia & Cook, 1988), GS would be more important in ammonium assimilation than NADPH-GDH. This investigation has shown that host tissue from *Anemonia viridis* incorporates nitrogen from [^{15}N]ammonium. If the host was a site of ammonium assimilation, then one would predict that the host should contain an assimilatory enzyme system with high affinity for ammonium. The available evidence suggests that GS could fill this role (Rees *et al.*, 1994; Yellowlees *et al.*, 1994; Ferrier *et al.*, 1996) and any further investigation should analyse the activity of GS in *Anemonia viridis*. However, animal cells are not thought to possess an enzyme system akin to GOGAT (Fowden, 1981) which can regenerate glutamate from glutamine and so provide a substrate for transamination to produce further amino acids (Stryer, 1988). This study has shown that *Anemonia viridis* has glutamine-linked GDH activity in the host fraction; a feature of other cnidarian GDH enzymes (Male & Storey, 1983). In theory, this provides a means by which the host could produce glutamate from glutamine,

although further work is needed to see if such activity exists at physiological concentrations of glutamine.

Past investigations have provided indirect evidence that zooxanthellae, in common with bacteria and plants, do possess GOGAT (Summons & Osmond, 1981; Summons *et al.*, 1986; Rahav *et al.*, 1989). Therefore nitrogen from ammonium assimilated by zooxanthellae via GS (Anderson & Burris, 1987) could be transferred via GOGAT to produce two molecules of glutamate. While one glutamate would be available to be used as a substrate by GS, the second would be available for transamination to produce other amino acids. In the present study, freshly isolated zooxanthellae from *Anemonia viridis* possessed GOGAT activity in the presence of a ferredoxin analogue. This suggested that the activity seen would be ferredoxin-dependent *in vivo*. In plant systems, ferredoxin is reduced by electrons generated in photosystem I by the light reactions of photosynthesis. However, since zooxanthellae are able to assimilate ammonium in darkness, there must be catabolic reactions capable of ferredoxin reduction in the dark (Syrett, 1981).

Many workers have interpreted symbiotic zooxanthellae populations as nitrogen-deficient or even nitrogen-limited (see section 1.8). The great increases seen in the zooxanthellae population of corals when supplied with ammonium provided dramatic evidence for this hypothesis (Hoegh-Guldberg & Smith, 1989; Muscatine *et al.*, 1989; Dubinsky *et al.*, 1990; Stambler *et al.*, 1991; Hoegh-Guldberg, 1994; Muller-Parker *et al.*, 1994). It has also been suggested that the host restricts access to nitrogen as a means of controlling the rate of zooxanthellae growth, perhaps by reducing its rate of deamination (Rees, 1987; Rees & Ellard, 1989; Rees, 1989b; Szmant *et al.*, 1990). However, as discussed in section 1.8, much of the evidence for the hypothesis that zooxanthellae growth is nitrogen-limited is contradictory. Any nitrogen-deficiency shown by the *Chlorella* symbionts of *Hydra* seems to reflect their limited capacity to assimilate nitrogen. Since a large proportion of the carbon fixed is translocated to the host as maltose, the *Chlorella* cells may lack carbon skeletons to act as amino group acceptors during ammonium assimilation (Rees, 1990; McAuley, 1992; McAuley, 1996). However, as the results presented in chapter 5 demonstrate, zooxanthellae from *Anemonia viridis* seem to have ample capacity to assimilate ammonium and synthesise amino acids. At the light levels used in these experiments, the zooxanthellae would be expected to translocate between 36 and 77% of their fixed carbon (Tytler, 1982). Despite this carbon loss, the zooxanthellae are still capable of nitrogen assimilation. This confirms the interpretation

of McAuley (1996) that the way in which carbon and nitrogen metabolism is linked in zooxanthellae may differ from that in *Chlorella*.

In all experiments in which *Anemonia viridis* was incubated with [^{15}N]ammonium, the zooxanthellae became enriched at a greater rate than the host, indicating that zooxanthellae assimilate ammonium more actively. However, following a pulse label with ^{15}N, the total nitrogen enrichment of zooxanthellae and host did not change over two hours. Therefore, over the time scale of the experiments carried out, there was no evidence that decreasing zooxanthellae enrichment was mirrored by increasing host enrichment which could have indicated nitrogen transfer from zooxanthellae to host. This pattern was only seen in the *Tridacna gigas* symbiosis over 245 hour time period (Hawkins & Klumpp, 1995). However, although the duration of these experiments appears to have been too short to have shown any change in total nitrogen enrichment, ^{15}N label was transferred to host high molecular weight material. Whether this represents direct assimilation by the host or translocation of assimilated nitrogen from the zooxanthellae cannot be inferred from these data. For this reason, the initial amino acid products of ammonium assimilation were analysed in both zooxanthellae and host. If essential amino acids, which cannot be synthesised by conventional animal cell pathways, were enriched in the host, then this would have provided good evidence that the zooxanthellae assimilated ammonium and translocated these amino acids to the host. However, a recent study by FitzGerald and Szmant (1997) has suggested that three species of zooxanthellate reef corals and two species of non-zooxanthellate coral may have the capacity to synthesise eight amino acids which are regarded as essential in most animal cells. These authors dismissed the possibility that the essential amino acids could have been synthesised by the zooxanthellae and translocated to the coral host since no difference was found in the amino acid labelling between symbiotic and non-symbiotic corals.

Analysis of the zooxanthellae showed that over 96% of the amino acid enrichment detected was accounted for by the amino acids glutamate, glutamine and aspartate. This study also showed that the enrichment of glutamine was consistent with the GS/GOGAT cycle as the mechanism of ammonium assimilation (Summons & Osmond, 1981; Summons *et al.*, 1986). In addition, a number of essential amino acids incorporated nitrogen from [^{15}N]ammonium. However, the amino acid analysis of host tissue failed to give reliable enrichment data. The amino acid derivatization of host samples was

prevented by a contaminant which was not removed despite sample preparation which included lipid extraction, TCA precipitation, cation exchange purification and ultrafiltration. The fact that zooxanthellae analysis was successful whereas host analysis was not, indicates that this contaminant was present in the host material. Any further attempts to analyse amino acid ^{15}N enrichment from host material will require an improved sample preparation procedure to remove this contaminant.

When symbiotic anemones were incubated with [^{15}N]ammonium under two light levels the rate at which the host took up ^{15}N increased at the higher light level whereas that of the zooxanthellae remained unchanged (section 5.3.1.2). Since the rate of photosynthesis is likely to have been saturated at both these light levels (Tytler, 1982), it was suggested that the ability of the zooxanthellae to assimilate ammonium had also reached a maximum. However, a mechanism is needed to account for the increased host ^{15}N enrichment at the higher light level. It is suggested that the nitrogen content of translocated photosynthate could vary with irradiance. This mechanism was postulated by Muscatine *et al.* (1984) to take place in colonies of the coral *Stylophora pistillata* from different irradiance regimes. It was intended to examine this explanation by analysing the amino acid products of ammonium assimilation in the host. If this hypothesis was correct it would predict that their composition would vary with irradiance. However, as discussed above, host amino acid enrichment analysis was unsuccessful.

Unlike non-symbiotic anemones, *Anemonia viridis* has access to ammonium as a novel source of nitrogen. In the absence of holozoic feeding, ammonium uptake by this anemone can contribute significantly to the anemone's nitrogen budget. The two components of the symbiosis possess metabolic systems which could assimilate ammonium but the zooxanthellae appear to be the primary site of assimilation. Whether the appearance of ^{15}N from ammonium in the host represents amino acid translocation from the zooxanthellae (recycling) or assimilation by the host (conservation) is still open to interpretation. The central question which remains is whether or not essential amino acids appear in the host which could not have been synthesised there. This would provide direct evidence for the hypothesis of nitrogen recycling which is still a central assumption in many discussions of algal-invertebrate symbiosis. However, since the Cnidaria may show the capacity to synthesise some of the essential amino acids (FitzGerald & Szmant, 1997), such results must be interpreted with care. Completion of

the ^{15}N enrichment analysis of amino acids from the host, which was described in chapter 5, holds great potential to answer this question.

Bibliography

Achituv, Y., Ben-Zion, M. and Mizrahi, L. (1994). Carbohydrate, lipid, and protein composition of zooxanthellae and animal fractions of the coral *Pocillopora damicornis* exposed to ammonium enrichment. Pacific Sci. **48**: 224-233.

Ahmed, I. and Hellebust, J. A. (1988). Enzymology of ammonium assimilation in three green flagellates. New Phytol. **109**: 415-421.

Anderson, S. L. and Burris, J. E. (1987). Role of glutamate synthetase in ammonia assimilation by symbiotic marine dinoflagellates (zooxanthellae). Mar. Biol. **94**: 451-458.

Atkinson, M. J., Kotler, E. and Newton, P. (1994). Effects of water velocity on respiration, calcification and ammonium uptake of a *Porites compressa* community. Pacific Sci. **48**: 296-303.

De Bary, A. (1879). Die Erscheinung der Symbiose. Naturforschung Versammlung Cassel, LI, Tagebl. p. 121.

Beaver, R. (1996). Regulation of the population of symbionts in *Anemonia viridis*. PhD thesis, University of Glasgow. 168pp.

Berthelot, M. P. E. (1859). Repert. de Chim. Appl. **1**: 282-284.

Bishop, S. H., Ellis, L. L. and Burcham, J. M. (1983). Amino acid metabolism in molluscs. In: The Mollusca. Volume 1. Metabolic Biochemistry and Molecular Biomechanics (ed. Hochachka, P. W.) Academic Press, New York, pp. 243-327.

Bishop, S. H., Klotz, A., Drolet, L. L., Smullin, D. H. and Hoffman, R. J. (1978). NADP-specific glutamate dehydrogenase in *Metridium senile* (L.). Comp. Biochem. Physiol. **61**(B): 185-187.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brown (1973). A highly sensitive automated technique for the determination of ammonium nitrogen. J. Sci. Food Agriculture **24**: 1119-1123.

Burris, R. H. (1983). Uptake and assimilation of $^{15}NH_4^+$ by a variety of corals. Mar. Biol. **75**: 151-155.

Bythell, J. C. (1988). A total nitrogen and carbon budget for the elkhorn coral *Acropora palmata* (Lamarck). Proc. 6th International Coral Reef Symposium, Townsville, Australia. **2**: 535-540.

Caprioli, R. M. (1972). Use of stable isotopes In: Biochemical applications of mass spectrometry (ed. Waller, G. R.) Wiley-Interscience, London, pp. 735-787.

Cates, N. and McLaughlin, J. J. A. (1976). Differences of ammonia metabolism in symbiotic and aposymbiotic *Condylactus* and *Cassiopea* spp. J. Exp. Mar. Biol. Ecol. **21**: 1-5.

Catmull, J., Yellowlees, D. and Miller, D. J. (1987). $NADP^+$-dependent glutamate dehydrogenase from *Acropora formosa*: purification and properties. Mar. Biol. **95**: 559-563.

Chaves das Neves, H. J. and Vasconcelos, A. M. P. (1987). Capillary gas chromatography of amino acids including asparagine and glutamine: sensitive gas chromatographic-mass spectrometric and selected ion monitoring gas chromatographic-mass spectrometric detection of the N,O(S)-*tert*-butyldimethylsilyl derivatives. J. Chromatography **392**: 249-258.

Chia, F. -S. (1972). Note on the assimilation of glucose and glycine from seawater by the embryos of a sea anemone, *Actinia equina*. Can. J. Zool. **50**: 1333-1334.

Cook, C. B. and D'Elia, C. F. (1987). Are natural populations of zooxanthellae ever nutrient-limited? Symbiosis **4**: 199-212.

Cook, C. B., D'Elia, C. F. and Muller-Parker, G. (1988). Host feeding and nutrient sufficiency for zooxanthellae in the sea anemone *Aiptasia pallida*. Mar. Biol. **98**: 253-262.

Cook, C. B. and Fitt, W. K. (1989). Some effects of dissolved inorganic nutrients on the growth of zooxanthellae in the hydroid *Myrionema amboinense*. Endocytobiology **4**: 285-288.

Cook, C. B., Muller-Parker, G. and D'Elia, C. F. (1992). Ammonium enhancement of dark carbon fixation and nitrogen limitation in symbiotic zooxanthellae: Effects of feeding and starvation of the sea anemone *Aiptasia pallida*. Limnol. Oceanogr. **37**: 131-139.

Cook, C. B., Muller-Parker, G. and Orlandini, C. D. (1994). Ammonium enhancement of dark carbon fixation and nitrogen limitation in zooxanthellae symbiotic with the reef corals *Madracis mirabilis* and *Montastrea annularis*. Mar. Biol. **118**: 157-165.

Craggs, R. J., McAuley, P. J. and Smith, V. J. (1994). Batch culture screening of marine microalgal nutrient removal from primary sewage effluent. Hydrobiologia **288**: 157-166.

Crossland, C. J. and Barnes, D. J. (1977). Nitrate assimilation enzymes from two hard corals, *Acropora acuminata* and *Goniastrea australensis*. Comp. Biochem. Physiol. **57B**: 151-157.

D'Elia, C. F. (1977). The uptake and release of dissolved phosphorous by reef corals. Limnol. Oceanogr. **22**: 301-315.

D'Elia, C. F. and Cook, C. B. (1988). Methylamine uptake by zooxanthellae-invertebrate symbioses: Insights into host ammonium environment and nutrition. Limnol. Oceanogr. **33**: 1153-1165.

D'Elia, C. F., Domoter, S. L. and Webb, K. L. (1983). Nutrient uptake kinetics of freshly isolated zooxanthellae. Mar. Biol. **75**: 157-167.

D'Elia, C. F. and Webb, K. L. (1977). The dissolved nitrogen flux of reef corals. Proc. 3rd International Coral Reef Symposium, Miami **1**: 325-330.

Davies, L. M. (1988). Nitrogen flux in the symbiotic sea anemone *Anemonia viridis* (Forskål). PhD thesis, University of Glasgow. 226pp.

Davies, P. Spencer (1984). The role of zooxanthellae in the nutritional energy requirements of *Pocillopora eydouxi*. Coral Reefs **2**: 181-186.

Davies, P. Spencer (1992). Endosymbiosis in marine cnidarians. In: Plant-Animal Interactions in the Marine Benthos. (ed. John, D. M., Hawkins, S. J. and Price, J. H.) Clarendon Press, Oxford, pp. 511-540.

Day, R. J. (1994). Algal symbiosis in *Bunodeopsis*: sea anemones with 'auxiliary' structures. Biol. Bull. **186**: 182-194.

Deaton, L. E. and Hoffman, R. J. (1988). Hypoosmotic volume regulation in the sea anemone *Metridium senile*. Comp. Biochem. Physiol. **91C**: 187-191.

Degobbis, D. (1973). On the storage of seawater samples for ammonia determination. Limnol. Oceanogr. **18**: 146-150.

Douglas, A. E. (1987). Alga-invertebrate symbiosis. In: Biochemistry of Algae and Cyanobacteria. (ed. Rogers, L. J. and Gallon, J. R.) Oxford University Press, Oxford. pp. 297-310.

Douglas, A. E. (1994). Symbiotic Interactions. Oxford University Press, Oxford. 148pp.

Dubinsky, Z., Stambler, N., Ben-Zion, M., McCloskey, L. R., Muscatine, L. and Falkowski, P. G. (1990). The effect of external nutrient resources on the optical properties and photosynthetic efficiency of *Stylophora pistillata*. Proc. R. Soc. Lond. B. **239**: 231-246.

Dudler, N. and Miller, D. J. (1988). Characterisation of two glutamate dehydrogenases from the symbiotic microalga *Symbiodinium microadriaticum* isolated from the coral *Acropora formosa*. Mar. Biol. **97**: 427-430.

Dudler, N., Yellowlees, D. and Miller, D. J. (1987). Localisation of two L-glutamate dehydrogenases in the coral *Acropora latistella*. Arch. Biochem. Biophys. **254**: 368-371.

Dunlap, W. C. and Chalker, B. E. (1986). Identification and quantitation of near-UV absorbing compounds (S-320) in a hermatypic scleractinian. Coral Reefs **5**: 155-159.

Edmunds, P. J. and Davies, P. Spencer (1986). An energy budget for *Porites porites* (Scleractinia) Mar. Biol. **92**: 339-347.

Falkowski, P. G., Dubinsky, Z., Muscatine, L. and McCloskey, L. (1993). Population control in symbiotic corals. BioScience **43**: 606-611.

Falkowski, P. G., Greene, R. M. and Geider, R. J. (1992). Physiological limitations on phytoplankton productivity in the ocean. Oceanography **5**: 84-91.

Ferrier, M. D. (1991). Net uptake of dissolved free amino acids by four scleractinian corals. Coral Reefs **10**: 183-187.

Ferrier, M. D., McDonnell, T. A. and Hood, H. Dail. (1996). The effect of nutrient history on glutamine synthetase activity in *Aiptasia pallida* and its endosymbiotic algae Proc. 8th International Coral Reef Symposium, Panama City, Panama. (in press).

Fitt, W. K. (1984). The role of chemosensory behaviour of *Symbiodinium microadriaticum*, intermediate hosts, and host behaviour in the infection of coelenterates and molluscs with zooxanthellae. Mar. Biol. **81**: 9-17.

Fitt, W. K. and Cook, C. B. (1989) Some effects of host feeding on growth of zooxanthellae in the hydroid *Myrionema amboinense* in the laboratory and in nature. Endocytobiology **4**: 281-284.

Fitt, W. K. and Pardy, R. L. (1981). Effects of starvation, and light and dark on the energy metabolism of symbiotic and aposymbiotic sea anemones, *Anthopleura elegantissima*. Mar. Biol. **61**: 199-205.

Fitt, W. K., Rees, T. A. V., Braley, R. D., Lucas, J. S. and Yellowlees, D. (1993). Nitrogen flux in giant clams: size-dependency and relationship to zooxanthellae density and clam biomass in the uptake of dissolved inorganic nitrogen. Mar. Biol. **117**: 381-386.

FitzGerald, L. M. and Szmant, A. M. (1997). Biosynthesis of 'essential' amino acids by scleractinian corals. Biochem. J. **322**: 213-221.

Flynn, K. J., Dickson, D. M. J. and Al-Amoudi, O. A. (1989). The ratio of glutamine:glutamate in microalgae: a biomarker for N-status suitable for use at natural cell densities. J. Plankton. Res. **11**: 165-170.

Flynn, K. J. (1990). The determination of nitrogen status in microalgae. Mar. Ecol. Prog. Ser. **61**: 297-307.

Flynn, K. J. (1991). Algal carbon-nitrogen metabolism: A biochemical basis for modelling the interactions between nitrate and ammmonium uptake. J. Plankton Res. **13**: 373-387.

Fowden, L. (1981). Contrasts in nitrogen metabolism between animals and plants. In: Nitrogen metabolism in man (ed. Waterlow, J. C. and Stephen, J. M. L.) Applied Science Publishers, London. pp. 87-95.

Franzisket, L. (1973). Uptake and accumulation of nitrate and nitrite by reef corals. Naturwissenschaften **60**: 554.

Franzisket, L. (1974). Nitrate uptake by reef corals. Revue ges. Hydrobiol. **59**: 1-7.

Freudenthal, H. D. (1962). *Symbiodinium* gen. nov. and *Symbiodinium microadriaticum* sp. nov., a zooxanthella: taxonomy, life cycle and morphology. J. Protozool. **9**: 45-52.

Gedes, P. (1882). On the nature and functions of the 'yellow cells' of radiolarians and coelenterates. Proc. R. Soc. Edinburgh **11**: 377-396.

Gunnerson, J., Yellowlees, D. and Miller, D. J. (1988). The ammonium/methylamine uptake system of *Symbiodinium microadriaticum*. Mar. Biol. **97**: 593-596.

Harland, A. D. and Davies, P. Spencer (1994). Time-course of photoadaptation in the symbiotic sea anemone *Anemonia viridis*. Mar. Biol. **119**: 45-51.

Harvey, H. W. (1957). The chemistry and fertility of sea waters. Cambridge University Press, Cambridge.

Hawkins, A. J. S. and Bayne, B. L. (1985). Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. Mar. Ecol. Prog. Ser. **25**: 181-188.

Hawkins, A. J. S. and Bayne, B. L. (1991). Nutrition of marine mussels: factors influencing the relative utilizations of protein and energy. Aquaculture **94**: 177-196.

Hawkins, A. J. S. and Klumpp, D. W. (1995). Nutrition of the giant clam *Tridacna gigas* (L.). II. Relative contributions of filter-feeding and the ammonium-nitrogen acquired and recycled by symbiotic alga towards total nitrogen requirements for tissue growth and metabolism. J. Exp. Mar. Biol. Ecol. **190**: 263-290.

Hecht, U., Oelmuller, R., Schmidt, S. and Mohr, H. (1988). Action of light, nitrate and ammonium on the levels of NADH- and ferredoxin-dependent glutamate synthases in the cotyledons of mustard seedlings. Planta **175**: 130-138.

Herrera, F. C., Lopez, I., Egea, R. and Zanders, I. P. (1989). Short-term osmotic responses of the cells and tissues of the sea anemone, *Condylactis gigantea*. Comp. Biochem. Physiol. **92A**: 377-384.

Hochachka, P. W. and Somero, G. N. (1984). Biochemical Adaptation. Princeton University Press, Princeton, New Jersey. 537pp.

Hoegh-Guldberg, O. (1994). Population dynamics of symbiotic zooxanthellae in the coral *Pocillopora damicornis* exposed to elevated ammonium [$(NH_4)_2SO_4$] concentrations. Pacific Sci. **48**: 263-272.

Hoegh-Guldberg, O. and Smith, G. J. (1989). Influence of the population density of zooxanthellae and supply of ammonium on the biomass and metabolic characteristics of the reef corals *Seriatopora hystrix* and *Stylophora pistillata*. Mar. Ecol. Prog. Ser. **57**: 173-186.

Hoegh-Guldberg, O. and Davidson, R. (1996). The initial site of ammonium incorporation by symbiotic cnidarians. Proc. 8th International Coral Reef Symposium, Panama City, Panama. (in press).

Hoffman, R. J., Bishop, S. H. and Sassaman, C. (1978). Glutamate dehydrogenase from coelenterates NADP specific. J. Exp. Zool. **203**: 165-170.

Hofman, D. K. and Kremer, B. P. (1981). Carbon metabolism and strobilation in *Cassiopea andromedea* (Cnidaria: Scyphozoa): significance of endosymbiotic dinoflagellates. Mar. Biol. **65**: 25-33.

von Holt, C. and von Holt, M. (1968a). Transfer of photosynthetic products from zooxanthellae to coelenterate hosts. Comp. Biochem. Physiol. **24**: 73-81.

von Holt, C. and von Holt, M. (1968b). The secretion of organic compounds by zooxanthellae isolated from various types of *Zoanthus* Comp. Biochem. Physiol. **24**: 83-92.

Janssen, H. H. and Möller, H. (1981). Effects of various feeding conditions on *Anemonia sulcata*. Zool. Anz., Jena **206**: 161-170.

Jarret, H. W., Cooksy, K. D., Ellis, B. and Anderson, J. M. (1985). The separation of o-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica columns. Anal. Biochem. **153**: 189-198.

Johannes, R. E., Coles, S. L. and Kuenzel, N. T. (1970). The role of zooplankton in the nutrition of some scleractinian corals. Limnol. Oceanogr. **15**: 579-586.

Johannes, R. E. and Tepley, L. (1974). Examination of feeding of the reef coral *Porites lobata in situ* using time lapse photography. Proc. 2nd International Coral Reef Symposium, Brisbane **1**: 127-131.

Kahn, M. A. (1994). Studies on the measurement and availability of major plant nutrients in farm animal wastes. PhD Thesis, University of Glasgow. 249pp.

Kasschau, M. R., Ragland, J. B., Pinkerton, S. O. and Chen, E. C. M. (1984). Time related changes in the free amino acid pool of the sea anemone *Bunodosoma cavernata*. Comp. Biochem. Physiol. **79A**: 155-159.

Kawaguti, S. (1953). Ammonium metabolism of the reef corals. Biol. J. Okayama Univ. **1**: 171-176.

Kolber, Z., Zehr, J. and Falkowski, P. G. (1988). Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. Plant Physiol. **88**: 923-929.

Kreeger, D. A., Hawkins, A. J. S. and Bayne, B. L. (1996). Use of dual-labeled microcapsules to discern the physiological fates of assimilated carbohydrate, protein carbon, and protein nitrogen in suspension-feeding organisms. Limnol. Oceanogr. **41**: 208-215.

Kuhl, M., Cohen, Y. Dalsgaard, T., Jorgensen, B. B. and Revsbech, N. P. (1995). Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O_2, pH and light. Mar. Ecol. Prog. Ser. **117**: 159-172.

Lang, C. A. (1958). Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. **30**: 1692-1694.

Law, R. and Lewis, D. H. (1983). Biotic environments and the maintenance of sex- some evidence from mutualistic symbioses. Biol. J. Linn. Soc. **20**: 249-276.

Lea, P. J., Blackwell, R. D., Chen, F-L. and Hecht, U. (1990). Enzymes of Ammonia Assimilation. In: Methods in Plant Biochemistry. Vol. 3 (ed. Day, P. M. and Halborne, J. B.) Academic Press, London. pp. 257-276.

Lea, P. J., Blackwell, R. D., Murray, A. J. S. and Joy, K. W. (1989). In: Recent advances in Phytochemistry Vol. 23 (ed. Conn, E. E.) Plenum, New York. pp. 157-189.

Lewis, D. H. and Smith, D. C. (1971). The autotrophic nutrition of symbiotic marine coelenterates with special reference to hermatypic corals. I. Movement of photosynthetic products between the symbionts. Proc. R. Soc. Lond. B. **178**: 111-129.

Liddicoat, M. I., Tibbits, S. and Butler, E. I. (1975). The determination of ammonia in seawater. Limnol. Oceanogr. **20**: 131-132.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Male, K. B. and Storey, K. B. (1983). Kinetic characterisation of NADP-specific glutamate dehydrogenase from the sea anemone, *Anthopleura xanthogrammica*: control of amino acid biosynthesis during osmotic stress. Comp. Biochem. Physiol. **76B**: 823-829.

Manuel, R. L. (1981). British Anthozoa. Academic Press, London.

Markell, D. A. and Trench, R. K. (1993). Macromolecules exuded by symbiotic dinoflagellates in culture: amino acid and sugar composition. J. Phycol. **29**: 64-68.

Marubini, F. (1996). The physiological response of hermatypic corals to nutrient enrichment. PhD thesis, University of Glasgow. 192pp.

Marubini, F. and Davies, P. S. (1996). Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. Mar. Biol. **127**: 319-328.

McAuley, P. J. (1981). What determines the population size of the intracellular symbionts in the digestive cells of green hydra? Experimentia **37**: 346-347.

McAuley, P. J. (1986). Isolation of viable uncontaminated *Chlorella* from green hydra. Limnol. Oceanogr. **31**: 222-224.

McAuley, P. J. (1990). Uptake of ammonium by green hydra. Proc. R. Soc. Lond. B **242**: 45-50.

McAuley, P. J. (1992). The effect of maltose release on growth and nitrogen limitation of symbiotic *Chlorella*. Br. Phycol. J. **27**: 417-422.

McAuley, P. J. (1994). Amino acid content of zooxanthellae freshly isolated from *Pocillopora damicornis* Pacific Sci. **48**: 247-253.

McAuley, P. J. (1996). Effect of maltose release on uptake and assimilation of ammonium by symbiotic *Chlorella* (Chlorophyta). J. Phycol. 32: 839-846.

McAuley, P. J. and Cook, C. B. (1994). Effects of host feeding and dissolved ammonium on cell division and nitrogen status of zooxanthellae in the hydroid *Myrionema amboinense*. Mar. Biol. **121**: 343-348.

McIlwain, H. and Bachelard, H. S. (1971). Biochemistry and the central nervous system. Churchill Livingston, London. 616pp.

McLaughlin, J. J. and Zahl, P. A. (1966). Endozoic algae In: Symbiosis. Vol. I (ed. Henry, M. S.) Academic Press, New York. pp. 257-297.

McNally, K. L., Govind, N. S., Thome, P. E. and Trench, R. K. (1994). Small-subunit ribosomal DNA sequence analysis and a reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pyrrophyta). J. Phycol. **30**: 316-329.

Miflin, B. J. and Lea, P. J. (1977). Amino acid metabolism. Ann. Rev. Plant Physiol. **28**: 299-329.

Miller, D. J. and Yellowlees, D. (1989). Inorganic nitrogen uptake by symbiotic marine cnidarians: a critical review. Proc. R. Soc. Lond. B **237**: 109-125.

Miller, R. E. and Stadtman, E. R. (1972). Glutamate synthase from *Escherichia coli*. J. Biol. Chem. **247**: 7407-7419.

Möller, H. (1978). Investigations of the feeding ecology of *Anemonia sulcata*. Zool. Anz. **200**: 369-373.

Moore, S. and Stein, W. H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. **176**: 367-388.

Muller-Parker, G., McCloskey, L. R., Hoegh-Guldberg, O. and McAuley, P. J. (1994). Effect of ammonium enrichment on animal and algal biomass of the coral *Pocillopora damicornis*. Pacific Sci. **48**: 273-282.

Muscatine, L. (1961). Symbiosis in marine and freshwater coelenterates. In: The Biology of Hydra (ed. Lenhoff, H. and Loomis, W. F.) University of Miami Press, Miami. pp. 255-268.

Muscatine, L. (1980). Uptake, retention, and release of dissolved inorganic nutrients by marine alga-invertebrate associations. In: Cellular Interactions in Symbiosis and Parasitism (ed. Cook, C. B., Pappas, E. D. and Rudolph, E. D.) Ohio State University Press, Columbus. pp. 229-244.

Muscatine, L., Boyle, J. E. and Smith, D. C. (1974). Symbiosis in the acoel flatworm *Convoluta roscoffensis* with the alga *Platymonas convolutae*. Proc. R. Soc. Lond. B. **187**: 221-234.

Muscatine, L. and Cernichiari, E. (1969). Assimilation of photosynthetic products of zooxanthellae by a reef coral. Biol. Bull. **137**: 506-523.

Muscatine, L. and D'Elia, C. F. (1978). The uptake, retention, and release of ammonium by reef corals. Limnol. Oceanogr. **23**: 725-734.

Muscatine, L., Falkowski, P. G., Dubinsky, Z., Cook, P. A. and McCloskey, L. R. (1989). The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc. R. Soc. Lond. B **236**: 311-324.

Muscatine, L., Falkowski, P. G., Porter, J. W. and Dubinsky, Z. (1984). Fate of photosynthetically fixed carbon in light- and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. Proc. R. Soc. Lond. B **222**: 181-202.

Muscatine, L. and Marian, R. E. (1982). Dissolved inorganic nitrogen flux in symbiotic and nonsymbiotic medusae. Limnol. Oceanogr. **27**: 910-917.

Muscatine, L., Masuda, H. and Burnap, R. (1979). Ammonium uptake by symbiotic and aposymbiotic reef corals. Bull. of Mar. Sci. **29**: 572-575.

Muscatine, L., Pool, R. R. and Cernichiari, E. (1972). Some factors influencing selective release of soluble organic material by zooxanthellae from reef corals. Mar. Biol. **13**: 298-308.

Muscatine, L. and Pool, R. R. (1979). Regulation of numbers of intracellular algae. Proc. R. Soc. Lond. B. **204**: 131-139.

Parsons, T. R., Maita, Y. and Lalli, C. M. (1984). A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford. pp. 9-17.

Pierce, S. K., Jr. and Minasian, L. L., Jr. (1974). Water balance of a euryhaline sea anemone, *Diadumene leucolena*. Comp. Biochem. Physiol. **49A**: 159-167.

Porter, J. W. (1974). Zooplankton feeding by the Caribbean reef-building coral *Montastrea annularis*. Proc. 2nd International Coral Reef Symposium, Brisbane, Australia: 111-125.

Porter, J. W. (1976). Autotrophy, heterotrophy, and resource partitioning in Caribbean reef-building corals. American Naturalist **110**: 731-742.

Pound, R. (1893). Symbiosis and Mutualism. American Naturalist **27**: 509-520.

Preston, T. and Owens, N. J. P. (1983). Interfacing and automatic elemental analyser with an isotope ratio mass spectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. Analyst **108**: 971-977.

Preston, T. and Slater, C. (1994). Mass spectrometric analysis of stable-isotope-labelled amino acid tracers. Proc. Nutrition Soc. **53**: 363-372.

Pütter, A. (1911). Der Stoffwechsel der Aktinien. Z. allg. Physiol. **12**: 297-322.

Rahav, O., Dubinsky, Z., Achituv, Y. and Falkowski, P. G. (1989). Ammonium metabolism in the zooxanthellate coral, *Stylophora pistillata*. Proc. R. Soc. Lond. B **236**: 325-337.

Rands, M. L., Loughman, B. C. and Douglas, A. E. (1993). The symbiotic interface in an alga-invertebrate symbiosis. Proc. R. Soc. Lond. B **253**: 161-165.

Rees, T. A. V. (1987). The green hydra symbiosis and ammonium I. The role of the host in ammonium assimilation and its possible regulatory significance. Proc. R. Soc. Lond. B **229**: 299-314.

Rees, T. A. V. (1989a). The green hydra symbiosis and ammonium II. Ammonium assimilation and release by freshly isolated symbionts and cultured algae. Proc. R. Soc. Lond. B **235**: 365-382.

Rees, T. A. V. (1989b). Alga-invertebrate symbioses and nitrogen; recycling or conservation? Endocytobiology **4**: 4-8.

Rees, T. A. V. (1990). The green hydra symbiosis and ammonium III. Characteristics of ammonium release by a high maltose-releasing symbiotic strain of *Chlorella*. Proc. R. Soc. Lond. B **242**: 249-253.

Rees, T. A. V. (1991). Are symbiotic algae nutrient deficient? Proc. R. Soc. Lond. B **243**: 227-233.

Rees, T. A. V and Ellard, F. M. (1989). Nitrogen conservation and the green hydra symbiosis. Proc. R. Soc. Lond. B **236**: 203-212.

Rees, T. A. V., Fitt, W. K. and Yellowlees, D. (1994). Host glutamine synthetase activities in the giant clam-zooxanthellae symbiosis: effects of clam size, elevated ammonia and continuous darkness. Mar. Biol. **118**: 681-685.

Roman, M. R. (1983). Nitrogenous nutrition of marine invertebrates. In: Nitrogen in the marine environment (eds. Carpenter, E. J. and Capone, D. G.) Academic Press, London. pp. 347-384.

Roth, L. E., Jeon, K. and Stacey, G. (1988). Homology in endosymbiotic systems: the term 'symbiosome'. In: Molecular genetics of plant-microbe interactions (ed. Palacios, R. and Verma, D. P. S.) APS Press, St. Paul, Minnesota. pp. 220-225.

Rowan, R. (1996). Ecological correlates of specificity in dinoflagellate symbiosis. Proc. 8th International Coral Reef Symposium, Panama City, Panama. (in press).

Rowan, R. and Powers, D. A. (1991). A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbioses. Science **251**: 1348-1351.

Ryther, J. H. and Dunstan, W. M. (1971). Nitrogen, phosphorus and eutrophication in the coastal marine environment. Science **171**: 1008-1013.

Schlichter, D. (1978). On the ability of *Anemonia sulcata* (Coelenterata: Anthozoa) to absorb charged and neutral amino acids simultaneously. Mar. Biol. **45**: 97-104.

Schlichter, D. (1980). Adaptations of cnidarians for integumentary absorption of dissolved organic matter. Rev. Can. Biol. **39**: 259-282.

Schlichter, D. (1982). Nutritional strategies of cnidarians: the absorption, translocation and utilization of dissolved nutrients by *Heteroxenia fuscescens*. Am. Zool. **22**: 659-669.

Schlichter, D., Bajorat, K. H., Buck, M., Eckes, P., Gutknecht, P. K., Krisch, H. and Schmitz, B. (1987). Epidermal nutrition of sea anemones by absorption of organic compounds dissolved in the oceans. Zool. Beitr. N. F. **30**: 29-47.

Schmitz, K. and Kremer, B. P. (1977). Carbon fixation and analysis of assimilates in a coral-dinoflagellate symbiosis. Mar. Biol. **42**: 305-313.

Searle, P. L. (1984). The Berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. Analyst **109**: 549-568.

Sebens, K. P. (1980). The regulation of asexual reproduction and indeterminate body size in the sea anemone *Anthopleura elegantissima* (Brandt). Biol. Bull. **158**: 370-382.

Shashar, N., Cohen, Y. and Loya, Y. (1993). Extreme diel fluctuations of oxygen in diffusive boundary layers surrounding stony corals. Biol. Bull. **185**: 455-461.

Shick, J. M. (1975). Uptake and utilization of dissolved glycine by *Aurelia aurita* scyphistomae: temperature effects on the uptake process; nutritional role of dissolved amino acids. Biol. Bull. **148**: 117-140.

Shick, J. M. (1976). Ecological physiology and genetics of the colonizing actinian *Haliplanella luciae*. In: Coelenterate Ecology and Behavior (ed. Mackie, G. O.) Plenum, New York. pp. 137-146.

Shick, J. M. (1991). A Functional Biology of Sea Anemones. Chapman & Hall, London. 395pp.

Slater, C., Preston, T., McMillan, D. C., Falconer, J. S. and Fearon, K. C. H. (1995). GC/MS analysis of [$^2H_5$]phenylalanine at very low enrichment: measurement of protein synthesis in health and disease. J. Mass Spectrometry **30**: 1325-1332.

Smith, D. C. and Douglas, A. E. (1987). The Biology of Symbiosis, Contemporary Biology Series, Edward Arnold, London. 302pp.

Smith, E. L., Austen, B. M., Blumenthal, K. M. and Nye, J. F. (1975). Glutamate dehydrogenases. In: The Enzymes (Volume 11) (ed. Boyer, P. D.) Academic Press, New York. pp. 293-367.

Snidvongs, A. and Kinzie, R. A. III (1994). Effects of nitrogen and phosphorous enrichment on *in vivo* symbiotic zooxanthellae of *Pocillopora damicornis*. Mar. Biol. **118**: 705-711.

Solórzano, L. (1969). Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. **14**: 799-801.

Stahl, E (Ed.) (1965). Thin-layer chromatography. A laboratory handbook. Academic Press, New York.

Stambler, N., Popper, N., Dubinsky, Z. and Stimson, J. (1991). Effects of nutrient enrichment and water motion on the coral *Pocillopora damicornis*. Pacific Sci. **45**: 299-307.

Steele, R. D. (1975). Stages in the life history of a symbiotic zooxanthella in pellets extruded by its host *Aiptasia tagetes* (Duch and Mich) (Coelenterata, Anthozoa). Biol. Bull: **149**: 590-600.

Steele, R. D. (1976). Light intensity as a factor in the regulation of the density of symbiotic zooxanthellae in *Aiptasia tagetes* (Coelenterata, Anthozoa). J. Zool. **179**: 387-405.

Steele, R. D. (1977). The significance of zooxanthellae-containing pellets extruded by sea anemones. Bull. Mar. Sci. **27**: 591-594.

Steen, G. R. and Muscatine, L. (1987). Low temperature evokes rapid exocytosis of symbiotic algae by a sea-anemone. Biol. Bull. **172**: 246-263.

Stephens, G. C. (1962). Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. Biol. Bull. **123**: 648-659.

Stewart, G. R., Mann, A. F. and Fenten, P. A. (1980). Enzymes of glutamate formation: glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. In: The Biochemistry of Plants (ed. Miflin, B. J.), vol. 5, Academic Press, London. pp. 271-327.

Stimson, J. and Kinzie, R. A. (1991). Temporal patterns and release rates of zooxanthellae from *Pocillopora damicornis* under nitrogen enriched and control conditions. J. Exp. Mar. Biol. Ecol. **153**: 63-74.

Stryer, L. (1988). Biochemistry, 3rd Edition, W. H. Freeman and Company, New York. 1089pp.

Summons, R. E., Boag, T. S. and Osmond, C. B. (1986). The effect of ammonium on photosynthesis and the pathway of ammonium assimilation in *Gymnodinium microadriaticum in vitro* and in symbiosis with tridacnid clams and corals. Proc. R. Soc. Lond. B **227**: 147-159.

Summons, R. E. and Osmond, C. B. (1981). Nitrogen assimilation in the symbiotic marine alga *Gymnodinium microadriaticum*: direct analysis of ^{15}N incorporation by GC-MS methods. Phytochemistry **20**: 575-578.

Syrett, P. J. (1981). Nitrogen metabolism of microalgae. In: Physiological bases of phytoplankton ecology. (ed. Platt, T) Bulletin 210, Department of Fisheries and Oceans, Ottawa. pp. 182-210.

Szmant, A. M., Ferrer, L. M. and FitzGerald, L. M. (1990). Nitrogen excretion and O:N ratios in reef corals: evidence for conservation of nitrogen. Mar. Biol. **104**: 119-127.

Szmant-Froelich, A. and Pilson, M. E. Q. (1977). Nitrogen excretion by colonies of the temperate coral *Astrangia danae* with and without zooxanthellae. Proc. 3rd International Coral Reef Symposium, Miami. **1**: 417-424.

Taylor, D. L. (1969). On the regulation and maintenance of algal numbers in zooxanthellae-coelenterate symbiosis, with a note on the relationship in *Anemonia sulcata*. J. Mar. Biol. Ass. U.K. **49**: 1057-1065.

Titlyanov, E. A., Titlyanov, T. V., Leletkin, V. A., Tsukahara, J., Vanwoesik, R. and Yamazato, K. (1996). Degradation of zooxanthellae and regulation of their density in hermatypic corals. Mar. Ecol. Prog. Ser. **139**: 167-178.

Trench, R. (1971a). The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates I. The assimilation of photosynthetic products of zooxanthellae by two marine coelenterates. Proc. R. Soc. Lond. B. **177**: 225-235.

Trench, R. K. (1971b). The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates II. Liberation of fixed ^{14}C by zooxanthellae *in vitro*. Proc. R. Soc. Lond. B. 177: 237-250.

Trench, R. K. (1971c). The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates III. The effect of homogenates of host tissues on the excretion of photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates. Proc. R. Soc. Lond. B. **177**: 251-264.

Trench, R. K. (1974). Nutritional potentials in *Zoanthus sociatus*. Helgolander Wissenschaftliche Meeresunters **26**: 174-216.

Trench, R. K. (1987). Dinoflagellates in non-parasitic symbiosis. In The Biology of Dinoflagellates. (ed. Taylor, F. J. R.) Blackwell, Oxford. pp. 530-570.

Trench, R. K. (1993). Microalgal-invertebrate symbioses: A review. Endocytobiosis & Cell Res. **9**: 135-175.

Trench, R. K. and Blank, R. J. (1987). *Symbiodinium microadriaticum* Freudenthal, *S. goreauii* sp. nov., *S. kawaguti* sp. nov. and *S. pilosum* sp. nov.: gymnodinoid dinoflagellate symbionts of marine invertebrates. J. Phycol. **23**: 469-481.

Turpin, D. H. and Harrison, P. J. (1978). Fluctuations in free amino acid pools of *Gymnodinium simplex* (Dinophyceae) in response to ammonia perturbation: evidence for glutamate synthetase pathway. J. Phycol. **14**: 461-464.

Tytler, E. M. (1982). The contribution of zooxanthellae to the energy requirements of the sea anemone, *Anemonia sulcata* (Pennant). PhD thesis, University of Glasgow. pp.105.

Tytler, E. M. and Davies, P. Spencer (1983). A method of isolating clean and viable zooxanthellae by density gradient centrifugation. Limnol. Oceanogr. **28**: 1266-1268.

Tytler, E. M. and Davies, P. Spencer (1986). The budget of photosynthetically derived energy in the *Anemonia sulcata* (Pennant) symbiosis. J. Exp. Mar. Biol. Ecol. **99**: 257-269.

Urich, K. (1990). Comparative Animal Biochemistry. Springer Verlag, Berlin. pp. 403-462.

Wallsgrove, R. M., Harel, H., Lea, E. and Miflin, P. J. (1977). Studies on glutamate synthase from leaves of higher plants. J. Exp. Bot. **28**: 588-596.

Waterlow, J. C., Garlick, P. J. Millward, D. J. (1978). Protein turnover in mammalian tissues and in the whole body. North Holland Publishing Company, Oxford. pp.804.

Webb, K. L. and Weibe, W. J. (1978). The kinetics and possible regulatory significance of nitrate uptake by several algal-invertebrate symbioses. Mar. Biol. **47**: 21-27.

Wilkerson, F. P. and Kremer, P. (1992). DIN, DON and PO_4 flux by a medusa with algal symbionts. Mar. Ecol. Prog. Ser. **90**: 237-250.

Wilkerson, F. P. and Muscatine, L. (1984). Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. Proc. R. Soc. Lond. B **221**: 71-86.

Wilkerson, F. P. and Trench, R. K. (1986). Uptake of dissolved inorganic nitrogen by the symbiotic clam *Tridacna gigas* and the coral *Acropora* sp. Mar. Biol. **93**: 237-246.

Yellowlees, D., Rees, T. A. V. and Fitt, W. K. (1994). Effect of ammonium-supplemented seawater on glutamine synthetase and glutamate dehydrogenase activities in host tissue and zooxanthellae of *Pocillopora damicornis* and on ammonium uptake rates of the zooxanthellae. Pacific Sci. **48**: 291-295.

Zamer, W. E. and Shick, J. M. (1987). Physiological energetics of the intertidal sea anemone *Anthopleura elegantissima* II. Energy balance Mar. Biol. **93**: 481-491.

Zamer, W. E. and Shick, J. M. (1989). Physiological energetics of the intertidal sea anemone *Anthopleura elegantissima* III. Biochemical composition of body tissues, substrate-specific absorption, and carbon and nitrogen budgets. Oecologia **79**: 117-127.