NITROGEN FLUX IN THE SYMBIOTIC SEA ANEMONE *ANEMONIA VIRIDIS* (FORSKAL)

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CONTENTS

ABSTRACT

The aim of this study was to investigate factors affecting the uptake of dissolved inorganic nitrogen (DIN) by the temperate symbiotic sea anemone, *Anemonia viridis*. Laboratory experiments were used to test DIN uptake rates of symbiotic and aposymbiotic (alga-free) individuals, to enable the prediction of the magnitude of DIN flux rates in anemones in their natural environment, and to allow the construction of a model of the DIN fluxes in the association. It was also hoped to assess the potential for fully autotrophic growth in this species.

Uptake rates were measured by following depletion of DIN from small-volume incubation chambers that had been enriched to produce DIN concentrations across the reported range for temperate coastal waters.

At light levels of 190 $\mu E.m^{-2}.s^{-1}$, ammonia was taken up by symbiotic anemones from all concentrations tested, whilst aposymbionts lost ammonia to the surrounding sea water. Nitrate was not taken up by any of the anemones.

The relationship between the weight-specific ammonia flux rate and ambient ammonia concentration was linear for both symbionts and aposymbionts. In all cases the slope of the relationship was positive, symbionts showing increasing rates of uptake and aposymbionts showing decreasing rates of efflux with increasing ammonia concentration. There was no evidence of Michaelis-Menten uptake kinetics across the range of concentrations used, despite extending this to 30 $\mu g\text{-at } NH_3\text{-N}.l^{-1}$ in an attempt to show saturation of uptake.

In darkness, the elevation of the rate/concentration relationship was depressed for symbionts, but not for aposymbionts, so that symbionts showed efflux of ammonia at concentrations below 4 $\mu g\text{-at } NH_3\text{-N}.l^{-1}$. The elevation increased with light intensity up to

50 $\mu E.m^{-2}.s^{-1}$, whilst the slope of the relationship remained constant. Above 50 $\mu E.m^{-2}.s^{-1}$ the elevation could not increase, but the slope became greater.

Feeding the anemones with squid mantle tissue three days prior to experiments had variable effects on ammonia flux rates, but there was a tendency for the elevation of the rate/concentration relationship to drop in recently fed anemones, compared to starved anemones at the same light intensity. Effects are likely to be more marked over the first 24 hours after feeding, and ammonia flux during this period may have significant consequences for the nitrogen balance of the association.

Freshly isolated zooxanthellae took up ammonia, and showed Michaelis-Menten type uptake kinetics, with k_s between 3 and 7 $\mu g\text{-at } NH_3\text{-N}.l^{-1}$, and φ_{max} between 0.025 and 0.043 $\mu g\text{-at } NH_3\text{-N}.(\mu g\ chl_a)^{-1}.h^{-1}$. Uptake rates were not affected by light level, and were an order of magnitude higher than those of the intact association over the range of experimental concentrations used.

Incubation of the intact association with $^{15}NH_3$-enriched sea water resulted in the ^{15}N being incorporated into the zooxanthellae, and not the host tissue, over 30 minutes exposure to the labelled ammonia. This showed directly that the algae are responsible for the ammonia uptake of the association.

The results obtained are consistent with a two-component uptake system, diffusion across the animal cell membrane being driven by a concentration gradient produced by algal ammonia assimilation. The observed kinetics are the result of two antagonistic processes; the catabolic production of ammonia by the animal cell, and the active uptake of ammonia by the algae. It appears that algal ammonia uptake is saturated at irradiances of 50 $\mu E.m^{-2}.s^{-1}$ and below, and unsaturated at higher irradiances.

Light levels and ammonia concentrations were measured in the vicinity of a natural population of *Anemonia viridis* in Loch Sween, Argyll. The laboratory experiments were used to predict the net metabolic gain or loss of DIN experienced by anemones under the measured field conditions. Ambient ammonia concentrations were negligible around the anemones in Loch Sween throughout the year, and under such conditions a net loss of nitrogen would have occurred, making a heterotrophic input of nitrogen necessary for growth. Net nitrogen gain may, however, occur at ambient ammonia concentrations that could exist in rockpools further south in Britain, where *A. viridis* is frequently the dominant animal species.

A laboratory experiment was conducted to test for the possibility of autotrophic growth in the presence of adequate levels of DIN. Unfortunately, experimental conditions did not consistently provide high enough concentrations of ammonia for net nitrogen gain to be predicted, but anemones exposed to elevated levels of DIN for 8 weeks lost significantly less weight than anemones kept in sea water with negligible ammonia concentrations. The weight loss of the DIN-"fed" anemones corresponded closely to the weight of lost nitrogen predicted from the flux experiments, after conversion to equivalent weight of protein.

It remains to be seen if *Anemonia viridis* can survive and grow autotrophically, but even in the nitrogen-poor environment of a Scottish sea loch, the algae can potentially supply 86-90% of the associations basal nitrogen requirements. The capacity for DIN uptake is clearly an important factor in the success of this species in temperate waters.

CHAPTER 1: INTRODUCTION

1.1 Algal-invertebrate associations

Intimate physiological associations between invertebrates and unicellular algae are widespread and well-documented in the marine environment. Although the algal partners include representatives from at least three algal classes, and host species are found in five or more of the major invertebrate phyla (Droop, 1963; Taylor, 1973; 1974), the association that has provoked the largest proportion of investigations is that between dinoflagellate algae and sessile coelenterates, as typified in the reef-building scleractinian corals.

The coelenterate-dinoflagellate association is commonly found in other groups of tropical anthozoans, and less commonly in anemones, zoanthids and scleractinians from temperate and boreal waters. The association is similarly not restricted to shallow, well-lit water, although it is at its most abundant in such environments, and coelenterate polyps with dense concentrations of dinoflagellate algae have been collected from depths of 180-210 m in the Atlantic (Zahl and McLaughlin, 1959).

The algae, which are located in vacuoles in the cells of the hosts' gastrodermis, were originally called zooxanthellae by Brandt (1881). Until relatively recently it was assumed that zooxanthellae from all hosts were of the same species (Taylor, 1967), described as *Symbiodinium microadriaticum* by Freudenthal (1962), and renamed *Gymnodinium microadriaticum* in benthic hosts by Taylor (1971). Taylor (1968) had previously named zooxanthellae from the anemone *Anemonia sulcata* "aff. *Symbiodinium microadriaticum*", indicating an affinity with the zooxanthellae described by Freudenthal. The latter, which had been isolated from two scyphozoan species, had not been examined with

an electron microscope, unlike those of Taylor, and so Kevin *et al.* (1969) amended the type description of *S. microadriaticum* Freudenthal after E.M. examination. They concluded that the zooxanthellae described by Freudenthal and Taylor were one and the same species, leading to the current general use of *Symbiodinium* as the generic name. There is now a growing literature that suggests that zooxanthellae in anthozoans belong to different strains (Schoenberg and Trench, 1980a; 1980b; Kinzie and Chee, 1982; Chang *et al.*, 1983) or species (Blank and Trench, 1985; Blank, 1987). The differences between strains, or species, may manifest themselves in morphological, biochemical or physiological variation (Blank and Trench, 1985), and so the results of similar physiological experiments using different symbioses may be expected to give some differences in results.

1.2 Ecological and physiological bases for the association

The coelenterate-algal association is generally recognized as a mutualistic symbiosis (Muscatine and Porter, 1977; Trench, 1980), defined by Trench (1979) as "a system of cells and tissues composed of two genetically distinct cell types which proliferate harmoniously, resulting in a stable unit". This implies that the metabolisms of the symbiotic partners must complement each other, to the mutual benefit of both, i.e. that the biological fitness of each partner whilst in association must be greater than the fitness of each in isolation from the other (Law and Lewis, 1983).

There are a number of ways in which the activities of each partner can operate to benefit the other, and these are chiefly due to the ability of the association for polytrophy, based upon the algal cells' capacity for autotrophic nutrition, and the hosts' capacity for

heterotrophic nutrition. Thus the advantages are largely quantifiable in terms of growth, following Muscatine's (1973) definition of nutrition as "the acquisition and processing of essential organic and inorganic bodily constituents that are utilized as energy sources and nutrients in promoting growth and maintainance". The autotrophic partner is able to grow and maintain itself completely by the utilization of inorganic nutrients, whilst the heterotrophic host can utilize nutrients in organic form.

The specific advantages of the symbiosis to either or both of the partners may be divided into three categories: provision of oxygen, enhancement of calcification (in those symbioses with calcium carbonate skeletons), and the improved acquisition of nutrients. These are briefly reviewed below.

Oxygen provision, from algal photosynthesis, was the first of these advantages to be noted by biologists investigating the symbiosis (Geddes, 1882; Pütter, 1911). More recent research has suggested that O_2 evolution by the zooxanthellae is of benefit to the host under conditions of environmental hypoxia. This has been shown to be the case in small rockpools at low tide, where algal-derived O_2 prevents the host from incurring an oxygen debt (Shick and Brown, 1977), and in dense clonal colonies, where symbiotic anemones appear to be able to crowd more closely than aposymbiotic (algal-free) conspecifics, and hence utilize space-limited habitats more efficiently (Fredericks, 1976). Except in such situations, which may be relatively uncommon in nature, it seems unlikely that O_2 evolution should be of major benefit to the host, because of the ease with which gas exchange can occur in an organism consisting of only two cell layers. It should be noted, moreover, that the potentially advantageous photosynthetically-produced oxygen may also be a disadvantage to the host because of the toxic effects of oxygen (Fridovich, 1977). To counteract this

toxicity, several species of symbiotic coelenterate have relatively high activities of the enzymes superoxide dismutase and catalase, which remove superoxide radicals and hydrogen peroxide respectively (Dykens and Shick, 1984; Shick and Dykens, 1985), in addition to behavioural strategies that prevent over-exposure to sunlight (Shick and Dykens, 1984; Dykens and Shick, 1984).

Calcification rates of zooxanthella-bearing hermatypic (reef-building) corals are known to be higher in light than in dark (Goreau and Goreau, 1959; Pearse and Muscatine, 1971), and considerably higher than those of aposymbiotic corals (Goreau, 1959; Goreau and Goreau, 1959). The increased deposition rate of calcium carbonate has been ascribed to various mechanisms. These include the favourable alteration of the equilibrium between calcium ions and calcium carbonate by the photosynthetic removal of CO_2 by the algae (Goreau, 1963), the effective scavenging of inorganic phosphate (a potential crystal poison for $CaCO_3$) by the zooxanthellae (Simkiss, 1964), or the provision of metabolites, either for the organic matrix (Wainwright, 1963) or as energy for calcification (Goreau *et al.*, 1979). High calcification rates in corals, allowing rapid increase in surface area for light or zooplankton capture, and preventing overgrowth by other reef organisms, are a major reason for the dominant position corals have in the ecology of tropical reefs.

Rapid calcification rates and oxygen provision to the host during environmental hypoxia may not benefit the zooxanthellae directly, although increased surface area for light capture will be one result of skeletal growth. The most directly mutualistic benefits of the association lie in the enhanced provision of nutrients for organic growth of both partners. Such mutualistic interdependance was originally not thought to exist in symbiotic coelenterates. Hermatypic

reef corals were considered to be effective heterotrophs (Yonge, 1930a; 1930b; Yonge and Nicholls, 1930; 1931b), and even relatively recently were described as "superbly efficient and voracious carnivores" (Goreau *et al.*, 1971). Yonge and Nicholls (1931b) showed experimentally that, in the absence of light, corals survived well if fed with zooplankton, whilst corals starved of zooplankton in the light died, and so concluded that the zooxanthellae were only commensal. Previous work had gone further and suggested that zooxanthellae were of direct nutritional benefit to the host because they were digested (Boschma, 1925), and some further evidence has been produced that agrees with this (e.g. Steele and Goreau, 1977; Szmant-Froelich and Pilson, 1980; Janssen and Möller, 1981). Other workers, however, have maintained that the evidence is more likely to indicate removal of degenerating algal cells than a systematic "farming" of the healthy zooxanthellae (Taylor, 1969b), and that the current consensus is that host nutrition by digestion of zooxanthellae remains speculative (e.g. Muscatine, 1973).

The concept of the association as a mutualistic symbiosis relies entirely upon the exchange of metabolically-useful compounds between the partners. In order for the host to benefit nutritionally from the algae, it has to receive the products of photosynthesis that are surplus to the algal cells' requirements for growth and maintainance. Yonge *et al.* (1932) showed that photosynthetic oxygen production could exceed the respiratory demands of the entire association. The significance of this was not appreciated at the time, because of insufficient understanding of the relationship between photosynthetic carbon production and oxygen evolution. Muscatine and Hand (1958) succeeded in showing that ^{14}C from labelled carbon dioxide in the incubation water appeared in the animal tissues of the symbiotic anemone *Anthopleura elegantissima*, proving that photosynthetically

fixed carbon was utilized by the host. Later studies were therefore able to show that the work of Yonge *et al.* (1932) indicated that more photosynthetically fixed carbon was being produced than was being consumed in respiration (Roffman, 1968).

The repertoire of carbon compounds that are translocated from the algae has been investigated in isolated zooxanthellae, and it was found to be varied, including, amongst other compounds, glycerol, alanine, glucose, fumaric acid, succinic acid, glycolic acid, acetate, lactate, succinate and citrate (Muscatine, 1967; von Holt and von Holt, 1968; Trench, 1971b). It was also found that rates of release of these compounds could be stimulated by incubating the isolated algae with host tissue homogenate (Muscatine, 1967; Taylor, 1969a; Trench, 1971c; Muscatine *et al.*, 1972), and that similar compounds were released by the algae *in vivo* (Trench, 1971a; Lewis and Smith, 1971). More recently some interest has been focussed on the back-translocation of organic compounds, from host to algae, in order to evaluate benefits derived by the algae, rather than the host, in the symbiosis. Isolated zooxanthellae have been shown to be capable of taking up taurine, cysteine, methionine and alanine (Deane and O'Brien, 1981; Carroll and Blanquet, 1984a; 1984b), and can grow at irradiances below compensation when cultured with glycerol, glycolate, acetate, malate or propionate (Steen, 1987). Whilst these *in vitro* experiments have demonstrated the algal cells' capacity for utilization of organic compounds from the host, other experiments have shown that isotopically-labelled carbon (Taylor, 1984) and sulphur (Cook, 1971; Steen, 1986) from organic compounds fed to the host appear in the algae, suggesting the *in vivo* utilization of host-derived CO_2 and amino acids by the algal symbionts.

Algal-coelenterate symbioses were therefore shown to be

potentially self-sufficient, at least in terms of fixed carbon. Increasing numbers of studies showed that, contrary to the assertion of Yonge and Nicholls (1931b), corals could survive by the photosynthetic products of the algae. Franzisket (1969; 1970), in experiments similar to those of Yonge and Nicholls, found that hermatypic corals kept in the light, in zooplankton-free sea water, grew as fast as those in unfiltered sea water, and that corals kept in darkness died or atrophied, despite being supplied with unfiltered sea water. This showed that, not only could the corals survive on the photosynthetically-produced compounds of the zooxanthellae, but that they could not survive solely on the heterotrophic input available to them. Other studies have estimated that the waters surrounding some corals do not contain sufficient quantities of zooplankton for the corals to survive on (Johannes *et al.*, 1970; Porter, 1974), implying that those corals must rely predominantly upon zooxanthellar photosynthesis. The available zooplankton was, however, considered a potentially important part of the nutritional requirements of the corals, through the provision of scarce nutrients (Johannes *et al.*, 1970).

Having shown that the host may survive on the translocated products of the algae, much work has concentrated upon quantifying the relative importance of the provision of nutrients from algal autotrophy and host heterotrophy. This has generally taken the form of determinations of the contribution of the zooxanthellae to the respiration of the host (e.g. Muscatine and Porter, 1977; Muscatine *et al.*, 1981) or the respiration and growth of the entire association (e.g. Tytler, 1982; Davies, 1984; Tytler and Davies, 1986). The latter type of study has commonly shown that photosynthetic carbon production may, under certain conditions, exceed the growth and respiratory demands of the entire symbiosis (Davies, 1984; Muscatine *et al.*, 1984;

Tytler and Davies, 1986; Edmunds and Davies, 1986). This excess carbon is assumed to be lost from the association, possibly as mucus (Davies, 1984), or as low molecular weight compounds (Edmunds, 1986), and overproduction on "ideal" days has been proposed as a means of surviving "less-than-ideal" days (Edmunds and Davies, 1986).

One possible reason for the over-production of fixed carbon might be the requirement for a particular minimum algal photosynthetic activity in order to supply nutrients other than carbon to the host. This is based upon the idea that, since free-living algae can utilize dissolved inorganic nutrients for growth, zooxanthellae might also be expected to be able to do the same thing. Algal growth rates in the association are limited by the host, and so much of the algal production may not be required for the maintainance and growth of the zooxanthellae. The host, therefore, could induce the release of compounds containing environmentally-scarce nutrients from the algae, and hence benefit from autotrophic sources of the nutrients. In this system, carbon would be available in excess and some other nutrient would be limiting. Nitrogen is a strong contender for this postion, since it is frequently the limiting nutrient in marine ecosystems (Ryther and Dunstan, 1971), and is potentially made available to the symbiosis in significant amounts from the activities of the endosymbiotic algae.

Nitrogen availability to the algae could be from two sources: free dissolved inorganic nitrogen in the surrounding sea water, or intracellular nitrogen as an end-product of the animal cells' nitrogen excretion pathways. The former source would potentially include ammonia, nitrate and nitrite, since these are relatively common inorganic nitrogen compounds in sea water (Sharp, 1983), and the latter would consist of metabolically-produced ammonia, the major

nitrogen excretory product in coelenterates (Pütter, 1911; Muscatine, 1980).

The uptake of excretory nitrogen from the host by symbiotic algae was suggested very early on in the study of the symbiosis, Geddes noting in 1882 that the algae may perform some "intracellular renal function". In confirmation of this, decreased rates of ammonia excretion have been found in symbiotic species of anemone, coral and scyphozoan, when compared with related aposymbiotic species (e.g. Pütter, 1911; Kawaguti, 1953; Cates and McLaughlin, 1976; Muscatine *et al.*, 1979).

Uptake of dissolved inorganic nitrogen (DIN), in the form of ammonia, from the surrounding water by symbiotic individuals has been reported for corals (e.g. Kawaguti, 1953; Szmant-Froelich and Pilson, 1977; Muscatine and D'Elia, 1978; Propp, 1981; Burris, 1983; Wilkerson and Trench, 1986), anemones (Pütter, 1911; Wilkerson and Muscatine, 1984), scyphozoans (Muscatine and Marian, 1982), and even cultured vertebrate cells infected with coral zooxanthellae (Taylor, 1978b). If this phenomenon is considered a net uptake by the symbiosis, then Geddes' "renal function" for the algae must represent a conservation or retention of nitrogen by the symbiosis. The uptake and retention of ammonia has been directly attributed to the algae, because it only occurs when they are present, because it is generally considered a property of plant, but not animal cells (although some aquatic invertebrates are capable of ammonia uptake under certain environmental conditions (Taylor *et al.*, 1987)), and also because the uptake or conservation has been shown to be affected by light intensity (Kawaguti, 1953; Cates and McLaughlin, 1976; Szmant-Froelich and Pilson, 1977; Muscatine *et al.*, 1984). Several studies have demonstrated the capacity of isolated zooxanthellae for ammonia uptake (e.g. Muscatine *et al.*, 1979; Muscatine and Marian, 1982; D'Elia *et*

al., 1983; Domotor and D'Elia, 1984), although the relevance of this to *in situ* algal DIN uptake has been disputed (Rees, 1987). It has been suggested, however, that the ammonia uptake may in fact be due to the animal tissues, although this has been based upon work with a different symbiosis, that of hydra and zoochlorellae (Rees, 1987). Whilst this freshwater association may have similarities with the anthozoan-dinoflagellate symbioses discussed here, there are significant physiological differences, such as the pH control of maltose release (the major translocatory product in the green hydra symbiosis (Muscatine, 1965)) from the zoochlorellae, which does not occur in zooxanthellae.

Nitrate has also been shown to be taken up by symbiotic coelenterate species (Franzisket, 1973; 1974; Webb and Wiebe, 1978; Cates and McLaughlin, 1979; Propp, 1981), although the phenomenon seems less universal than ammonia uptake, some species being able to take up ammonia but not nitrate (Muscatine and Marian, 1982; Wilkerson and Muscatine, 1984; Muscatine *et al.*, 1984). When ammonia and nitrate are both made available to algal cells, nitrate uptake is generally suppressed (Syrett, 1981), but some corals may be able to take up both ammonia and nitrate simultaneously (Anderson and Burris, 1987).

Although inorganic nitrogen has been the subject of most studies on nutrient supply to the symbiosis, there is also evidence to show that inorganic phos phorus may also be provided by the algae in much the same way as nitrogen (D'Elia, 1977; Cates and McLaughlin, 1979).

As was the case with carbon (see above), if the host is to benefit from DIN uptake by the algae, translocation of nitrogen-containing compounds is necessary. Glycerol was believed to be the principal compound translocated from zooxanthellae (see review of Cook, 1983), whilst more recent work attaches more importance to lipid

release (Battey and Patton, 1984). Muscatine *et al.* (1972), however, showed that alanine was the most abundant product released by zooxanthellae from the coral *Agaricia agaricites*, whilst Lewis and Smith (1971) found that increased ammonia availability resulted in greater release of alanine from the zooxanthellae of *Porites divaricata*. The recapture of waste nitrogen from the host by the algae, followed by recycling back to the host (and, ultimately, the host's excretory metabolism) of the nitrogen in useful organic compounds is therefore possibly one of the best demonstrations of the close metabolic integration that forms the basis of the mutualistic symbiosis.

1.3 Objectives of the study

The evidence for significant benefits to the nitrogen requirements of the symbiotic association is somewhat fragmentary, and much of it is contradictary. The first aim of this study was therefore, using the temperate symbiotic anemone *Anemonia viridis*, to investigate a number of factors that could affect the rates of supply of dissolved inorganic nitrogen (DIN) to the symbiosis. The effects of ambient DIN concentration and light intensity on DIN uptake and retention were considered, because the studies that have investigated them previously have produced conflicting results. Very few studies have looked at the effects of feeding of the host on DIN uptake or retention, despite the intuitive observation that protein fed to the host ought to result in some change in availability of excretory ammonia to the retention mechanism of the association. It was therefore intended that uptake rates of fed and starved anemones should be compared, to evaluate the effects of feeding.

Throughout the study symbiotic and aposymbiotic *A. viridis* were

compared, so that the effects of the presence of zooxanthellae on DIN flux could be quantitatively expressed. Such comparisons, based upon the knowledge of the flux of ammonia in anemone tissue alone, under a range of environmental conditions, allowed the construction of a model of DIN flux in the intact association. Although suggestions have been made for the mechanism of nitrogen uptake in this type of symbiosis, they have not generally been based upon the evidence from a single symbiotic species, or taken into consideration the dynamics of nitrogen flux in aposymbiotic individuals. Experiments investigating DIN uptake by the zooxanthellae, both freshly isolated and *in situ*, were used to confirm some of the assumptions made in this, and previous models.

Although methods for carbon budgeting have advanced in recent years, virtually no attempt has been made to adapt the approach for nitrogen. Szmant-Froelich and Pilson (1984) showed that 54% of the daily basal nitrogen requirements of the temperate coral *Astrangia danae* could be provided by the zooxanthellae, but this figure was the mean value for starved and fed corals, and was calculated for an unspecified ambient DIN concentration (>0 μg-at NH_3-N.l^{-1}) under a single set of laboratory light conditions. Since light and exogenous DIN availability are both likely to affect DIN uptake rates, there is considerable room for a more realistic evaluation of nitrogen balance for the symbioses in the field. The second aim of the study was therefore to make some quantitative predictions of the benefit of DIN uptake and retention to symbiotic *A. viridis* under field conditions of irradiance and DIN concentration. These were based upon the model derived for nitrogen flux rates, applied to DIN concentrations and light intensities measured for a population of *A. viridis* on the West coast of Scotland.

Finally, it was hoped to test the hypothesis that the *Anemonia viridis* symbiosis could be fully autotrophic under appropriate conditions of light and nitrogen availability.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

A number of techniques were used throughout this study, or were common to several of the sections described subsequently. To avoid repetition or excessive cross-referencing these techniques are described in full in this chapter.

2.2 The experimental stocks

2.2.1 Maintainance of symbiotic *Anemonia viridis*

The *Anemonia viridis* used in this study were obtained from subtidal populations in Loch Sween, Argyll, by divers either snorkelling or using SCUBA. Initially, specimens were collected from Bagh na Doide (see Chapter 3), but as that population proved unreliable as a source of large individuals, the majority of anemones were obtained from the Pinnacle site (see Chapter 3). *A. viridis* from the latter site were all of the brown colour morph, and may have been from a single clone. The anemones were transferred to tanks in the recirculating sea water system at the Department of Zoology, Glasgow University, where they were maintained at a temperature of 10 $\pm$ 1^{o}C.

The anemones were divided into 3 stocks; fed symbionts, starved symbionts, and aposymbiotic animals (see section 2.2.2).

"Fed" anemones were fed weekly on squid mantle tissue, to emulate the feeding protocol of Tytler (1982). "Starved" individuals were given no solid food, although no attempt was made to filter the circulation water in which they were kept since the water appeared to have little or no suspended material in it.

Starved and fed stocks of symbiotic *A. viridis* were kept under the same lighting regime as Tytler's (1982) High Light Stock, using four 65-80 W, 1500 mm "Northlight" (Thorn) fluorescent strips, giving an irradience of 140 $\mu E.m^{-2}.sec^{-1}$. The lights were connected to a time switch, giving a 12 hour light/12 hour dark cycle.

2.2.2 Production and maintenance of aposymbiotic *Anemonia viridis*

Anemonia viridis does not naturally occur without endosymbiotic zooxanthellae (Taylor, 1969b). Thus, in order to conduct experiments on the animal host alone, aposymbiotic (algal-free) anemones must be created artificially. This has been done for a number of symbiotic coelenterate species, and methods have been based on the host's control of its algal population, in which non-photosynthesizing algal cells tend to be expelled from the association (Taylor, 1969b; Pardy, 1976). Various methods have been used to induce such expulsion, including exposure of the association to heat (Pearse, 1974a), cold (Steen, 1986; Steen and Muscatine, 1987), intense light (Pardy, 1976) and prolonged darkness (Smith, 1939).

Initially the proceedure of Tytler (1982) was followed, incubating symbiotic anemones in static sea water tanks in the presence of $5x10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Penicillin and streptomycin were added to counteract bacterial action in the static tank, and the anemones were left for a period of five weeks under normal illumination, during which time they were starved. This method produced high mortalities, and surviving anemones looked unwell and would not feed normally. Douglas and Smith (1983) reported similar effects when treating *Hydra* with DCMU, and further showed that aposymbiotic *Hydra* incubated with DCMU had reduced growth rates

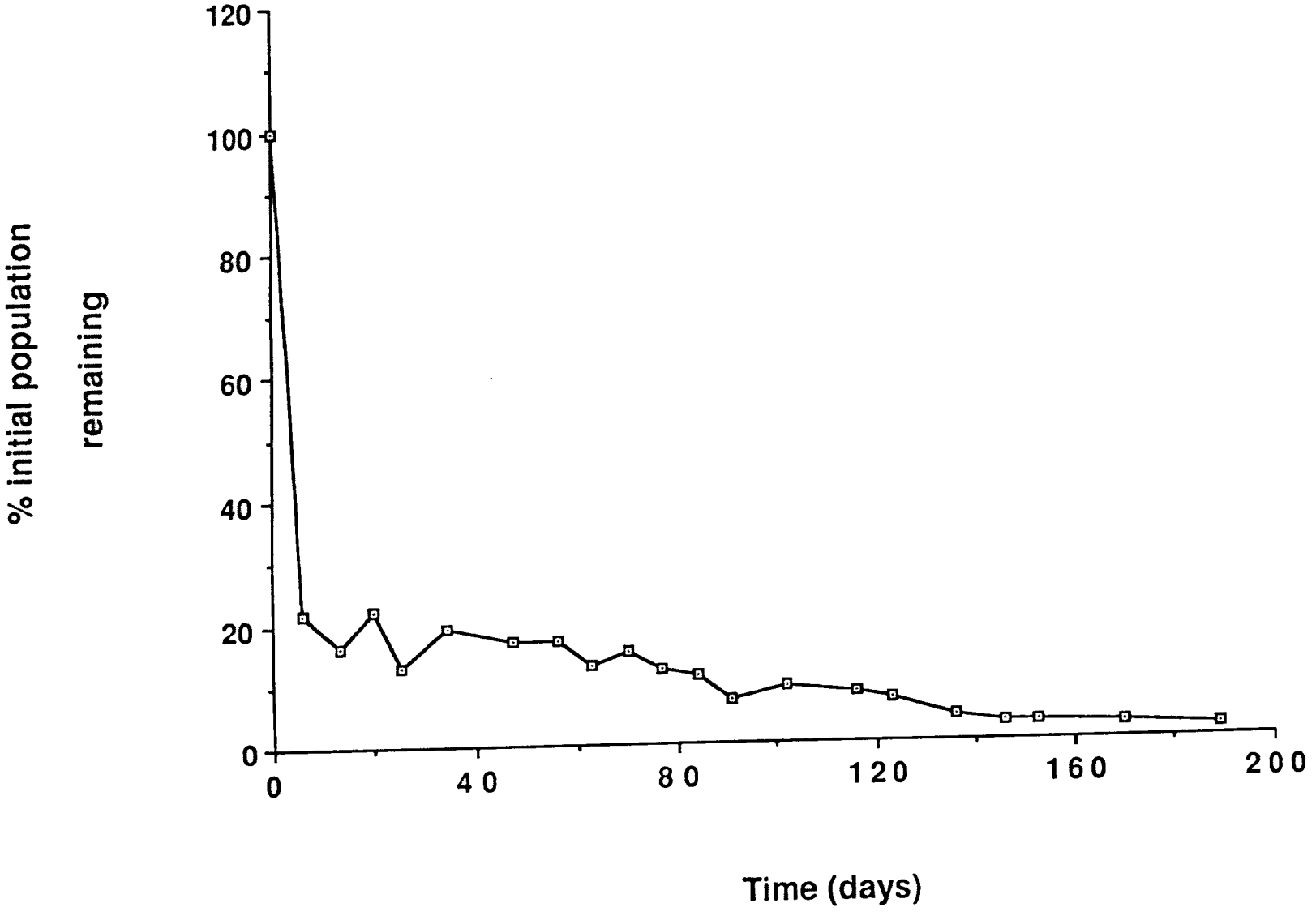
compared with incubations containing no DCMU (although Vandermeulen *et al.* (1972) found no harmful effects of DCMU when used on a variety of invertebrates in symbiosis with zooxanthellae).

Because of the problems associated with chemical interference with photosynthesis, other methods were investigated. The cold stripping technique of Steen (1986) proved unsuccessful, even after reducing the temperature to 0.5^{o}C for several hours. Attempts using Pearse's (1974a) methods were also unsuccessful, but this may have been due to the narrow band of effective times and temperatures reported by Pearse for *Anthopleura elegantissima* being suboptimal for *Anemonia viridis*.

The final, and most successful method tried was that of Smith (1939), in which symbiotic *A. viridis* were kept in total darkness and starved. Single tentacles were excised from three anemones at approximately weekly intervals, for homogenization and counting of zooxanthellae. After six days zooxanthellae numbers had dropped to 21% of the initial value, the rate of zooxanthella loss slowing after this, to give 4% of the initial population on day 136 (Figure 2.1). Numbers continued to decline slowly, but even after 190 days the anemones were still not totally devoid of zooxanthellae. For experimental purposes anemones were considered to be effectively aposymbiotic from day 136 onwards, since less than 5% of the original population of zooxanthellae remained. The anemones appeared to show no ill effects, feeding voraciously when offered squid mantle tissue. All aposymbiotic anemones obtained in this way were maintained in total darkness in the circulating sea water system, and were fed once weekly unless starved individuals were required, when food was witheld for a minimum of three weeks prior to the experimental use of the animals.

Figure 2.1 *Anemonia viridis*: decline in numbers of zooxanthellae in anemones maintained in darkness. Relative algal population size was estimated from counts of zooxanthellae from three tentacles excised from one of the 20 anemones maintained in darkness, and expressed as a percentage of the number of zooxanthellae in three tentacles from an anemone not kept in darkness. Anemones were considered effectively aposymbiotic (alga-free) when less than 5% of the original zooxanthella population was estimated to remain, i.e. from day 136 onwards.

Figure 2.1


% initial population remaining
120
100
80
60
40
20
0
0
40
80
120
160
200
Time (days)

2.3 The isolation and purification of zooxanthellae

In order to observe the flux of dissolved inorganic nitrogen in zooxanthellae, either *in situ* or in isolation from the host, it was necessary to have samples that were free from host contamination. Standard methods of zooxanthellae extraction (e.g. Muscatine, 1967) are not adequate when applied to *A. viridis*, as the large amounts of mucus produced by this species cause clumping of zooxanthellae and animal debris, with subsequent contamination of the algal fraction, particularly with nematocysts. To counter this, a modification of the ultracentrifugation method of Tytler and Davies (1983) was adapted for use in some experiments, whilst an extended and modified method based on that of Muscatine (1967) was used to provide zooxanthellae for ammonia flux experiments, when the buffers used in the ultracentrifugation method caused interference with assays (see below, and section 2.5.1.2).

2.3.1 Zooxanthella isolation by isopycnic density gradient centrifugation

The method of Tytler and Davies (1983) produced a cleaner zooxanthella fraction than any other isolation procedure investigated. Douglas (1987) found that, after isolation of *Chlorella* from green hydra by this method, 90% of the protein in the zoochlorella preparation was algal. This compared with nearly 50% of protein being of host origin after isolation with multiple washing.

The zoxanthella preparations produced by this method were found to be unsuitable for ammonia flux measurements because of contamination with the Tris buffer used in the salines, which interferes with the colour development of the phenol-hypochlorite

reaction used to assay ammonia (see section 2.5.1.2). The method was suitable, however, for the isolation of zooxanthellae from anemones exposed to ^{15}N-labelled ammonia, during the tracer study (section 6.2). Because the zooxanthellae separated were to be freeze-dried immediately it was not necessary to buffer the salines to exact pH, and so, in order to minimize the amount of non-zooxanthellar nitrogen in the samples, the tris buffers and EDTA of Tytler and Davies' (1983) TBSAS and TBSPS salines were omitted, and final washes were done with filtered sea water.

2.3.2 Zooxanthella isolation by repeated washing

Normal methods for isolating zooxanthellae consist of homogenization in sea water followed by filtering through a mesh to remove large debris, then repeated centrifugation at a single speed and resuspension in filtered sea water (e.g. Muscatine, 1967; D'Elia *et al.*, 1983). When these methods are applied to *A. viridis*, the zooxanthella pellet is invariably heavily contaminated with nematocysts and other animal debris, visible as a white layer on top of the pellet.

This problem was circumvented by first spinning at high speed, to produce a pellet of algal and animal material, with a supernatent containing most of the mucus, discarding the supernatent and resuspending and centrifuging the pellet material at a lower speed. This avoids the problem of algal clumping, and, in practice, if the proceedure is repeated at successively lower speeds (maintaining the same duration) a cleaner and cleaner zooxanthella pellet is obtained. It was found that the best protocol was initially to centrifuge the crude homogenate for 10 minutes at 1,300 x g, to repeat the procedure

using that speed until a clean supernatent was obtained, then to continue by successively centrifuging at 780, 270 and 95 x g for the same duration, repeating each speed until a clean supernatent resulted. After a total of seven or eight spins the final pellet had no visible white layer of animal material on its surface, and under microscopic investigation proved to contain only zooxanthellae with a few nematocysts. The method worked best when only tentacles were homogenized, because of the problems caused by the large amount of tough muscular tissue and excessive mucus production of the column.

This method, as with the density gradient separation, did not give a 100% yield of zooxanthellae, some being lost in discarded supernatents.It did, however, give samples of viable zooxanthellae with relatively little animal contamination, which could be used for nitrogen flux experiments.

2.4 Biomass determinations

2.4.1 Buoyant weighing

The reference unit of biomass used in this study was organic weight (ash-free dry weight), to allow direct comparison with the results of Tytler (1982) and Tytler and Davies (1984; 1986).

Since the direct determination of organic weight requires the drying and ashing of an anemone, it was necessary to use a non-destructive method of animal weighing that could be related to organic weight. To this end, wet weighing is unsatisfactory, because of the variable amount of water retained in the coelenteron even after blotting individuals on paper towels, and so weight in water, or buoyant weight, was the value taken for animals used in experiments.

The weight of an anemone in water of a given density should be

due to the density of the organic matter in the anemone and the density of the water and salts in the cells and in the coelenteron. If the intracellular and coelenteronic water and salts have the same density as the surrounding water, the buoyant weight will be due to the organic matter alone, and a direct relationship between organic and buoyant weights can be expected.

Buoyant weights were obtained in artificial sea water of known density. 45.2 g of Tropic Marin aquarium artificial sea salt was dissolved in, and made up to 1 litre with distilled water, to give a final density of 1.022 at 10°C (calculated from the weights in air and artificial sea water of a glass slide of known specific gravity). Artificial sea water was used in preference to circulation sea water because of the fluctuating density of the latter. Such fluctuations would have caused problems because the relationship between organic and buoyant weights is dependent upon the specific gravity of the water used for the buoyant weighing. The anemones were suspended in the water from a perspex trapeze set across the weighing pan of a Mettler H15 balance, by means of a length of very fine wire terminating in a stainless steel frog heart clip. The clip allowed the anemone to be held without damage by the edge of the basal disc, whilst the wire was fine enough to be held taut by the weight of the anemone, minimizing variable surface tension effects by ensuring that the wire was always perpendicular to the water surface. The balance was supported on a platform above the beaker of sea water, and the apparatus was shrouded in polythene sheeting to prevent perturbation of the trapeze by draughts. In this way, it was possible to weigh anemones to the nearest 0.1 mg. All anemones were kept overnight in artificial sea water of identical density to the sea water in which they were weighed, to ensure that water in the coelenteron was also of

the same density. All weighings and overnight incubations were carried out at 10°C to prevent any variation in the specific gravity of the sea water due to temperature fluctuation.

In order to derive the relationship between organic and buoyant weights, fifteen starved and fifteen fed symbiotic *Anemonia viridis* (see section 2.2.1) were buoyant weighed as described above, and then sacrificed to obtain their organic weight values. After buoyant weighing, anemones were placed on preweighed aluminium trays and dried to constant weight at 60°C, weights being recorded to the nearest 0.1 mg. The anemones were then ashed in a muffle furnace at 500°C for 6 hours, allowed to cool in a desiccator, and weighed again, the ash weight being subtracted from the dry weight to give the organic, or ash-free dry weight. Figure 2.2 shows the relationship between buoyant and organic weights for both groups of anemones. Both fed and starved symbiotic *A. viridis* showed significant correlation coefficients ($r=0.994$ and 0.976 respectively, $P<0.001$ for both, with 13 d.f.). Covariance analysis indicated that there was no significant difference in the slopes and elevations of the two regression lines ($F_{slopes}=$ 0.644 with 1 and 26 d.f., $P>0.25$, N.S.; $F_{elevations}=$ 0.138 with 1 and 27 d.f., $P>0.50$, N.S.).

A potential criticism of the buoyant weighing technique is that diurnal variations in the chemical composition of the anemones, due to storage products being laid down or utilized, might lead to fluctuating densities and hence unreliable buoyant weights. The most extreme example of this should be seen when comparing regularly-fed animals with those that have been starved for months, and since this does not produce a significantly changed relationship, it is assumed that smaller-scale daily changes in chemical composition are not likely to influence buoyant weight values.

Figure 2.2 *Anemonia viridis*: the relationship between buoyant and ash-free dry (organic) weights for starved and fed symbiotic anemones. The buoyant weights were obtained as given in the text, in artificial seawater of specific gravity 1.022 at a temperature of 10°C. The equations of the lines are:

Fed

$$w_d = 4.826w_b + 0.0657$$

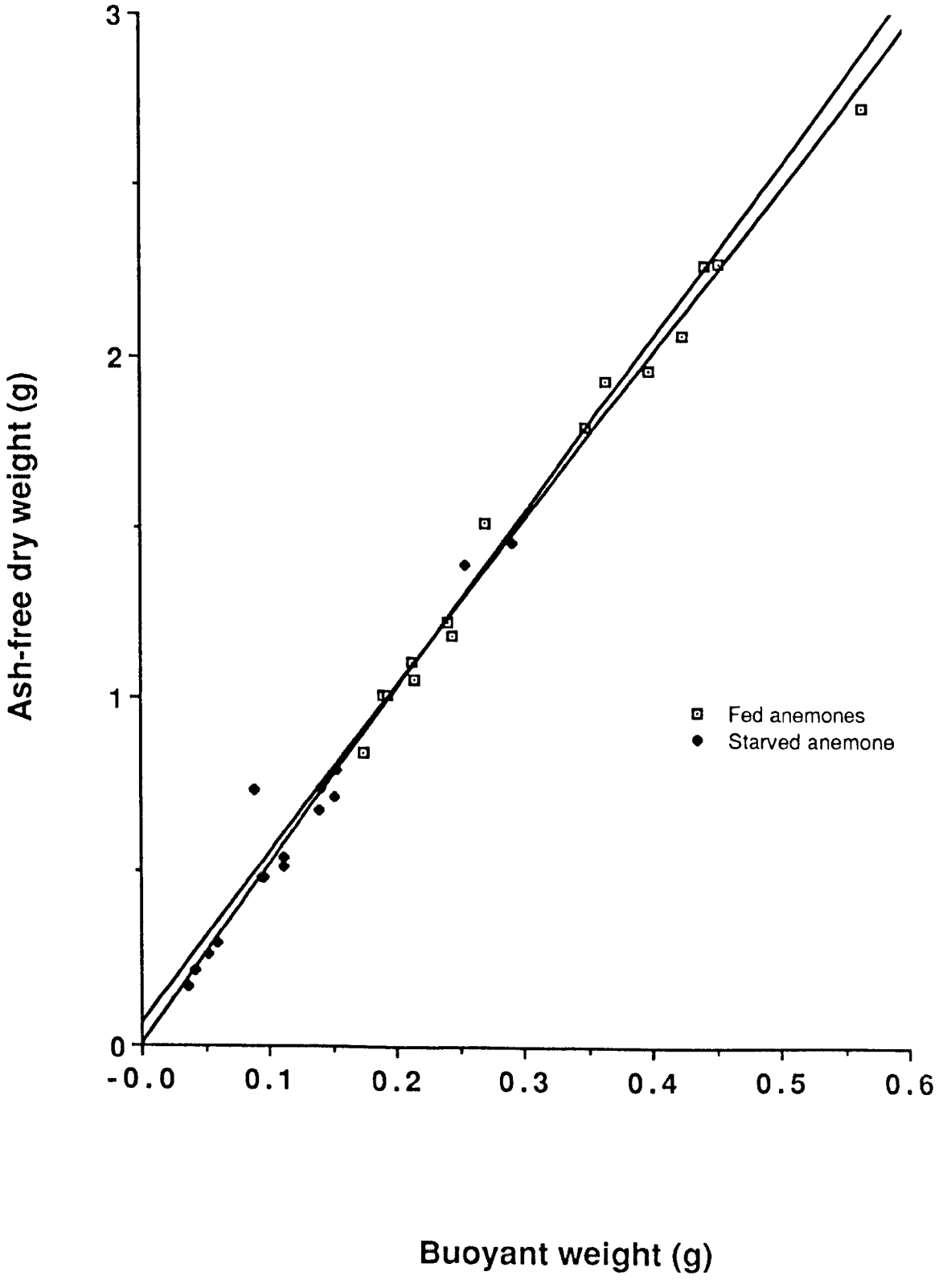
Starved

$$w_d = 5.087w_b + 0.0056$$

Where w_d = ash-free dry weight in grams

w_b = buoyant weight in grams

Figure 2.2


Ash-free dry weight (g)
3
2
1
0
-0.0
0.1
0.2
0.3
0.4
0.5
0.6
Fed anemones
Starved anemone
Buoyant weight (g)

2.4.2 Other biomass estimations

Some studies have used biomass units other than organic weight, such as weight of protein (e.g. Crossland and Barnes, 1977), chlorophyll *a*, numbers of algal cells and nitrogen content (e.g. D'Elia *et al.*, 1983). To allow comparison of the results in this study with others, it was therefore desirable to investigate the relationships between organic weight and chlorophyll *a*, protein and nitrogen content and algal population size.

Ten anemones from the starved symbiotic *A. viridis* stock were taken for the calibration, together with the ten anemones from the dissolved inorganic nitrogen-starved stock of the growth experiment (see Chapter 8). Figure 2.3 outlines the fractionation and analysis proceedures used on each anemone in order to obtain values for organic weight, zooxanthella population size, and chlorophyll *a* content. Buoyant weights were taken as described in section 2.4.1, and the analytical techniques for the other determinations are described below. The results of the calibrations are given in Appendix I.

2.4.2.1 Zooxanthella separation and counting

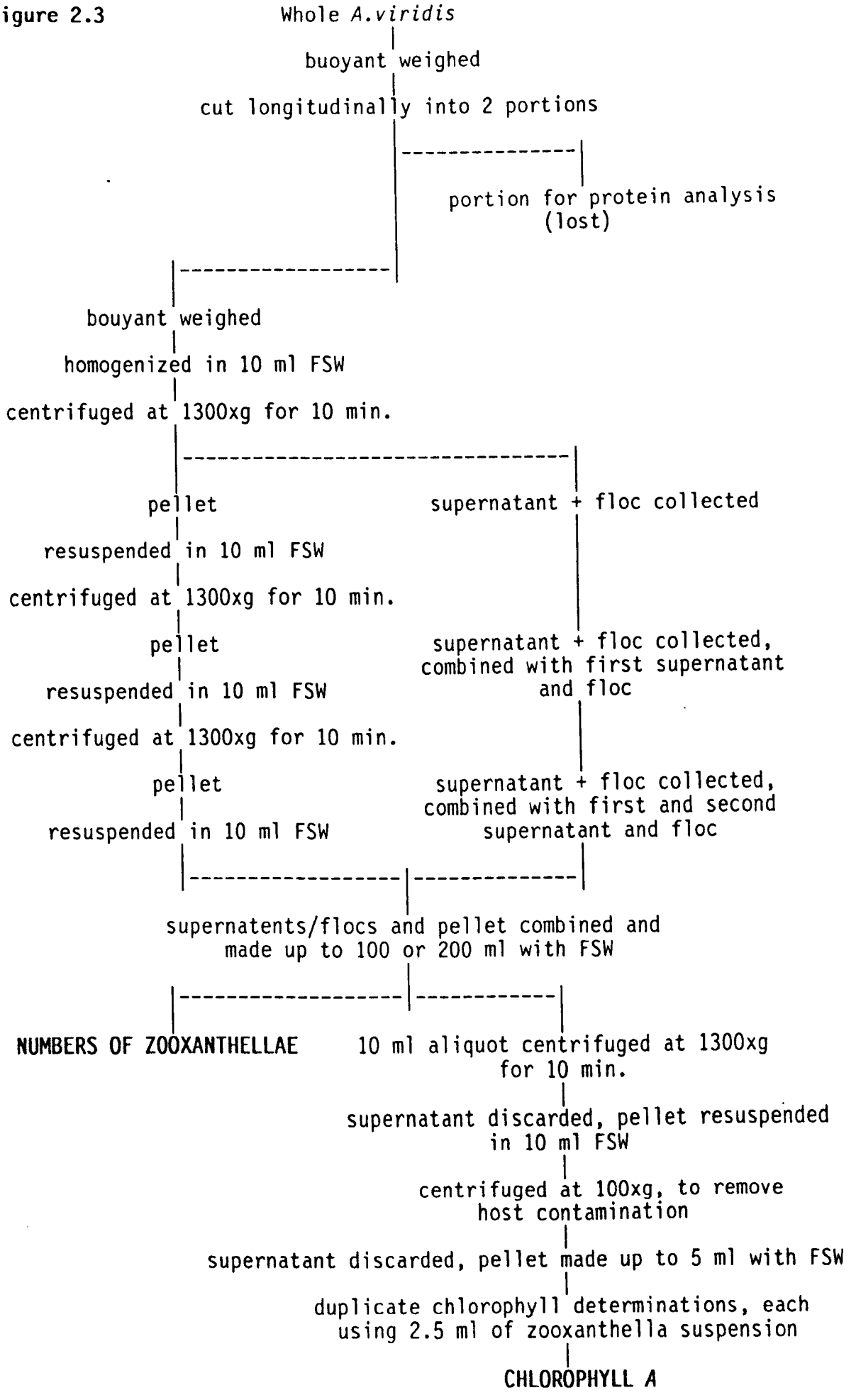
Zooxanthellae were separated from the animal host tissue by a proceedure similar to that decribed in section 2.3.2, except that all fractions were retained and were ultimately recombined, since all contained some zooxanthellae.

The anemones were cut into portions, as detailed in figure 2.3, and were homogenized in a motor-driven glass/PTFE potter homogenizer after being cut into smaller pieces with a scalpel. Centrifugation produced a pellet of zooxanthellae with some animal debris, a milky supernatent and a buoyant floc consisting of zooxanthellae and animal

Figure 2.3 *Anemonia viridis*: outline of the procedure for fractionating anemones to obtain values for total zooxanthella numbers and chlorophyll *a* content (see section 2.4.2). The anemones were initially cut into two portions in order to obtain the latter two values, and also protein content, but the samples for protein analysis were lost.

FSW = membrane-filtered seawater (5 μm pore size)

Figure 2.3


Whole A.viridis
buoyant weighed
cut longitudinally into 2 portions
portion for protein analysis (lost)
bouyant weighed
homogenized in 10 ml FSW
centrifuged at 1300xg for 10 min.
pellet
resuspended in 10 ml FSW
centrifuged at 1300xg for 10 min.
pellet
resuspended in 10 ml FSW
centrifuged at 1300xg for 10 min.
pellet
resuspended in 10 ml FSW
supernatant + floc collected
supernatant + floc collected, combined with first supernatant and floc
supernatant + floc collected, combined with first and second supernatant and floc
supernatents/flocs and pellet combined and made up to 100 or 200 ml with FSW
NUMBERS OF ZOOXANTHELLAE
10 ml aliquot centrifuged at 1300xg for 10 min.
supernatant discarded, pellet resuspended in 10 ml FSW
centrifuged at 100xg, to remove host contamination
supernatant discarded, pellet made up to 5 ml with FSW
duplicate chlorophyll determinations, each using 2.5 ml of zooxanthella suspension
CHLOROPHYLL A

material. The supernatent and floc were collected and shaken vigorously, to resuspend the floc particles. The pellet was resuspended in membrane-filtered sea water (5.0 μm pore size) by rapid passage through a 19g hypodermic needle attached to a 5 ml syringe, and then centrifuged again after making up to 10 ml with filtered sea water. The process, including collection of floc and supernatent, was repeated until the zooxanthellae had been centrifuged three times. The pellet was then resuspended as before, placed in a 100 ml volumetric flask, and the combined flocs and supernatents were added and made up to 100 ml with filtered sea water. This mixture was shaken thoroughly, then mixed further by forcing through a 19g hypodermic needle with a 10 ml syringe until no clumps of animal or algal material remained. In some cases, with larger anemone portions, this suspension was further diluted to 200 ml with filtered sea water to aid counting. Ten replicate counts of each preparation were carried out on an Improved Neubauer haemocytometer, with resuspension of the preparation between paired counts. The total population of zooxanthellae for each anemone (Z) was calculated from the equation:

$$Z = \frac{W_b}{W_b'} \times 10^4 \times \frac{100}{\text{or } 200} \times Z_h$$

Where W_b is the buoyant weight of the whole anemone

W_b' is the buoyant weight of the anemone portion

Z_h is the mean number of zooxanthellae per 0.1 mm^3 of diluted homogenate

2.4.2.2 Chlorophyll determination

Chlorophyll determinations were carried out on zooxanthellae from

the suspension described in section 2.4.2.1, using a method modified from McCloskey and Muscatine (1984). 10 ml of the suspension were centrifuged at 1,300 x g for 10 minutes, and the supernatent was discarded (no buoyant floc was produced during this, or subsequent centrifugations). The pellet, containing all of the zooxanthellae and some animal material, was resuspended and made up to 10 ml with filtered sea water and centrifuged again at 100 x g for 10 minutes. This lower speed caused the zooxanthellae to pellet, whilst the animal material remained in the supernatent, and the purified pellet of zooxanthellae was resuspended in 5 ml of filtered sea water. Chlorophyll determination was done in duplicate on this preparation, by filtering a 2.5 ml aliquot onto a Whatman GF/C filter, followed by rinsing with 2.5 ml of distilled water (with a drop of magnesium carbonate suspension added, to prevent acidic decomposition of the chlorophyll). Each filter was placed in a test tube with 5 ml of 100% acetone, the test tube was sealed with Parafilm, covered with aluminium foil, and left overnight in a refrigerator at $5^{o}C$. After centrifugation, the absorbances of chlorophyll extracts were measured at 630 and 663 nm in a Pye Unicam PU8600 spectrophotometer, correcting for the absorbance of acetone at these wavelengths. The formulae of Jeffrey and Humphrey (1975) were used to calculate mass of chlorophyll a in the acetone extraction solution. Since each sample consisted of 2.5 ml of algal suspension, at twice the algal concentration in the anemone homogenate, and the acetone volume was 5 ml, the calculated mass of chlorophyll, in $\mu g.ml^{-1}$ acetone, is also equivalent to the mass of chlorophyll per millilitre of anemone homogenate. To obtain the mass of chlorophyll *a* in the entire anemone, therefore, the following equation was used:

$$Chl_a = \frac{W_b}{W_b'} \times v \times Chl_{a\ h}$$

Where Chl_a is the mass of chlorophyll *a* in the whole anemone

$Chl_{a\ h}$ is the mass of chlorophyll *a* in 1 ml of anemone homogenate

W_b and W_b' are as in section 2.4.2.1

v is the volume of diluted anemone homogenate

2.5 Nutrient analysis

2.5.1 Determination of ammonia concentrations in sea water

The accurate determination of small concentrations of nutrients in sea water was central to this study, and since the relevent ranges of nutrient concentrations are at the lower limits of detection by standard chemical and electrochemical techniques, additional care had to be taken in the selection, testing and operation of the analytical methods used. It is only relatively recently that convenient and precise methods of ammonia determination in sea water have been developed, despite the well-established methodology for the analysis of ammonia in fresh water samples (see D'Elia, 1983), and even now there is controversy over the relative merits of the various techniques used.

Ammonium (NH_4^+) and ammonia (NH_3) form an acid-base pair with a pK_a value of 9.3, and so the dominant species at pH values less than 8.2, and hence most sea water, is the ammonium ion. Despite this, concentrations in this study are expressed in terms of ammonia, since all standard analytical techniques for ammonia/ammonium in sea water involve buffering the pH at levels where ammonia is the only species

present. The values cited therefore represent the combined concentrations of both species, after the convention of Riley (1975).

There are two main methods currently used for the determination of ammonia in sea water;

1) A spectrophotometric method, using the phenol-hypochlorite reaction.

2) An electrochemical method, using a gas-permeable ion-specific electrode.

Both of these methods were investigated. A third method, recently suggested by Willason and Johnson (1986), using the principle of flow-injection analysis (FIA), was also considered because of its apparent simplicity and suitability to constant monitoring of ammonia concentrations, but this was taken no further because of the prohibitive cost of the apparatus.

2.5.1.1 Sea water analysis using an ammonia electrode

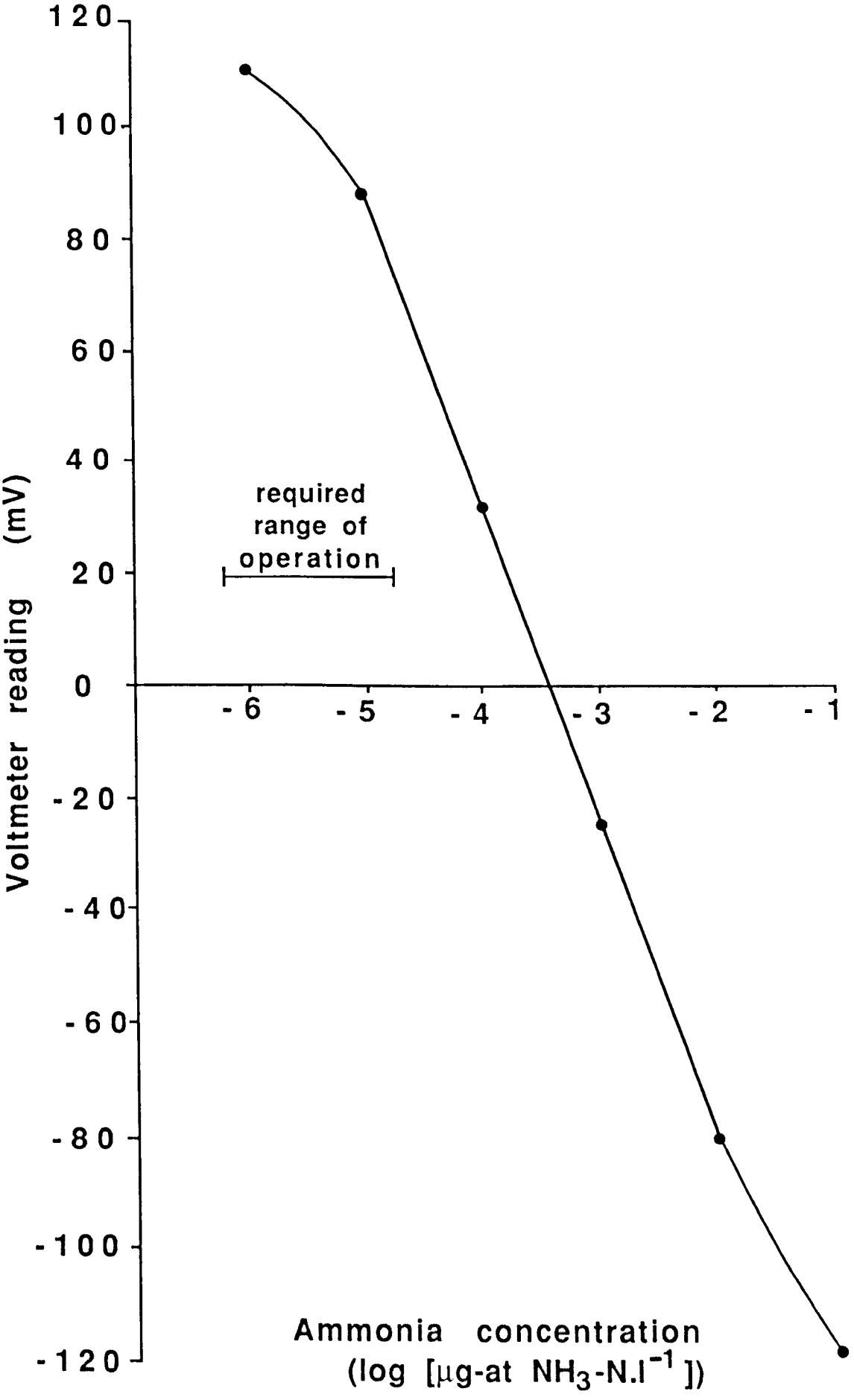
The use of an ammonia-sensing electrode for ammonia determinations in sea water was first evaluated by Thomas and Booth in 1973, soon after the introduction of such an electrode by Orion Research Inc., Cambridge, Mass. The electrode comprised a combination pH electrode, separated from the sample solution by a hydrophobic gas-permeable membrane. Between the membrane and the electrode was a solution that changed pH as ammonia diffused across the membrane from the sample solution, the pH change being proportional to the concentration of OH^- ions produced as the NH_3 was reduced to NH_4^+. The electrode was reported to exhibit Nernstian behaviour down to 0.02 μg NH_3-N.l^{-1} (approximately 2 μg-at NH_3-N.l^{-1}) in samples, and to

be as or more precise than an automated version of the phenol-hypochlorite method of ammonia determination (Thomas and Booth, 1973). Since then several studies have used the ammonia electrode (e.g. Simeonov *et al.*, 1979; Sayer and Davenport, 1987), with reported useful ranges down to those required by this study, and so a similar technique was tested, with the aim of *in situ* field measurements using portable pH meters, without the necessity of sample preservation (see section 2.5.1.2).

The electrode tested was a Corning ammonia combination electrode (Corning Scientific Instruments, Medfield, MA, USA). The specified protocol for testing samples was to immerse the tip of the electrode in the untreated sample solution and then to add 1 ml of 10N NaOH for each 100 ml of sample solution. This raised the pH so that all the ammonium ions were reduced to ammonia, which then diffused across the electrode membrane and caused the meter reading to deflect. This proceedure worked well for standards made up in distilled water, but was unsuccessful in sea water as the high pH caused the precipitation of hydroxides which appeared to clog the electrode membrane. A modified alkalizing agent, as used by Davenport and Sayer (1986), containing 0.93 M EDTA as a chelating agent prevented precipitate formation, and the electrode produced a calibration line as shown in figure 2.4. It can be seen that the relationship between millivolts and log ammonia concentration is linear across a large range of ammonia concentrations, but that the linearity decreases below 10^{-5} M NH_3 (10 µg-at NH_3-N.l^{-1}), which is at the upper end of the range required for this study. In addition, the equilibrium times after alkalizing reagent introduction were protracted, ranging from three minutes for the 10^{-1} M NH_3 standard to twenty minutes for the 10^{-5} M NH_3 standard. The reduction of sample analysis rate to three per hour

Figure 2.4 Ammonia analysis: typical calibration curve for the analysis of ammonia in seawater, using an ammonia-sensitive electrode.

Figure 2.4


120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
Voltmeter reading (mV)
required range of operation
-6
-5
-4
-3
-2
-1
Ammonia concentration
(log [µg-at NH_3-N.l^{-1}])

at typical ammonia concentrations, compared with up to four times this rate for the spectrophotometric method (see below), was unacceptable.

The ionic strength of the electrode fill solution should be approximately the same as that of the sample solution (Proelss and Wright, 1973), and so the osmolarity of both solutions was checked to discover whether an imbalance in these might be the cause of the slow response time. The osmolarity of the fill solution supplied with the electrode, as determined by freezing-point depression, was approximately 91 mOsmoles, whilst the osmolarity of a sample of 10^{-6} M NH_3 in artificial sea water with alkalizing reagent added was approximately 1,000 mOsmoles. This ten-fold difference was clearly undesirable, and so a new fill solution was made up, following the proceedure of Proelss and Wright (1973), consisting of 0.1 M NH_4Cl, 0.25 M sodium sulphate and 100 mg.litre^{-1} methyl orange (for leak detection), with an osmolarity of 650 mOsmoles. This succeeded in reducing equilibration times slightly, but not to the level at which the method would have been as efficient as spectrophotometric determination. This, along with the curvilinear calibration and variable accuracy at low concentrations, led to the ammonia electrode being rejected for the purposes of this study.

2.5.1.2 Spectrophotometric determination of ammonia

Almost all current methods for the spectrophotometric determination of ammonia are based on the reaction of phenol and hypochlorite in the presence of ammonia, first reported by Berthelot in 1859. The reaction involves the formation of chloramine, which reacts with phenol in the presence of a catalyst to form quinonechloramine. This in turn reacts with a further molecule of phenol to form indophenol, a compound which dissociates at high pH to

give a blue colouration (Bolleter *et al.*, 1961).

Since the reactions take place at high pH, the use of this technique for the analysis of ammonia in sea water has been confounded by the precipitation of magnesium and calcium compounds. This problem can be overcome by the addition of a complexing agent, usually sodium citrate, before or as the solution is made alkaline (Solórzano, 1969). Variations in the methods published since Solórzano have involved changing the catalyst, the source of hypochlorite ions, and the length and type of incubation required to achieve maximum colour development. Initially the catalyst used was sodium nitroprusside, with a commercial bleach solution as a source of hypochlorite (Solórzano, 1969), but these reagents have been found to give erratic results (e.g. McCarthy and Kamykowski, 1972; Liddicoat *et al.*, 1975). The method of Liddicoat *et al.* (1975) uses a potassium ferrocyanide catalyst, and sodium dichloroisocyanurate as the hypochlorite donor, because of the instability and consequent erratic hypochlorite content of stock hypochlorite solutions. A further criticism of the Solórzano method was the potential for different intensities of colour development when samples were exposed to different sources of illumination, shown by varying blank readings on bright and cloudy days. Various types of incubation have been proposed to counteract this, from colour development under UV light, in order to standardize the light intensity and wavelength (Liddicoat *et al.*, 1975), to development in darkness at room temperature (Gravitz and Gleye, 1975) or in darkness at elevated temperature (e.g. Mostert, 1983).

The method adopted for use in this study was initially that of Liddicoat *et al.* (1975) with the dark colour development of Gravitz and Gleye (1975). The latter modification proved to produce extremely low absorbances, and so the UV colour development of Liddicoat *et al.*

was used, except that 1 cm pathlength cells were substituted for 10 cm cells, because of the limitations of the spectrophotometers available.

Reagents:

1) Phenol-alcohol solution: 5 g of phenol dissolved in 50 ml of 95% ethyl alcohol.

2) Oxidizing solution: 20 g of trisodium citrate dissolved in 40 ml of ammonia-free water, mixed with a solution of 1.6 g of sodium hydroxide in 40 ml of water, followed by the addition of 0.2 g of sodium dichloroisocyanurate and adjustment of volume to 100 ml with ammonia-free water.

3) Catalyst: 0.5 g of potassium ferrocyanide dissolved in 100 ml of ammonia-free water. Stored in an amber bottle.

All chemicals used were of analytical grade (BDH Chemicals, Poole, Dorset) unless general purpose grade was the highest purity available. The water used was the cleanest obtainable - this was generally de-ionized, but double-distilled and reagent-grade water (from a Milli-Q purification system, Millipore) were also used when available, or when blank values using de-ionized water were high. All reagents were made up daily, and stored in a refrigerator when not required for analyses.

Five millilitre samples for analysis were pipetted into 2-dram stoppered glass vials, and 0.2 ml of reagent 1, 0.5 ml of reagent 2, and 0.2 ml of reagent 3 were added in succession, shaking between each addition. The resultant samples were placed in a drum lined with aluminium foil, in the centre of which a 175 W long wave (365 nm wavelength) ultraviolet bulb was suspended, and were exposed to ultraviolet irradiation for 40 minutes. Absorbance was immediately read at 640 nm in 1 cm glass cuvettes against a distilled water blank,

using a Pye Unicam SP6-550 UV/VIS spectrophotometer, since the colour appeared not to be stable, particularly at higher concentrations. Correction was made for a reagent blank, using artificial sea water (31 g sodium chloride, 10 g magnesium sulphate and 0.02 g sodium bicarbonate dissolved in 1 litre of de-ionized or reagent-grade water). A saline blank was necessary because there is a salinity effect in this reaction, fresh water giving as much as 30% higher absorbance than sea water of a similar ammonia concentration (Koroleff, 1983). The samples analysed, however, did not vary in salinity sufficiently to require corrections for salt content. The analysis was calibrated by the use of ammonium sulphate standards in the range 0.75 to 10.5 μg-at NH_3-N.l^{-1}, made up in artificial sea water (see above).

The method described above gave consistent and reliable results, down to a lower detection limit of 0.1 μg-at NH_3-N.l^{-1}, but required extreme care to prevent contamination. All glassware used was washed in Decon-90 laboratory detergent, rinsed with de-ionized water, soaked for a minimum of 2 hours in 15% hydrochloric acid, and finally rinsed three times in de-ionized water. Any tobacco smoke in areas adjacent to the laboratory had deleterious effects on the analysis, as did the exposure to air of solutions containing higher concentrations of ammonia than those of the samples under analysis. Optimum accuracy was achieved by processing no more than nine samples in an hour (i.e. per batch), although results were acceptable with up to twelve samples per batch, each batch containing at least one standard for reference (the calibration was constructed each day, rather than checking against a general calibration).

Various constraints upon the method presented themselves during the course of the study. Turbid samples, rarely encountered, were

allowed to settle, rather than being filtered, because of the prevalence of ammonia contamination in available filters (Marvin *et al.*, 1972), and the interferance of certain primary and secondary amines with colour development prevented the use of the Tris-based buffers used in zooxanthellae isolation by ultracentrifugation (Ngo *et al.*, 1982; see also section 2.3.1). Samples that could not be analysed immediately were either placed in a refrigerator in darkness, for analysis within an hour, frozen slowly in a freezer at -15°C or rapidly in a container full of dry ice, or fixed by addition of the phenol-alcohol reagent, to be analysed within a week (Degobbis, 1973). Frozen samples were rapidly thawed in a 50°C waterbath and analysed immediately afterwards. Field samples were invariably subject to slightly more contamination than laboratory samples, because of increased handling of the collection glassware and prolonged periods between sampling and analysis, and so the lower limit of reliable detection for such samples was approximately 0.5 µg-at NH_3-N.l^{-1}.

2.5.2 Determination of nitrate concentrations in sea water

The standard method for the determination of nitrate in sea water involves the reduction of nitrate, in the presence of a catalyst, to nitrite, which is then assayed by means of the formation of an azo dye by reaction with sulphanilamide in the presence of N-(1-naphthyl)-ethylenediamine (see Grasshoff, 1983). Concentrations so obtained represent the combined concentrations of nitrate and nitrite in the sample, and so nitrate concentration has to be calculated by subtracting the separately-determined original nitrite concentration.

Initial attempts at nitrate analysis used a version of this method devised by Gardner *et al.* (1976), in which the reductor is a copper-coated cadmium wire inside a narrow-bore teflon tube. 5 ml

samples buffered with ammonium chloride were pumped through this reductor by a Watson-Marlow MHRE peristaltic pump, collected, and nitrite concentrations determined by the addition of sulphanilamide and N-(1-naphthyl)-ethylenediamine, with absorbance read at 543 nm in a spectrophotometer. Unfortunately the yield of nitrite from known concentrations of nitrate (calculated by passing standards of both ions through the column) was often low, and always variable, ranging from 46 to 90% of expected values. This was probably due to over-, rather than under-reduction, the nitrate being reduced beyond nitrite, and consequently giving a low yield of nitrite-azo dye complex, the control of reduction being difficult when using this type of column (Nydahl, 1976).

Because of these difficulties, the nitrate to nitrite reduction method was abandoned, although a copperized cadmium granule column was successfully set up and used on a Technicon AutoAnalyser II (with reduction yields of >98% of expected values) to analyse samples taken from rockpools in South Devon (see section 3.3). As an alternative, a method of nitrate determination using the nitration of a phenolic compound in the presence of a strong acid was adapted from the method of Velghe and Claeys (1985).

Reagents:

1) 5% resorcinol: 5 g of AnalaR resorcinol dissolved in 100 ml of de-ionized water.

2) 36N sulphuric acid: AristaR-grade sulphuric acid was used, because the relatively large acid:sample ratio required minimal nitrogen contamination levels.

50 µl of 5% resorcinol solution was added to 1 ml of sample in a 15 ml test tube. 1.3 ml of 36N sulphuric acid was carefully added, and

the sample was vortex-mixed for 15 seconds. After cooling to room temperature, the absorbance was read at 360 nm in a Pye Unicam SP6-550 UV/VIS spectrophotometer. Blanks consisted of 1 ml of artificial sea water (see section 2.5.1.2). Calibration was by potassium nitrate standards, made up in artificial sea water, between 0.9 and 50 µg-at NO_3^--N.l^{-1}. Absorbances were low, and consequently the limit of detection for this method was approximately 0.5 µg-at NO_3^--N.l^{-1}. Despite this, the assay was consistent, if a little insensitive, and considerably quicker than the copper-cadmium reduction method of Gardner *et al.* (1976).

CHAPTER 3: FIELD OBSERVATIONS

3.1 Introduction

The flux of dissolved inorganic nitrogen (DIN) in algal-coelenterate symbioses is known to be affected by environmental factors such as light and ambient concentrations of DIN (Muscatine, 1980; see Chapter 4). In addition, the recent nutritional history of the association, which is influenced by availability of prey items, may also affect DIN flux rates (Szmant-Froelich and Pilson, 1977; 1984; see Chapter 5). It was therefore important to gain some knowledge of the conditions experienced by *A. viridis* in the field, to ensure the relevance of laboratory-produced experimental conditions.

There is abundant available literature concerning physical, chemical and biological conditions in shallow waters around the British coasts, including values of dissolved inorganic nitrogen concentrations (e.g. Postma, 1978; Butler, 1979; Foster *et al.*, 1985). Relatively little of the data, however, concerns very shallow-water environments such as rock pools or the shallow subtidal, which are the favoured habitats of *A. viridis* (Gosse, 1860; Stephenson, 1935; Manuel, 1981), and this is particularly the case for measurements of light and nutrient levels. Because of this, the primary aim of the fieldwork presented here was to provide information about the environmental conditions to which anemones were exposed in the field.

The secondary aim of the fieldwork was to provide data on seasonal variations in environmental parameters so that in subsequent work nitrogen budgets, constructed from laboratory experiments, can be extrapolated to field populations. This approach has already worked well for carbon/energy budgets in corals, where carbon fixation rates have been measured in the laboratory under a range of light

intensities and extrapolated to animals kept on the reef in normal environmental conditions (Davies, 1984; Edmunds and Davies, 1986).

3.2 The study sites and populations

3.2.1 Sites

The study area chosen had to satisfy two basic criteria: it had to support more than one population of *A. viridis* (or a single large population), so that animals could be collected without disturbing the observed population, and the populations had to be readily accessible by land, since light monitoring equipment was to be set up on the shore and used over the course of a week in any one study period.

The nearest area satisfying both of these requirements was Loch Sween, Argyll (Figure 3.1), where Lewis and Powell (1960) recorded *Anemonia sulcata* (=*viridis*) from almost all shores of the loch and its associated arms, Linne Mhuirich and Caol Scotnish. Several sites were investigated for the purposes of observation and collection (see Figure 3.1a), with varying densities of *A. viridis* and with a range of accessiblities. Lewis and Powell (1960) indicated that the greatest densities of *A. viridis* were to be found in the Taynish Narrows, shallow tidal rapids between the Taynish peninsula and the Ulva Islands. Large specimens were found there, but subsequent investigations in June 1985 located a larger population with easier access, on the eastern shore of Linne Mhuirich (National Grid reference NR724 836, 55^{o} 59.49'N 5^{o} 38.98'W, Admiralty chart 2397), and this was chosen to be the observed population. Because this population comprised mainly small individuals, a second population at Bàgh na Doide, on the eastern shore of Loch Sween, where individuals of a wider size range were found, was examined as a source of

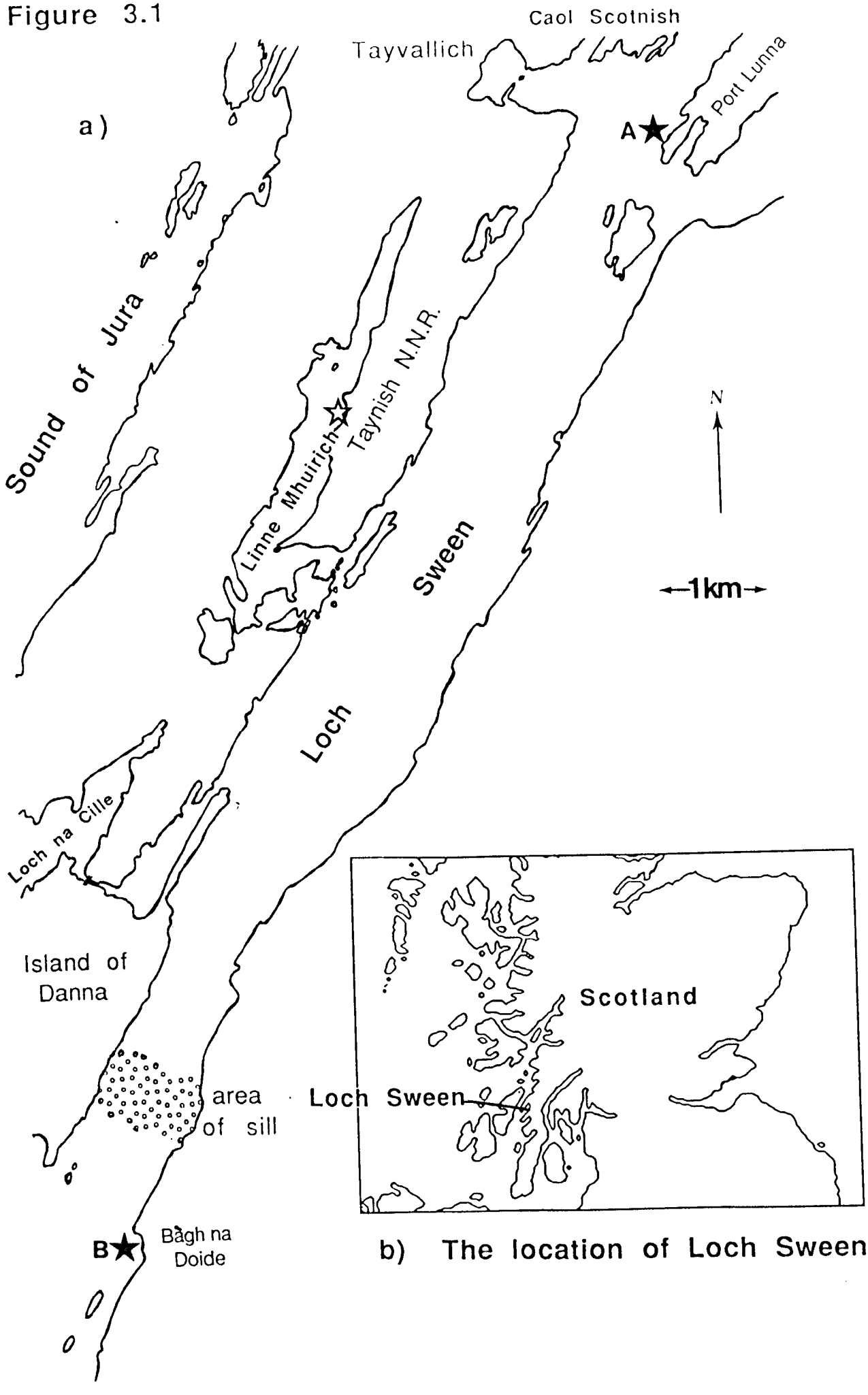
Figure 3.1 The study site: a) Loch Sween and it s associated arms, Linne Mhuirich and Caol Scotnish. The location of the study site, in Linne Mhuirich, and the collection sites, at Bàgh na Doide and the submerged pinnacle near Port Lunna, are marked. b) the West coast of Scotland, showing the location of Loch Sween.

☆= observation site.

A★= collection site (pinnacle).

B★= collection site (Bàgh na Doide).

Figure 3.1


Caol Scotnish
Tayvallich
Port Lunna
A
a)
Sound of Jura
Taynish N.N.R.
Linne Mhuirich
Sween
N
1km
Loch
Loch na Cille
Island of Danna
area of sill
B
Bàgh na Doide
Scotland
Loch Sween
b) The location of Loch Sween

laboratory animals. This site suffered from poor access and was relatively exposed, making collection in poor weather difficult, and so the site from which most laboratory anemones were collected was eventually a submerged pinnacle to the west of Rubha na Marraidh (pinnacle position 56° 1.05'N 5° 35.90'W, Admiralty chart 2397). This pinnacle at a water depth of 3 m supported a dense population of large *A. viridis*, all of the brown colour morph.

The study site in Linne Mhuirich was relatively sheltered, with boulder and sandy mud substrates descending to approximately 2 m depth within 15 m of the shoreline. At most points there was a mixed band of *Fucus spp.* and *Ascophyllum nodosum* (the tidal range here is extremely small - see section 3.6 - and somewhat erratic, causing unusual or blurred zonation of algae) on pebbles and stones, followed by a band of *Zostera marina* on mud, and then finally a band of *Laminaria digitata* on larger stones and boulders. Although surrounded by hills, the site was exposed to a 3 km fetch when the wind was blowing from the south west (the prevailing direction), which was enough to cause a choppy surface and to resuspend quantities of sediment in the water.

The pinnacle collection site was a bedrock ridge rising from depths of 18 m below chart datum to 3 m, running south west to north east and having at least two well-defined "peaks". *Anemonia* were present at these tops and down the sides to a depth of 10-12 m. Algal cover was largely in the form of an algal turf, with scattered *Laminaria* and some *Chorda filum*. No currents were noted on the pinnacle, which is in fairly sheltered water, and surface turbulence was never seen to extend to the depth of the anemones.

3.2.2 Population densities

The *Anemonia viridis* population at the Linne Mhuirich study site was surveyed monthly by taking three transect lines normal to the shore, extending to 14 m from the estimated extreme low water spring tide mark, to the edge of the dense *Laminaria* belt. Population surveys were conducted whilst snorkelling, by counting the *Anemonia* in consecutive 1 m^2 quadrats along the transects. Initially *Anemonia* were present and conspicuous, with mean densities of 1.9 to 2.3 anemones per square metre, but the numbers fell over the two years until very few anemones could be found (mean densities for the transects of 0 to 0.3 anemones.m^{-2}). This population decline was not due to collection, since none was carried out on this site, and was unlikely to be due to disturbance from the surveying. Some human disturbance was possibly experienced on the site, since oysters were periodically collected from this shore of Linne Mhuirich by visitors to the area, but the presence of mature oysters on the site throughout the study suggests that this pressure was not excessive. A second population, at the head of Linne Mhuirich, was noted during the study period. This population with densities of 5 to 6 anemones.m^{-2}, indicated that environmental conditions, in Linne Mhuirich as a whole, were not inappropriate for the growth of this species.

The pinnacle collection site had far higher densities of *Anemonia* than the study site (although densities on the pinnacle were estimated without quadrat counts). The anemones covered the substrate entirely in many places, giving estimated densities of more than 100 anemones.m^{-2}. In the most densely-populated areas anemones were found on the macroalgae to within 3 m of the water surface. The individuals on this site were much larger than in any other population observed in the Loch Sween area.

3.3 Dissolved inorganic nitrogen levels

Samples for nutrient analysis were collected monthly, from the study site, in acid-washed screw-cap glass bottles of 50 ml volume. Samples were taken in triplicate at a depth of approximately 1.5 m below the water surface. Immediately after collection the samples were either frozen in a polystyrene box containing dry ice, or (if for ammonia analysis) were fixed by the addition of 2 ml phenol-alcohol reagent (see Section 2.5.1.2). Samples were analysed as soon as possible after returning to the laboratory, by the methods described in Chapter 2.

Initially, both nitrate and ammonia levels were determined, but since the concentrations appeared to be too low for the relatively insensitive nitrate assay used (see Section 2.5.2), and because laboratory experiments appeared to show that *A. viridis* does not utilize nitrate (see Section 4.2), subsequent analyses were confined to ammonia alone. Nitrate concentrations recorded in Linne Mhuirich in August and September 1985 were below the limit of detection of 1 µg-at NO_3^--N.l^{-1}. Data collected by the Scottish Marine Biological Association from near the head of Loch Sween (fairly close to the pinnacle site) gave nitrate values at 5 m depth of 0.73, 0.09 and 1.96 µg-at NO_3^--N.l^{-1} in May, August and October of 1985 respectively (K. Jones, pers. comm.).

The results of monthly sampling for ammonia on the Linne Mhuirich study site are given in table 3.1, together with the data collected in 1985 by the Scottish Marine Biological Association.

Very few of the monthly ammonia concentration values were above the limit of detection of 0.5 µg-at NH_3-N.l^{-1}, and the highest values, of 1.3 and 2.2 µg-at NH_3-N.l^{-1}, occurring in September and October

Table 3.1 The study site: Monthly ammonia concentrations at 1.5 m depth on the study site. Ammonia concentrations at 5 m depth in Salean Mor, Loch Sween (see figure 3.1a), are also shown (Salean Mor data provided by the Scottish Marine Biological Association).

Month	Ammonia, Linne Mhuirich ($\mu g\text{-at } NH_3\text{-}N.l^{-1}$)	Ammonia, Salean Mor ($\mu g\text{-at } NH_3\text{-}N.l^{-1}$)
March, 1985	-	0.18
April,	-	-
May,	-	<0.1
June,	-	
July,		
August,	0.55 ± 0.09	
September,	1.31 ± 0.38	
October,	2.20 ± 1.09	
November,	<0.5	
December,	<0.5	
January, 1986	-	
February,	-	
March,	-	
April,	<0.5	
May,	<0.5	
June,	<0.5	
July,	<0.5	
August,	<0.5	
September,	<0.5	
October,	<0.5	
November,	<0.5	
December,	0.77 ± 0.06	
January, 1987	<0.5	
February,	<0.5	
March,	<0.5	
April,	0.58 ± 0.13	
May,	1.10 ± 0.90	
June,	<0.5	
July,	<0.5	

1985, are probably erroneous since the assay was not giving consistant results by these dates. Highest concentrations would have been expected during the winter months, when primary productivity is at its lowest, but whilst the December 1986 value of 0.77 µg-at NH_3-N.l^{-1} is high, the other values above detection limits were in April and May 1987, when the vernal phytoplankton bloom should have been keeping nutrient concentrations low. The S.M.B.A. data for March and May 1985 also show low levels of ammonia, and so it appears that the shallow water in Loch Sween and Linne Mhuirich seldom, if ever, contains appreciable concentrations of ammonia, always being at the lower end of the estimated concentration range for coastal waters (0-25 µg-at NH_3-N.l^{-1} (Sharp, 1983)).

A small number of ammonia concentrations was obtained by sampling rock pools on Start Point, South Devon (National Grid reference 826 371) in July 1986, as a comparison with the concentrations in Loch Sween. Water samples were taken from open water and from several rockpools containing large numbers of *A. viridis*. The open seawater contained 0.85 µg-at NH_3-N.l^{-1}, whilst rockpools that had been isolated from open water for approximately 30 minutes had levels of ammonia of between 2.48 and 2.92 µg-at NH_3-N.l^{-1}. It was unfortunately not possible to acquire further data from South coast rockpools, but these preliminary results suggest that *A. viridis* in rockpools may, for at least part of the tidal cycle, be exposed to considerably higher ammonia concentrations than the anemones in subtidal habitats in Scottish sea lochs. Since *A. viridis* are more numerous in the rockpool habitat, more information on the range of nutrient levels in this environment would be useful.

3.4 Light levels

Light levels were monitored at a depth of approximately 1 m on the Linne Mhuirich study site for one week in February and one week in July in 1986, and during the same weeks in 1987. The sensor of a Li-Cor light meter (LI 185B meter with LI 182SB sensor, designed to measure photosynthetically active radiation of wavelengths 400 to 700 nm) was fastened vertically on a pole that could be driven into the sediment adjacent to an area containing *A. viridis*, so that the sensor head was level with the anemones and pointing at the water surface. Light readings were made at hourly intervals, and the mean values for the pooled data for winter and summer from both years are shown in figures 3.2 and 3.3 respectively. Also shown in figures 3.2 and 3.3 are the potential maximum irradiances available to the anemones, estimated from the highest reading obtained for each hour, on the assumption that this corresponded to a clear sky. Thus the maximum light curve represents the illumination on a hypothetical "ideal" day, whilst the mean light curve represents that of an average (cloudy) day. Other factors that would affect the transmission of light to the substrate, such as tide height, water turbidity and increased reflection from a choppy water surface, are considered to have been included in the mean light curve. It is likely that an occasion when all of these conditions were optimal for maximum light transmission never occurred, and so the maximum light curve probably underestimates the true maximum irradiances experienced by *A. viridis* on this site.

The results, which represent photosynthetically active radiation (PAR), can be compared by taking the median PAR value for each hourly interval, converting the instantaneous values (in units of $\mu E.m^{-2}.s^{-1}$) to absolute values (in units of $E.m^{-2}.h^{-1}$), and summing these to

Figure 3.2 The study site: Light levels on the study site, taken at the level of the anemones on days in February. Light curves are shown for an "average" and an "ideal" day. Values for "average" days are the mean irradiances for the time of day, taken from a week in 1986 and the same week in 1987. Values for "ideal" days are maximum recorded irradiances for the time of day, taken from the data that provided the mean values. The curves were eye-fitted to the data.

Figure 3.2

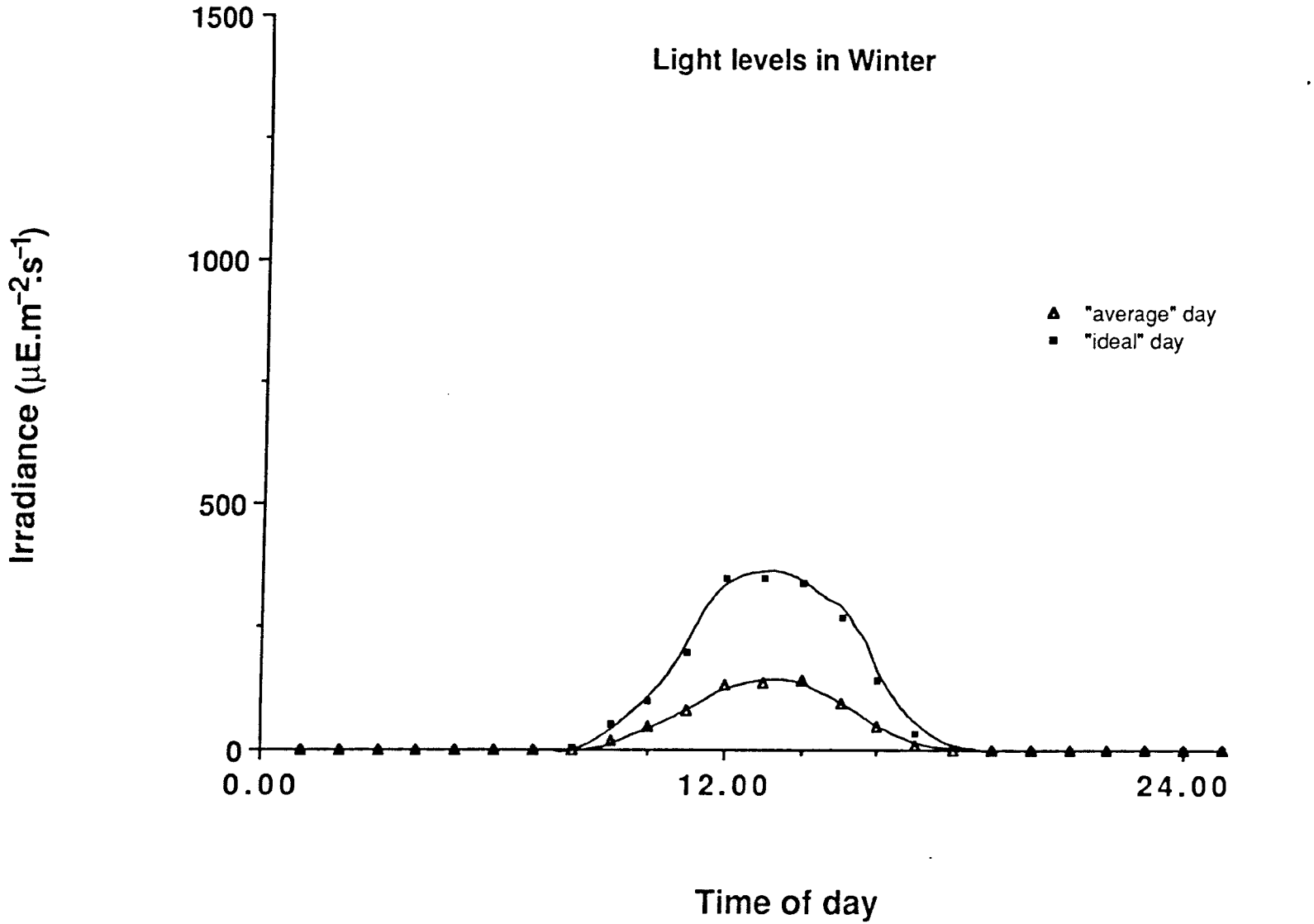
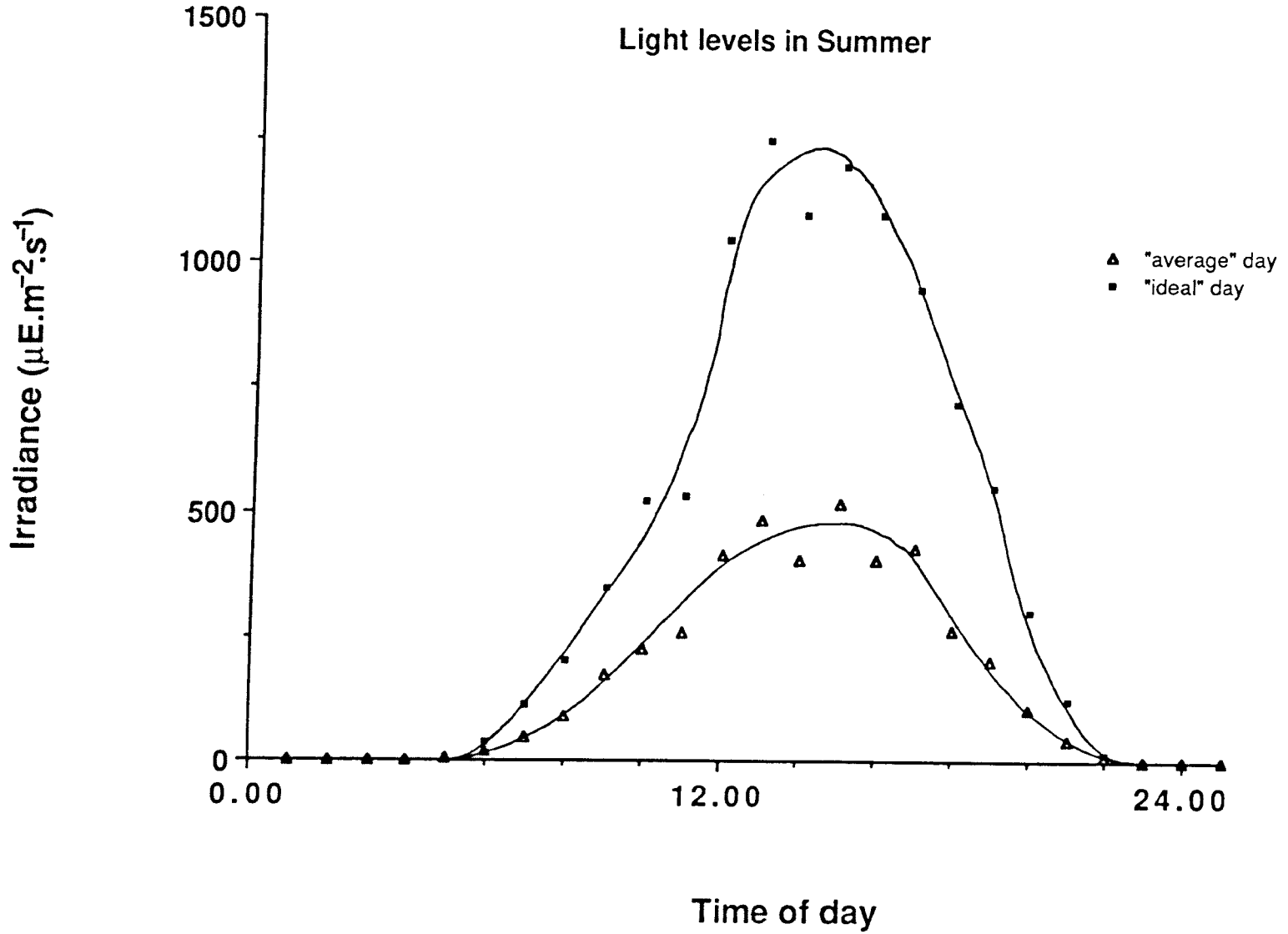

Light levels in Winter
1500
1000
500
0
Irradiance (μE.m-2.s-1)
"average" day
"ideal" day
0.00
12.00
24.00
Time of day

Figure 3.3 The study site: Light levels on the study site, taken at the level of the anemones on days in July. Light curves are shown for an "average" and an "ideal" day. Values for "average" days are the mean irradiances for the time of day, taken from a week in 1986 and the same week in 1987. Values for "ideal" days are maximum recorded irradiances for the time of day, taken from the data that provided the mean values. The curves were eye-fitted to the data.

Figure 3.3


Light levels in Summer
1500
1000
500
0
0.00
12.00
24.00
Irradiance (μE.m-2.s-1)
Time of day
"average" day
"ideal" day

obtain the integrated daily PAR.

Both winter and summer PAR curves roughly follow sine-wave forms, with departure from this due to cloud cover. A truly clear day was not observed at any time, and so the maximum PAR curves are composites of values from several days, from both years. In the summer, maximum light levels (1260 $\mu E.m^{-2}.s^{-1}$) were reached at approximately 1330 hours BST, with an integrated daily PAR of 37.6 $E.m^{-2}.d^{-1}$, whilst in winter maximum levels (350 $\mu E.m^{-2}.s^{-1}$) were achieved between 1130 and 1330 hours GMT, with a much lower integrated daily PAR of 6.7 $E.m^{-2}.d^{-1}$. The summer maximum estimations are likely to be close to the true maxima on a cloudless day since they were recorded with frequent breaks in the cloud, but the winter estimates are likely to be lower than actually occur, because neither winter period included any of the still, clear days that often appear in February on the West coast of Scotland.

3.5 Observations on heterotrophic feeding

Although largely outside the scope of this study, some preliminary observations were made on the prey availability to, and capture by *A. viridis* in Loch Sween. It was desirable to have some idea of the nutritional state of the anemones, since this may influence their nitrogen turnover rates (see Chapter 5).

To assess prey availability, plankton samples were taken on several occasions during monthly visits to the study site, since *A. viridis* in Lough Hyne, Eire, are recorded as being indiscriminate plankton feeders, with coelenteron contents reflecting the composition of the plankton available (J.R. Turner, pers. comm.). Samples of the plankton available to the anemones were collected by means of an electric pump (powered by a 12 v battery) attached to a plastic hose.

The end of the hose was secured above the sediment surface in 1.5 m of water and had a coarse (3 mm mesh) filter to prevent clogging with macroalgae. The outflow of the pump was directed into a small plankton net (mesh size 200 μm) with a collection bottle at its base. The pump raised 11 litres of seawater per minute, and was run for one hour to give a sampled water volume of 660 litres. The plankton samples were examined microscopically, and were found to contain relatively few (less than 10 per sample) recognizable zooplankton and very large amounts of plant detritus and filamentous green algae. The quantity of detritus increased markedly during or after strong winds, and was due to the resuspension of detrital organic matter by wave action. The quantity of zooplankton was always small, which may reflect a genuinely low availability of such potential food, but may also be due to inadequacies in the collection method, since scyphozoans and hydromedusae, which were preyed on by *A.viridis* (see below), were absent from all samples despite being present in the water column during sampling. Similar low densities of plankton were found when samples were obtained by passing 80 litres of seawater, collected by bucket, through the plankton net. This may indicate that the plankton net was the source of the low counts, since it was used for both methods, or that the plankton availability was genuinely low. The results differ greatly from those of Gauld (1950), who found up to 500 organisms.l^{-1} in summer zooplankton samples from Kyle (Caol) Scotnish, an arm of Loch Sween opening into the main loch to the north east of Linne Mhuirich (see figure 3.1a).

Direct observation of feeding was rare, but on several occasions *A. viridis* on the pinnacle site were noted capturing and ingesting scyphozoans (*Aurelia aurita* and *Cyanea capillata*). The prey individuals were all relatively small, never more than 10 cm bell diameter, and prey at the upper limit of this size range were often

captured by several *Anemonia* simultaneously. Such capture of relatively large prey was only observed when large numbers of the scyphozoans were present, in early and late summer, and no other large prey types were ever noted.

In February and June 1986 twenty *A. viridis* were collected from the pinnacle site, placed in 10% formalin (which was also injected directly into their coelenterons) and later dissected to examine coelenteron contents. Three of the forty anemones contained chitinous remains that could have been copepod carapaces, whilst several others had small quantities of organic material. Apart from one individual which was found to contain a ball of mesoglea-like jelly, which was probably the remains of a scyphozoan, no other recognizable food particles were found.

These observations are incomplete, but suggest that planktonic food may not be abundant for much of the year in Linne Mhuirich, and whilst detritus resuspended from the bottom might provide a larger source of particulate matter, the proportion of such particulate matter which might be of nutritional value to the study species is unknown.

Anemonia viridis is reported as being a voracious carnivore (Gosse, 1860; Mesnil, 1901; Smith, 1939), and in a study on the coast of Brittany, 97.5% of *Anemonia* examined had prey in their coelenteron (Möller, 1978). Prey types are reported as being varied, including a large proportion of crustaceans such as amphipods, isopods and copepods, and lesser amounts of gastropod molluscs, insects and polychaetes. Fish and adult decapod crustaceans are also represented in the diet of *Anemonia*, but both appear rather scarce (Möller, 1978), despite the anemones' ability to immobilize and kill these types of prey (Möller and Béress, 1975). The Brittany study also noted algal remains in the coelenterons of some *Anemonia*, and van Praët (1982;

1983) has suggested from digestive enzyme studies that microalgae and plant detritus may contribute substantially to the diet of anemones, even in species normally thought of as macrophagous.

In the light of such information, it appears surprising that so little evidence for heterotrophic input was found in this study. The sealoch environment, however, is unusual and has quite different hydrographic and biological conditions from rockpools on an open coast. It is perhaps significant that *A. viridis* was the only anthozoan species present on the study and collection sites and was the only anthozoan that could be found on most shores of Loch Sween. *Anemonia viridis* were found coexisting with other anthozoans, for example in the tidal rapids of Taynish Narrows (other species including *Actinia equina*, *Metridium senile*, *Sagartia elegans* and *Sagartiogeton laceratus*) and on the relatively exposed shore at Bagh na Doide (*Actinia equina* and *Urticina felina*), both of which sites experience substantially greater water movement than is the norm for Loch Sween. The distribution of these other, non-symbiotic, anthozoan species in the loch is possibly limited largely by food avialability (since abundant hard substrate is available on most shores of Loch Sween). *Anemonia*, when in areas of reduced water exchange such as the study site, may therefore be exploiting habitats that are barred to other anthozoan species by lack of food.

3.6 Summary

Populations of *Anemonia viridis* in Loch Sween were investigated to provide basic data on the environmental conditions experienced by this species in the field.

Loch Sween is a shallow type "B" fjordic sea loch, with an entrance sill of 8 m depth and an exceptionally small tidal range

(Milne, 1972), resulting in hydrographic conditions that are quite different from those of the open coast. A characterisic of such systems is that water temperatures follow air temperatures more closely than in open coastal water, with the result that winter temperatures tend to be markedly lower, and summer temperatures markedly higher than in the adjacent Sound of Jura. The combination of low tidal amplitude and shelter given by the surrounding hills ensures that the majority of the loch is not exposed to much water movement, and consequently water exchange may occur relatively slowly throughout much of the loch system. Linne Mhuirich, where the study site was situated, has even less water movement because of an extremely shallow (0.5-1 m) and restricted opening onto the main loch, although the opening itself is a tidal rapid and supports a rich community of filter feeding organisms. Nutrient levels around the anemones were uniformly low throughout the year, and light levels were frequently reduced by heavy cloud. There was a large seasonal difference in light levels, both in duration and intensity, with the integrated "maximum" PAR in winter being less than 20% of the summer value.

These conditions are probably atypical of the environment normally inhabited by *A. viridis*, which is nearing the northern limit of the species' distribution at this point. The apparent low abundance of food, depressed light levels and low winter temperatures are likely to mean that the study population is more stressed than populations in the south of Britain and the Mediterranean, and this may be reflected in the large population density fluctuations shown by the anemones on the study site.

Despite these environmental pressures, *A. viridis* remains the dominant anthozoan species in the shallow waters of the loch, and on some sites, such as the pinnacle collection site, it is the dominant macrofaunal species. The potential for polytrophic nutrition due to

the symbiosis with intracellular algae may well be a contributing factor in this species' ability to exploit habitats that related species cannot.

CHAPTER 4: DISSOLVED INORGANIC NITROGEN FLUX IN STARVED *Anemonia viridis*

4.1 Introduction

It is now a well-reported phenomenon that coelenterate species hosting zooxanthellae exhibit dissolved inorganic nitrogen (DIN) fluxes that differ markedly from those of non-symbiotic species. Whilst non-symbiotic species show a large net loss of ammonia (e.g. Pütter, 1911; Wilkerson and Trench, 1986), symbiotic species may demonstrate a decreased ammonia loss (Zamer and Shick, 1987) or ammonia and/or nitrate uptake (see Muscatine, 1980, for review). This retention or uptake of DIN by symbiotic individuals has been attributed directly to the activity of the zooxanthellae, which suggests that the process should be photo-linked because of the requirement of carbon and reductant for algal DIN uptake (Syrett, 1981). In addition to light-dependency, algal DIN uptake is also strongly concentration-dependent, with the relationship between concentration and uptake rate usually obeying Michaelis-Menten kinetics (e.g. Dugdale, 1967; Eppley and Coatsworth, 1968; MacIsaac and Dugdale, 1969). DIN uptake rates in algal-coelenterate symbioses have also been shown to be concentration-dependent (e.g. Wilkerson and Muscatine, 1984), but the rate-concentration relationship frequently departs from the Michaelis-Menten kinetics of free-living microalgae (Muscatine, 1980).

The *Anemonia viridis* symbiosis has been shown to be capable of fixed carbon production in excess of its needs (Tytler, 1982; Tytler and Davies, 1986;), suggesting that some other factor, such as the uptake and conservation of nitrogen may be of major importance in the maintenance of the symbiosis. As mentioned above, such uptake is

likely to be both DIN-concentration and light dependant. Because of this, any quantitative prediction of the advantage conferred upon the association by the enhanced supply of nitrogen would have to be based upon a knowledge of the ambient light and nutrient availability. Since both of these may change over a day in the field, it was necessary to test for nitrogen uptake and retention under a range of light intensities and DIN concentrations.

The aims of this section were therefore:

1) To confirm that the possession of algae allows nitrogen uptake and conservation in this association.
2) To determine the effects of different ambient external concentrations of DIN on the DIN flux of the association.
3) To investigate the effects of different light intensities on the DIN flux of the association.

The information obtained in these experiments was intended to provide the basis for a descriptive model of the fluxes of dissolved inorganic nitrogen to and from the animal and algal partners in the symbiosis. It was also anticipated that this model would permit quantitative estimates to be made of the provision of nitrogen to the symbiosis from the uptake and retention system, for anemones under field conditions.

4.2 Demonstration of DIN flux in symbiotic and aposymbiotic *A. viridis*

4.2.1 Introduction

The intention of this set of preliminary experiments was to investigate the existence of nitrogen uptake and conservation in symbiotic *A. viridis*. Nitrogen conservation of sorts takes place in

all cells, with nitrogenous end-products of metabolic pathways being recycled by other pathways. The term "conservation", as used here, becomes relative, and the aim of the experiments was to investigate the existence of nitrogen conservation beyond the capabilities of anemones containing no symbiotic algae. Experiments therefore took the form of comparing the DIN flux of symbiotic *A. viridis* with that of aposymbiotic individuals differing from the symbionts only by the absence of algae. All anemones used in these, and subsequent experiments in this section, had been starved for a minimum of three weeks prior to experimental use, to eliminate the possible variation in nitrogen metabolism rates that arise from unequal feeding or assimilation efficiencies (Szmant-Froelich and Pilson, 1977; 1984).

4.2.2 Methods

For each experiment, five beakers each containing 1 litre of sea water from the circulating sea water system at the Zoology Department, University of Glasgow were maintained at a constant temperature ($10\pm1^{o}C$). Three of the beakers each contained six *Anemonia*, the remaining beakers being left as controls. After the anemones had apparently settled, each of the five beakers was spiked with a small volume of ammonium sulphate or potassium nitrate solution (see below) to elevate the concentrations of inorganic nitrogen. The volume and concentration of the spike was controlled in order to give a starting concentration of 10 µg-at $N.l^{-1}$ in each beaker. After a brief period to allow for mixing, a 5 ml water sample was taken from each beaker. This sampling was repeated at hourly intervals for eight hours. Water samples were assayed for ammonia or nitrate concentration as described in sections 2.5.1.2 and 2.5.2 respectively.

Four different experimental treatments were used:

1) Aposymbiotic anemones in light, spiked with ammonia
2) Symbiotic " " " , " " "
3) " " " dark, " " "
4) " " " light, " " nitrate

During a day-long experiment a single treatment was therefore investigated, with experimental beakers in triplicate and control beakers in duplicate.

Treatments 1, 2 and 3, using ammonia, were spiked with 1.5 mM ammonium sulphate solution. All experiments were carried out in the animals' normal "daylight" period, light experiments using the standard aquarium illumination (140 $\mu E.m^{-2}.s^{-1}$, see section 2.2.1), whilst for dark experiments the lights were turned off. Since experiments commenced at the start of the aquarium daylight period, all anemones had been preconditioned with 12 hours of darkness prior to the start of the experiments (apparatus set-up was completed within ten minutes, during which time the anemones were exposed to full aquarium light levels).

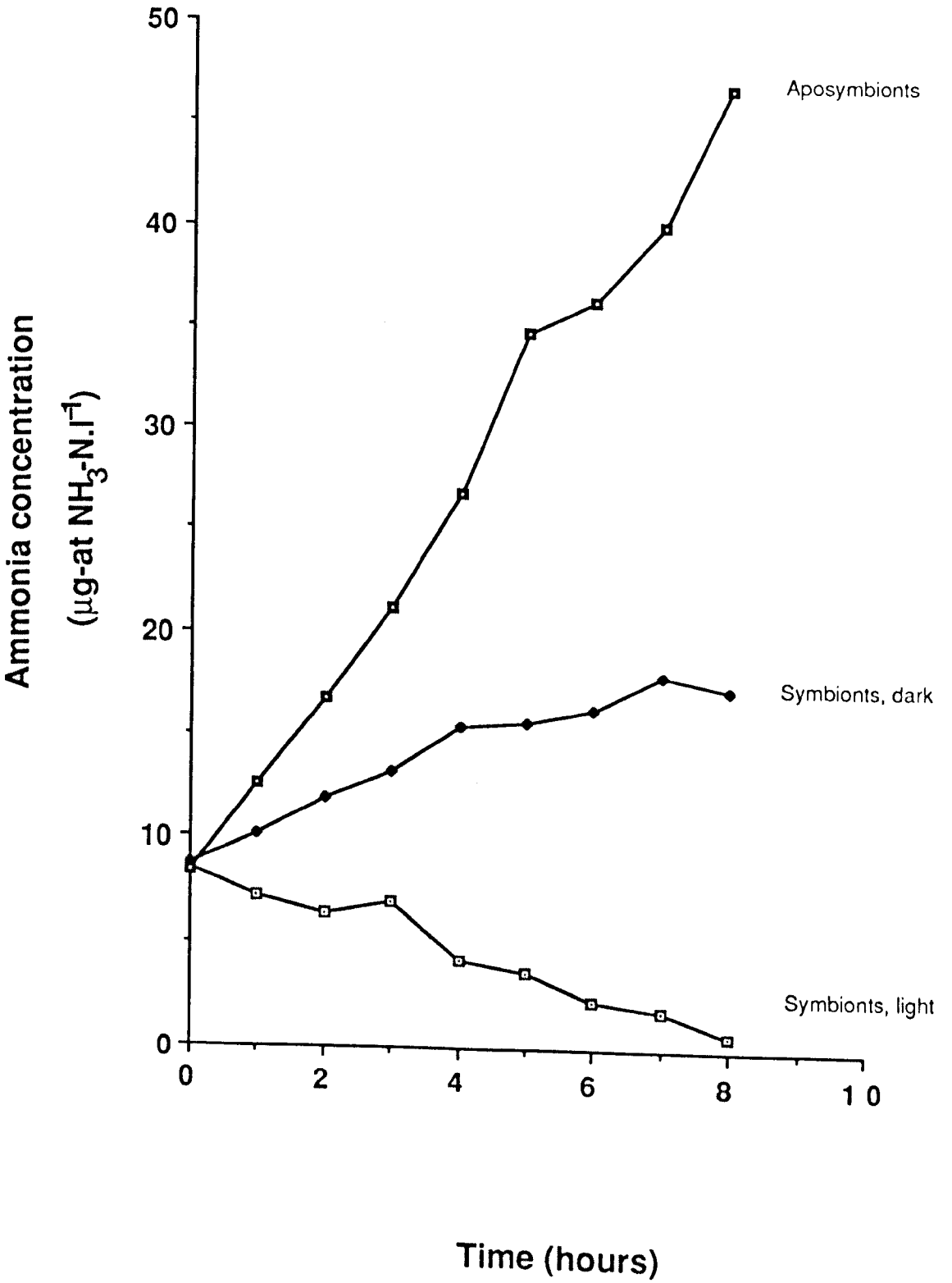
Following each experiment, the anemones were buoyant weighed (see section 2.4.1), to give the total organic weight of anemones in each beaker.

4.2.3 Results

Figure 4.1 shows results for each of the experimental conditions using ammonia. The results obtained from repeats of the same experiments often demonstrated far more variation than those depicted. This is considered to be due to some anemones not settling, and consequently having modified DIN flux which alters the net, observed

Figure 4.1 *Anemonia viridis*: typical time courses for ammonia concentrations in incubations of starved aposymbiotic and symbiotic anemones at an irradiance of 140 $\mu E.m^{-2}.s^{-1}$, and for symbionts in darkness. Starting concentrations were between 8 and 9 μg-at NH_3-$N.l^{-1}$. Each incubation beaker contained 1 litre of sea water and 6 anemones (approximate total organic weight of 6.0 g).

Figure 4.1


Ammonia concentration
(µg-at NH_3-N.l^{-1})
50
40
30
20
10
0
0
2
4
6
8
10
Aposymbionts
Symbionts, dark
Symbionts, light
Time (hours)

flux of the group. Unsettled anemones did not expand to the same extent as settled anemones and so had reduced surface areas, which was likely to reduce their rates of flux when compared with fully expanded anemones.

Nitrate levels, which were invariably higher than 10 µg-at NO_3^--N.l^{-1} in all beakers at the start of the experiments because of elevated ambient levels in the sea water supply, remained stable in both control and experimental beakers (figure 4.2). From these results it appears that *A. viridis* was not able to take up nitrate from the surrounding water.

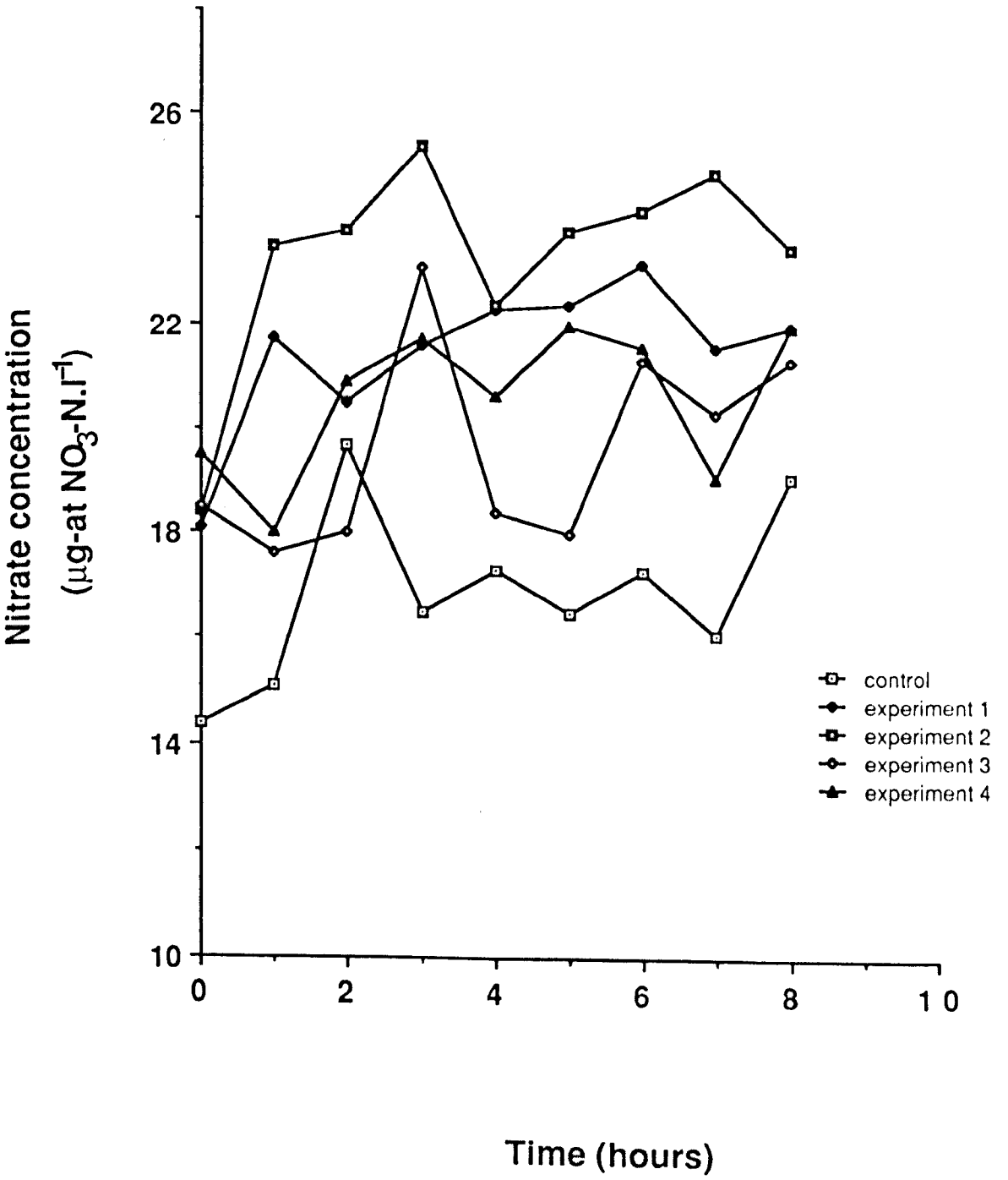
Symbiotic *A.viridis* in the light depleted ammonia levels in the beakers, often down to undetectable levels. The control beakers showed no such depletion, indicating that ammonia was not being lost to the atmosphere. The ammonia concentration of the water containing the aposymbiotic anemones in the light increased considerably, whilst in darkness, beakers containing symbiotic *A. viridis* also showed increases in ammonia levels, although these were not as marked as in the beakers containing aposymbionts.

If the observed changes in ammonia flux were due solely to the zooxanthellae, and not to alterations in the animal tissue nitrogen metabolism in the presence of algae, then the excretion rate of the animal tissue was represented by the aposymbiotic anemones. The fact that ammonia loss from symbiotic anemones in darkness was less than that of aposymbionts shows that the zooxanthellae were conserving at least some of the excretory nitrogen produced by the animal tissues. In the light the zooxanthellae were presumably able to conserve all such nitrogen, and in addition could utilize ammonia from the surrounding water.

Whilst demonstrating that uptake of ammonia, but not nitrate, does occur in symbiotic *A. viridis* in the light, further analysis of

Figure 4.2 *Anemonia viridis*: typical time courses for nitrate concentrations in incubations of starved symbiotic anemones at an irradiance of 140 $\mu E.m^{-2}.s^{-1}$. Starting concentrations were between 14 and 20 μg-at NO_3^--$N.l^{-1}$. Each incubation beaker contained 1 litre of seawater and 6 anemones (approximate total organic weight of 6.0 g).

Figure 4.2


Nitrate concentration
(µg-at NO_3-N.l^{-1})
26
22
18
14
10
0
2
4
6
8
10
Time (hours)
control
experiment 1
experiment 2
experiment 3
experiment 4

these results was not attempted because of the variation produced by the use of six anemones per incubation vessel. In order to investigate ammonia flux more closely, an apparatus was required that allowed the study of individual anemones, and this is described in the following sections.

4.3 The effects of ammonia concentration on flux rates

4.3.1 Introduction

The method commonly adopted in previous studies with algal-coelenterate symbioses to investigate the relationship between the concentration of a nutrient in the incubation medium and the rate of uptake, has been to produce a cumulative depletion curve as in the previous section. The curve is then divided into periods and the uptake rate (calculated either as a tangent to the curve, or as the difference between the concentrations at start and finish of a period) and mean nutrient concentration calculated for each period. This approach is equally applicable to intact associations or algae in isolation from their symbiotic partner (e.g. Wilkerson and Muscatine, 1984; D'Elia *et al.*, 1983). The method becomes inadequate, however, when the data is so variable that a depletion curve can not be fitted with any degree of confidence. This was the case in the experiments described in the previous section, and consequently the data were not analysed further. An alternative is to measure the concentrations at the start and finish of a short period and then to replace the incubation medium with sea water of a different concentration and repeat the experiment. In this way, single flux rate values are obtained for each incubation. This method has the advantage that any aberrant results can be retested rapidly without the need to repeat

lengthy incubations.

The aim of the experiments in this section was, using the modified procedure outlined above, to investigate the effects of ammonia concentration on the rates of ammonia flux in single *Anemonia viridis*.

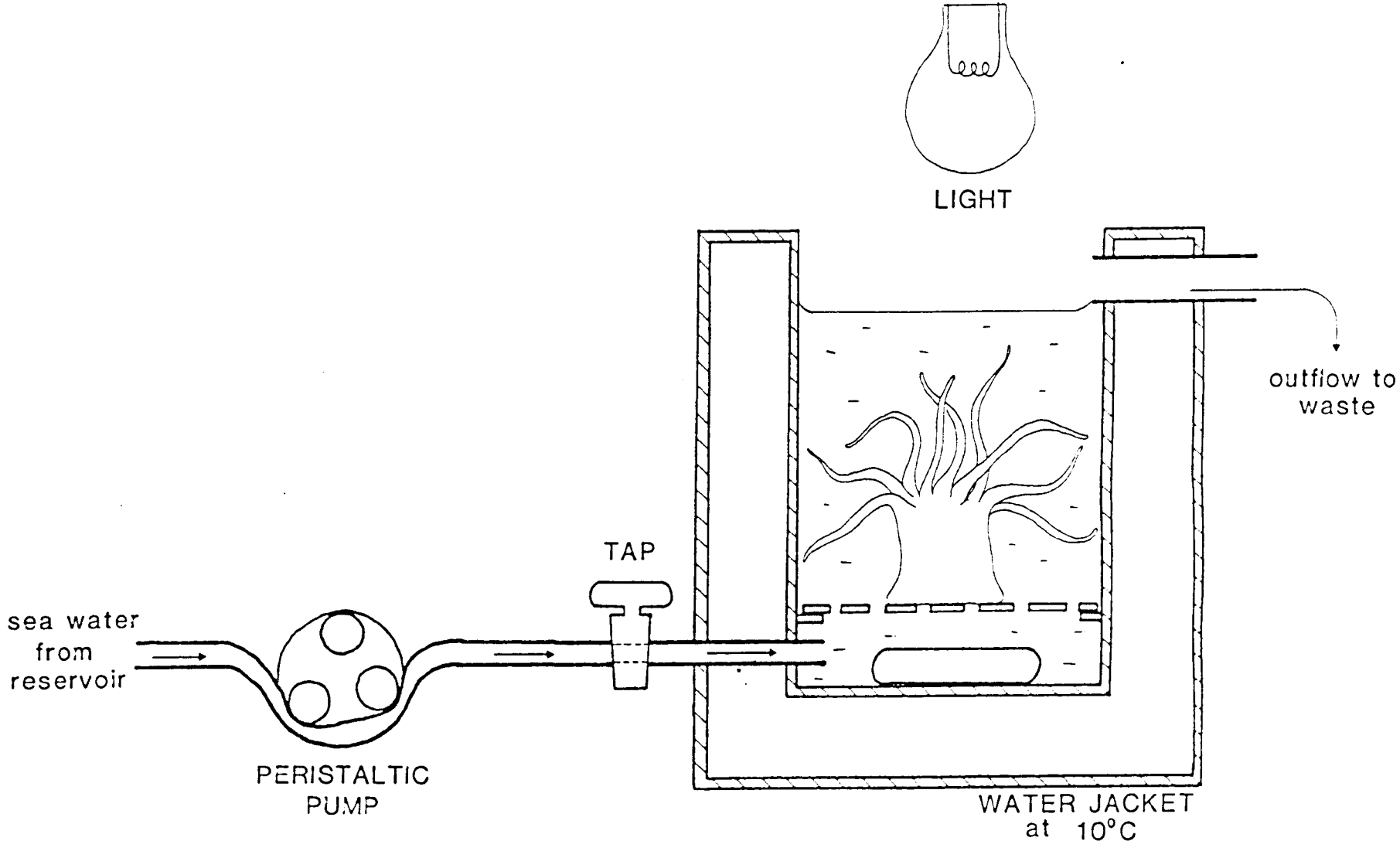
4.3.2 Methods

Incubation chambers were constructed from transparent perspex (figure 4.3). Three chambers, each 60 mm in diameter and 65 mm in height, were set into a water jacket through which water from a constant temperature water bath set at 10°C was circulated. Each chamber had a removable perforated platform supported above the floor of the chamber to allow a magnetic stir-bar to rotate underneath. Each incubation chamber had a volume of 185 ml, and could be supplied with a flow of sea water from a reservoir via a peristaltic pump. The reservoir consisted of 4 litres of sea water in a beaker secured in the water bath that supplied the water jacket of the experimental chambers. The inflow to each chamber could be isolated by a tap, and the outflow passed to waste. The water in the chambers was circulated by the magnetic stir-bars, which were rotated slowly by a low-speed d.c. electric motor fitted with a button magnet and mounted on the outside of the water jacket beneath each chamber.

In initial experiments two of the chambers contained anemones, with the third being treated and sampled in the same way (see below), but containing no anemone. This last chamber acted as a control against atmospheric losses or inputs of ammonia. In order to increase the sample size, however, the third chamber was subsequently also used as an experimental chamber. The control in these experiments was a clean 200 ml beaker, which was filled with 185 ml of water from the

Figure 4.3 Diagrammatic section through incubation chamber used to test DIN flux rates in *Anemonia viridis*.

Figure 4.3


LIGHT
outflow to waste
TAP
sea water from reservoir
PERISTALTIC PUMP
WATER JACKET at 10°C

outflows of the three experimental chambers just prior to their isolation, and sampled in the same way as the experimental chambers. Although the control beaker was not water-jacketed, the water temperature did not change appreciably (less than $1^{o}C$) over the hour of the incubation.

The chamber assembly was contained within a light hood (50 cm deep x 70 cm high x 85 cm wide) which had reflective inner walls for even lighting from all sides. A bank of fluorescent strip lights (20 W warm white, Thorn), at a height of 50 cm above the top of the chambers, could be controlled with a variable transformer to give light levels between 0 and 190 $\mu E.m^{-2}.s^{-1}$ at the water surface level. Higher light levels, to 300 $\mu E.m^{-2}.s^{-1}$ could be obtained by raising the chambers. Light intensity was measured with a Skye quantum sensor (Skye Instruments SKP 210, attached to SKP 200 meter, measuring photosynthetically active radiation at 400 to 700 nm) clamped at the same height as the incubation chambers.

To obtain data on hourly flux rates, single anemones were placed in each chamber and sea water from the Zoology Department circulating sea water system (ammonia levels less than 0.1 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$) was pumped through the chambers for two hours, to allow the anemones to settle. Any anemone not attaching to the platform or failing to expand fully was replaced or discounted. Once the anemones had settled, the low-ammonia sea water in the reservoir was replaced by sea water which had been spiked with ammonium sulphate to a predetermined level, and this was pumped through the chambers until at least a litre had passed through each. This took approximately 15 minutes and ensured that the chamber water was completely replaced with water of the required ammonia concentration without disturbing the anemones. The taps on the inflow line were then closed, and 5 ml water samples were collected from each chamber into acid-washed 2-dram glass vials. After one hour

the chambers were sampled a second time and the start and finish water samples were analysed for ammonia as described in section 2.5.1.2.

Hourly uptake rates were calculated from the following formula:

$$\rho = \Delta S \times v \times t^{-1} \times w^{-1} \qquad (1)$$

where:

ρ = the specific uptake rate, in μg-at NH_3-N.g organic $wt^{-1}.h^{-1}$.

ΔS = change in NH_3 concentration in chamber, in μg-at NH_3-N.l^{-1}.

v = volume of chamber in litres.

t = length of incubation in hours.

w = organic weight of anemone in grams.

Values of the specific uptake rate, ρ, were plotted against S, the mean chamber ammonium concentration during the incubation.

To obtain flux rates for a range of S values, the chambers were flushed through with sea water spiked to a different concentration of ammonia, and the experiment was repeated. In this way six incubations could be carried out within the normal 12 hour light period, and a typical set of incubations would have starting concentrations of 0, 2, 4, 6, 8 and 10 μg-at NH_3-N.l^{-1}. After such a series of experiments the anemones were buoyant weighed (section 2.4.1) and returned to the stock tanks. Both symbiotic and aposymbiotic anemones were tested using this method, with the ammonia efflux of aposymbionts being considered as a negative uptake.

The light level throughout the experiments was 190 $\mu E.m^{-2}.s^{-1}$, which exceeds the value of 161 $\mu E.m^{-2}.s^{-1}$, the lowest value for photosynthetic saturation in *A. viridis* recorded by Tytler and Davies (1984).

4.3.3 Results

The symbiotic anemones took up ammonia at all incubation concentrations tested, and the uptake rates were faster at higher ambient ammonia concentrations (figure 4.4). The relationships between uptake rate and incubation concentration appear to be linear over the range of concentrations used in the experiments. Least squares regressions were fitted to the data from each anemone, and the equations are given in table 4.1.

The calculated regression lines were compared by covariance analysis, using the method of Snedecor (1966), and no statistical difference was found, either in slopes or elevations (F ratios of 1.573, 5 and 24 d.f., and 1.812, 5 and 29 d.f. respectively, $P>0.1$ N.S. for both). This suggests that the data can be considered as six subsamples from a larger sample. Following the method of Snedecor (1966) further, the sums of squares and sums of products of the regression lines were pooled, to give a common regression line with the equation $y = 0.132x + 0.038$, the slope of which is significantly different from zero (t-test, $P<0.001$).

In phytoplankton and algal-coelenterate symbioses the relationship between nutrient uptake rate and nutrient concentration is often graphically described by a rectilinear hyperbola, the result of Michaelis-Menten uptake kinetics (Dugdale, 1967; Muscatine, 1980). The flattening out of the curve represents the uptake mechanism approaching a maximum uptake rate as it becomes saturated. None of the experiments described above showed any sign of decrease in slope at higher nutrient concentrations, and this may have been due to the maximum concentrations used being too low to allow uptake rates to approach maximum. A set of experiments was therefore carried out as above, but with much higher concentrations of ammonia in the

Figure 4.4 *Anemonia viridis*: weight-specific hourly ammonia flux rates at different incubation ammonia concentrations for six starved symbiotic anemones. Uptake rates were calculated from the difference in ammonia concentration over a 1 hour incubation. Light level was 190 $\mu E.m^{-2}.s^{-1}$. Positive values of ρ indicate net uptake of ammonia by the symbiosis, negative values indicate net loss of ammonia to the incubation water. The equations for linear regression lines fitted to the data for each anemone are given in table 4.1.

Figure 4.4

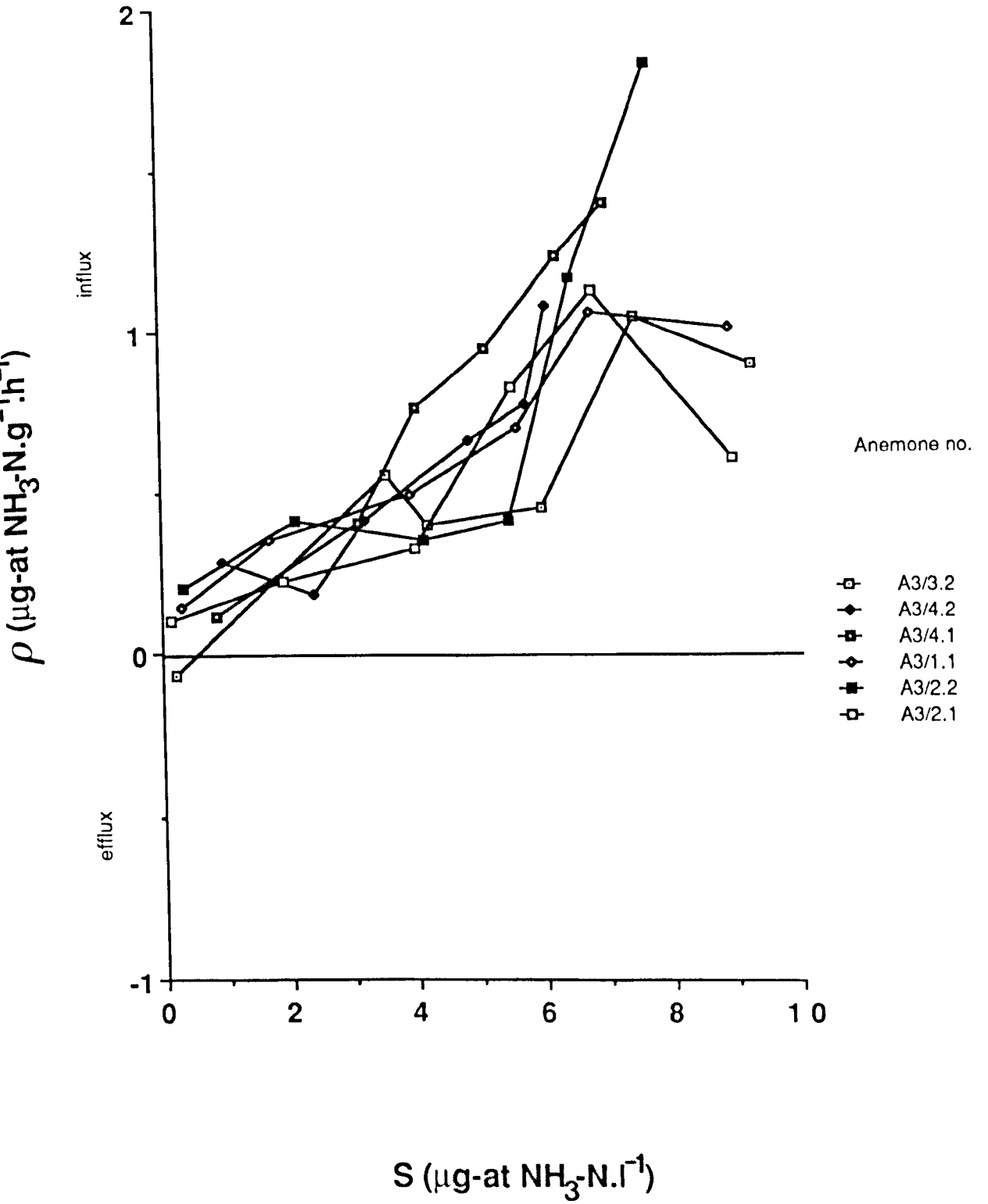

2
1
0
-1
0
2
4
6
8
10
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
A3/3.2
A3/4.2
A3/4.1
A3/1.1
A3/2.2
A3/2.1

Table 4.1 *Anemonia viridis*: equations of linear regression lines fitted to the ammonia uptake rate/ incubation concentration data shown in figure 4.4. Anemones were starved symbionts, and were exposed to an irradiance of 190 $uE.m^{-2}.s^{-1}$.

Anemone number	No. of points	Equation of regression line
A3/1.1	6	y = 0.109x + 0.127
A3/2.1	6	y = 0.090x + 0.119
A3/2.2	6	y = 0.188x - 0.099
A3/3.2	6	y = 0.111x - 0.026
A3/4.1	6	y = 0.213x - 0.137
A3/4.2	6	y = 0.150x - 0.023
Common line		y = 0.132x + 0.038

incubation medium. These concentrations exceeded by far the likely concentration range in the anemones' natural environment, and were conducted solely to attempt to elucidate the mechanism controlling ammonia uptake in this association. Five symbiotic *A. viridis* were exposed to mean ammonia concentrations between 5 and 30 µg-at NH_3-N.l^{-1} at a light intensity of 100 µE.m^{-2}.s^{-1}, and all produced linear relationships between uptake rate and concentration (figure 4.5). When compared using covariance analysis, there was no significant difference in slopes between the five anemones (F_{slopes}= 1.274, 4 and 20 d.f., P>0.25 N.S., B= 0.111). The slopes were compared with those of the other anemones tested at lower ammonia concentrations under the same light intensity (see section 4.4.3), and no statistical difference was found (F_{slopes}= 0.125, 1 and 53 d.f., P>0.50 N.S.). It therefore appears that saturation of ammonia uptake does not start to occur at concentrations below 30 µg-at NH_3-N.l^{-1}.

Aposymbiotic anemones under identical conditions to those used for the symbiotic anemones gave negative values of ρ, confirming that in the absence of zooxanthellae *A. viridis* excrete ammonia (figure 4.6). As with the symbiotic anemones, there appears to be a linear relationship between ρ and S, with efflux rate decreasing with increasing mean ammonia concentration. Table 4.2a gives the regression equations for the excretion rates. When the lines were compared by covariance analysis, no significant difference in slopes was found (F ratio = 1.849, 5 and 24 d.f. P>0.1 N.S.), and the common regression coefficient was significantly different from zero (B = 0.062, P<0.001). The elevations of the slopes, however, were significantly different (F = 60.451, 5 and 29 d.f., P<0.001).

The aposymbiotic anemones were able to produce relatively high concentrations of ammonia in the water when incubated for an hour, and this presented two potential problems. Firstly, it was not possible to

Figure 4.5 *Anemonia viridis*: ammonia uptake rates at different incubation ammonia concentrations for five starved symbiotic anemones exposed to irradiances of 100 $\mu E.m^{-2}.s^{-1}$. In an attempt to test for saturating uptake rates at high concentrations of ammonia, anemones were incubated in seawater with ammonia concentrations of between 5 and 35 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$. The equations of linear regressions fitted to the data for each anemone were:

$y = 0.093x - 0.205$

$y = 0.096x + 0.317$

$y = 0.109x - 0.088$

$y = 0.112x + 0.097$

$y = 0.120x - 0.280$

The slopes of these lines were compared by covariance analysis with those for starved symbiotic *A. viridis* under the same irradiance but tested at ammonia concentrations between 0 and 10 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$ (see table 4.3). Results of this comparison are presented in the text.

Figure 4.5

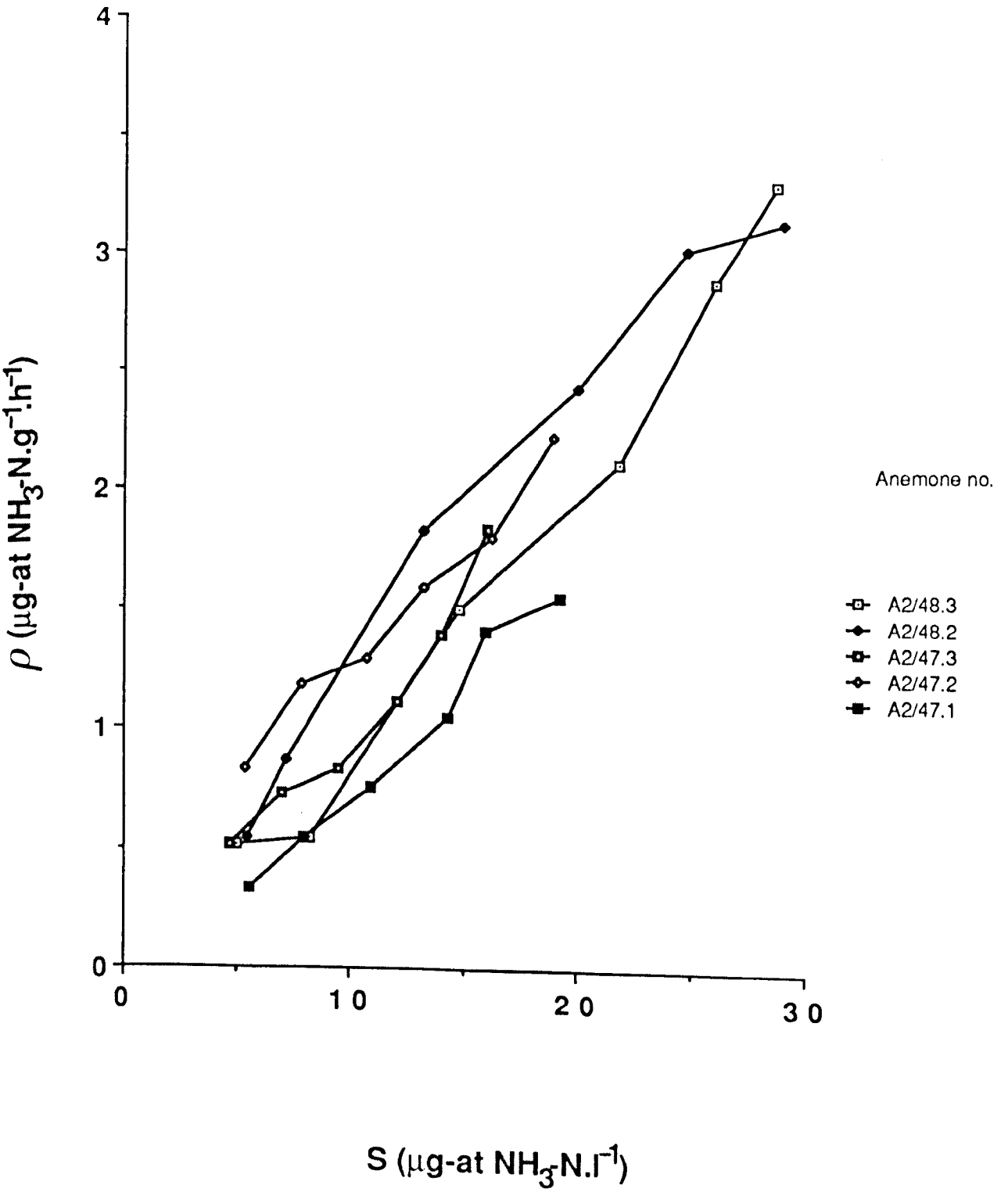

4
3
2
1
0
0
10
20
30
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
A2/48.3
A2/48.2
A2/47.3
A2/47.2
A2/47.1

Figure 4.6 *Anemonia viridis*: ammonia flux rates at different incubation ammonia concentrations for six starved aposymbiotic anemones. Light level was 190 $\mu E.m^{-2}.s^{-1}$. Negative values of ρ indicate net loss of ammonia from the symbiosis to the incubation water. The equations for linear regression lines fitted to the data from each anemone are given in table 4.2a.

Figure 4.6 ·

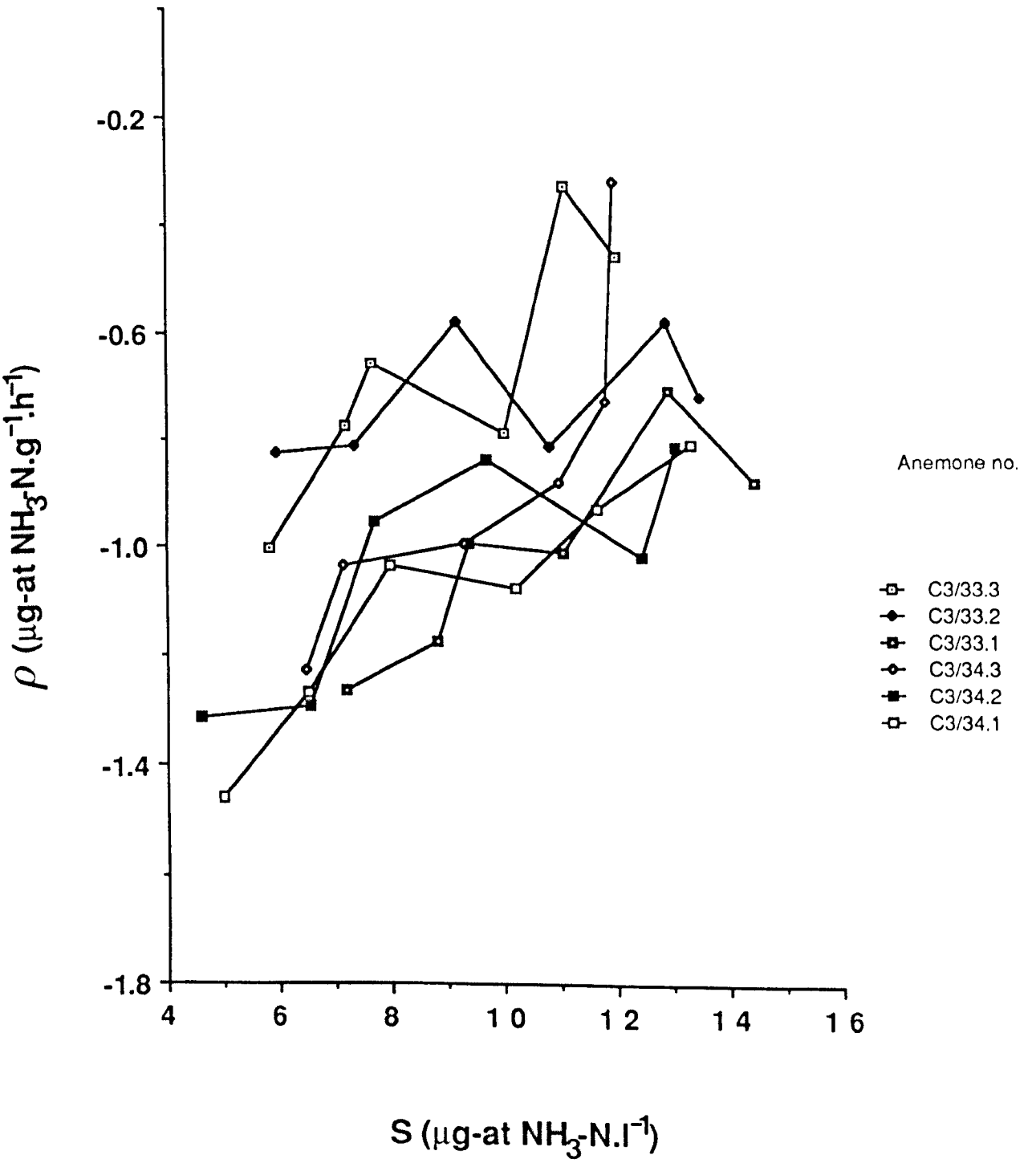

-0.2
-0.6
-1.0
-1.4
-1.8
4
6
8
10
12
14
16
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
C3/33.3
C3/33.2
C3/33.1
C3/34.3
C3/34.2
C3/34.1

Table 4.2 *Anemonia viridis*: equations of linear regression lines fitted to the ammonia uptake rate/incubation concentration data for starved aposymbiotic individuals at an irradience of 190 $\mu E.m^{-2}.s^{-1}$. a) Anemones incubated for 1 hour to obtain each value of (data shown in figure 4.6). b) Anemones incubated for 1/2 hour for each value of (data given in appendix II). The regression coefficients of all 12 anemones were compared by covariance analysis and were found not to differ significantly (result in text).

a) 1 hour incubations

Anemone number	No. of points	Equation of regression line
C3/33.1	6	y = 0.064x - 1.686
C3/33.2	6	y = 0.020x - 0.920
C3/33.3	6	y = 0.083x - 1.414
C3/34.1	6	y = 0.070x - 1.734
C3/34.2	6	y = 0.051x - 1.494
C3/34.3	6	y = 0.112x - 1.941

b) 1/2 hour incubations

Anemone number	No. of points	Equation of regression line
C3/50.1	6	y = 0.039x - 1.198
C3/50.2	6	y = 0.126x - 1.677
C3/50.3	6	y = 0.086x - 1.873
C3/51.1	6	y = 0.067x - 1.803
C3/51.2	6	y = 0.053x - 1.485
C3/51.3	6	y = 0.073x - 1.802

produce low S values, since even with an initial concentration of 0 μg-at NH_3-N.l^{-1}, a typical flux rate of -1.4 μg-at NH_3-N.g^{-1}.h^{-1} meant that the 185ml chamber would have a concentration of 7.57 μg-at NH_3-N.l^{-1} at the end of the hour, giving S a value of 3.78 μg-at NH_3-N.l^{-1}. Thus the relationship between φ and S close to S= 0 had to be extrapolated from points with much higher S values than those for the symbiotic anemones. Secondly, there was a possibility that efflux into higher than natural concentrations of ammonia might be affected by the high substrate build-up in the medium. Although this was, to an extent, what the experiments were designed to demonstrate, abnormally high extracellular ammonia concentrations may have unforseen effects on aspects of the cells' metabolism not directly related to the systems under investigation. To avoid these problems the experiments were repeated using half-hour incubation periods, and the equations for the regression lines obtained are given in table 4.2b. When tested using covariance there proved to be no significant differences in slopes ($F= 0.510$, 5 and 24 d.f., $P>0.75$ N.S.), and the common regression coefficient was significantly different from zero ($B= 0.071$, $P<0.001$). The elevations, however, varied significantly ($F= 25.517$, 5 and 29 d.f., $P<0.001$), as with the one hour incubations.

The combined slopes of the regressions from both sets of experiments were compared by further covariance analysis, and were not significantly different ($F= 0.208$, 1 and 58 d.f., $P>0.5$ N.S.). Elevations could not be compared, since these varied significantly within each set of experiments. It would therefore appear that there is little difference in the relationships between φ and S that can be attributed to length of incubation period. The shorter incubation period allowed smaller values of S to be produced, and hence more confident predictions of the value of φ when the regression lines

intercept the y-axis.

Comparison of figures 4.4, 4.5 and 4.6 suggests that there is a difference both in slopes and elevations between the φ and S relationships of symbiotic and aposymbiotic anemones. The difference for slopes was confirmed statistically by covariance analysis ($F= 13.255$, 1 and 88 d.f., $P<0.001$).

A single experiment was conducted to recheck the observation that symbiotic *A. viridis* do not take up nitrate (see section 4.2.3). Three symbiotic anemones were treated as in the above experiments, except that the concentration of nitrate, instead of ammonia, was varied between 10 and 3 µg-at NO_3^--N.l^{-1} (ammonia levels were undetectable during this experiment). Because of high ambient concentrations of nitrate in the circulating sea water system, low levels of nitrate were achieved by using freshly collected and filtered sea water. Although the nitrate assay was subject to more variation than that for ammonia, and consequently results were also more variable, there was no evidence of any nitrate flux at any concentration tested (data are given in appendix II). Regression analysis produced slopes that were not significantly different from zero, even when all the data were combined by covariance techniques, and the variation in flux rates was around the equilibrium point (φ= 0 µg-at NH_3-N.g^{-1}.h^{-1}).

4.4 The effect of light on ammonia flux rates

4.4.1 Introduction

Section 4.2 showed that symbiotic *A. viridis* exhibited ammonia uptake in light, and efflux in darkness. This difference was presumed to be due to the uptake mechanism being associated with the photosynthetic activity of the zooxanthellae. It is known that fixed

carbon is necessary for nitrogen fixation in microalgae, and that this carbon may be provided directly from photosynthesis or from storage products (Thacker and Syrett, 1972; Syrett, 1981; Elrifi and Turpin, 1987). Since storage products may be limited in quantity, photosynthesis would be expected to provide much of the fixed carbon for nitrogen uptake, at least during daylight hours. Photosynthetic rates of zooxanthellae *in situ* in *A. viridis* have been found to increase in direct proportion to light intensity between 34 and 120 $\mu E.m^{-2}.s^{-1}$ (Tytler and Davies, 1984). The relationship becomes curvilinear between 120 and 190 $\mu E.m^{-2}.s^{-1}$, presumably leading to saturation of photosynthesis at some intensity greater than 190 $\mu E.m^{-2}.s^{-1}$. It is therefore possible that DIN uptake rates also increase with light intensity, which would result in an anemone exposed to a natural light cycle exhibiting varying flux rates over a twenty four hour period.

The aim of this set of experiments was to investigate the effect of increasing light intensity on the relationship between mean substrate concentration and specific ammonia flux rate. The approach was intended partly to allow the prediction of uptake rates at known light intensities and ammonia concentrations, so that predictions of uptake rates could be made from measurements of light and nutrient levels in the field, and partly to provide information on the mechanism controlling ammonia fluxes in the intact association.

4.4.2 Methods

The procedure used was similar to that described in the previous section (4.3.2). Light levels of 5, 10, 20, 50, 100 and 300 $\mu E.m^{-2}.s^{-1}$ and darkness were tested, using a minimum of six starved symbiotic *A. viridis* at each light intensity. These numbers of anemones were

occasionally reduced by individuals that failed to settle. Because the protocol for this section was exactly the same as that used in section 4.3, the data obtained for anemones tested at 190 $\mu E.m^{-2}.s^{-1}$ in that section are included in the analysis of these results.

It was found in the experiments described in section 4.3 that anemones which did not settle or expand fully showed N flux rates that differed from well-expanded specimens. Symbiotic anemones of several species, including *A. viridis*, are known to have behavioural responses to light which result in daytime expansion and nighttime contraction (Pearse, 1974b; Sebens and DeReimer, 1977; Dorsett, 1984). Although aposymbiotic anemones, having no photosynthesizing algae, were expected to be unaffected by light intensity, they show a similar expansion/contraction cycle to symbionts. Because of this, six aposymbionts were tested, in the same way as symbionts, in darkness to determine the presence or absence of changed excretion rates due to state of expansion.

All experiments commenced at 0800 hours, which corresponded with the beginning of the normal 12 hour aquarium light period. Anemones were transferred in covered buckets from their holding aquaria and placed in the incubation chambers at the light intensity under which they were to be tested. They were allowed a two hour settling period before the commencement of the experiment. During dark incubations the laboratory was kept in darkness except during sampling, when the anemones were exposed briefly to a low intensity red light.

The experiments outlined above took place during the anemones' "daylight" period, irrespective of light level. Dark incubations were therefore carried out with 12 hours of pretreatment in darkness, and at a time when the anemones were normally under full aquarium illumination. To investigate the possibility that these conditions produced flux rates that differed from those actually occurring in

aquarium anemones during a normal 24 hour cycle of light and dark, experiments were carried out that tested the flux rates of anemones at a single value of S, at intervals over a complete light/dark cycle. A second aim of these experiments was to ascertain how long it took for the anemones to change from "dark" flux rates to "light" flux rates, or vice versa, when the lights were turned on or off.

The 24 hour experiments used the apparatus described in section 4.3.2, with a single symbiotic anemone in each incubation chamber. The anemones were allowed to settle for two hours (see section 4.3.2) before the first incubation. The first incubation, with an initial chamber concentration of 4 μg-at NH_3-N.l^{-1}, started between 1300 and 1400 hours (approximately 6 hours into the anemones normal light period), at a light intensity of 190 μE.m^{-2}.s^{-1}. The proce dure for the incubation, and the calculation of flux rates, was as described in section 4.3.2. After completion of the incubation and sampling of the final ammonia concentrations, the chambers were flushed through with sea water again containing 4 μg-at NH_3-N.l^{-1}, for 15 minutes, after which the chambers were isolated and a second incubation was carried out. This proce dure was repeated throughout the following 24 hours, one hour of incubation being followed by 15 minutes of flushing with sea water containing 4 μg-at NH_3-N.l^{-1}. At 2000 hours the lights were turned off, and they were turned back on again at 0800 hours the next morning, to simulate the light/dark cycle in the aquaria. Flux data for six symbiotic anemones were obtained in this way. A similar protocol was used for aposymbionts, except that half-hour incubations (see section 4.3.3) were used instead of one-hour incubations.

4.4.3 Results

The results of typical incubations under two different light

intensities are shown in figures 4.7 and 4.8, and the full results are given in appendix II. There was considerably more variation in the data for the lower light intensities than for the higher light intensities (50 $\mu E.m^{-2}.s^{-1}$ and above), particularly in the apparent elevations of the φ vs. S relationships for each anemone. Many of the data suggest a linear relationship between φ and S and table 4.3 gives the equations for regression lines fitted to the data from each anemone.

The regression lines for the group of anemones under each light intensity were compared using covariance analysis, and the results are given in table 4.4. Only the lines for anemones at 50, 190 and 300 $\mu E.m^{-2}.s^{-1}$ show no statistical difference in either slopes or elevations. The lines for symbiotic anemones in darkness and at 5, 10, 20 and 100 $\mu E.m^{-2}.s^{-1}$, and for aposymbiotic anemones in darkness and at 190 $\mu E.m^{-2}.s^{-1}$ show no differences in slope within each treatment, whilst giving statistically different within-treatment elevations.

Since there was no significant difference in the regression coefficients within each treatment, it was possible to calculate a common regression coefficient for each treatment as before (section 4.3.3). These values are given in table 4.4, and include the common regression coefficients determined for aposymbionts and symbionts at 190 $\mu E.m^{-2}.s^{-1}$ in section 4.3.3. To test the null hypothesis that light had no effect on the slopes of the lines relating flux to concentration, all of these regression coefficients were compared in a single covariance analysis. This test showed significant differences in slopes ($F= 2.054$, 10 and 333 d.f., $P<0.025$), and disproved the null hypothesis. The test could not, however, show if the differences were due to all or some of the treatments. Examination of the data in table 4.4 suggests a distinct discontinuity in the distribution of regression coefficients between 50 and 100 $\mu E.m^{-2}.s^{-1}$, thereby

Figure 4.7 *Anemonia viridis*: ammonia flux rates at different incubation ammonia concentrations for nine starved symbiotic anemones in darkness. Experiments took place after 14 hours pretreatment in darkness, during periods when the anemones would normally have been exposed to full aquarium illumination (140 $\mu E.m^{-2}.s^{-1}$). Positive values of ρ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water. The equations for linear regression lines fitted to the data are given in table 4.3.

Figure 4.7

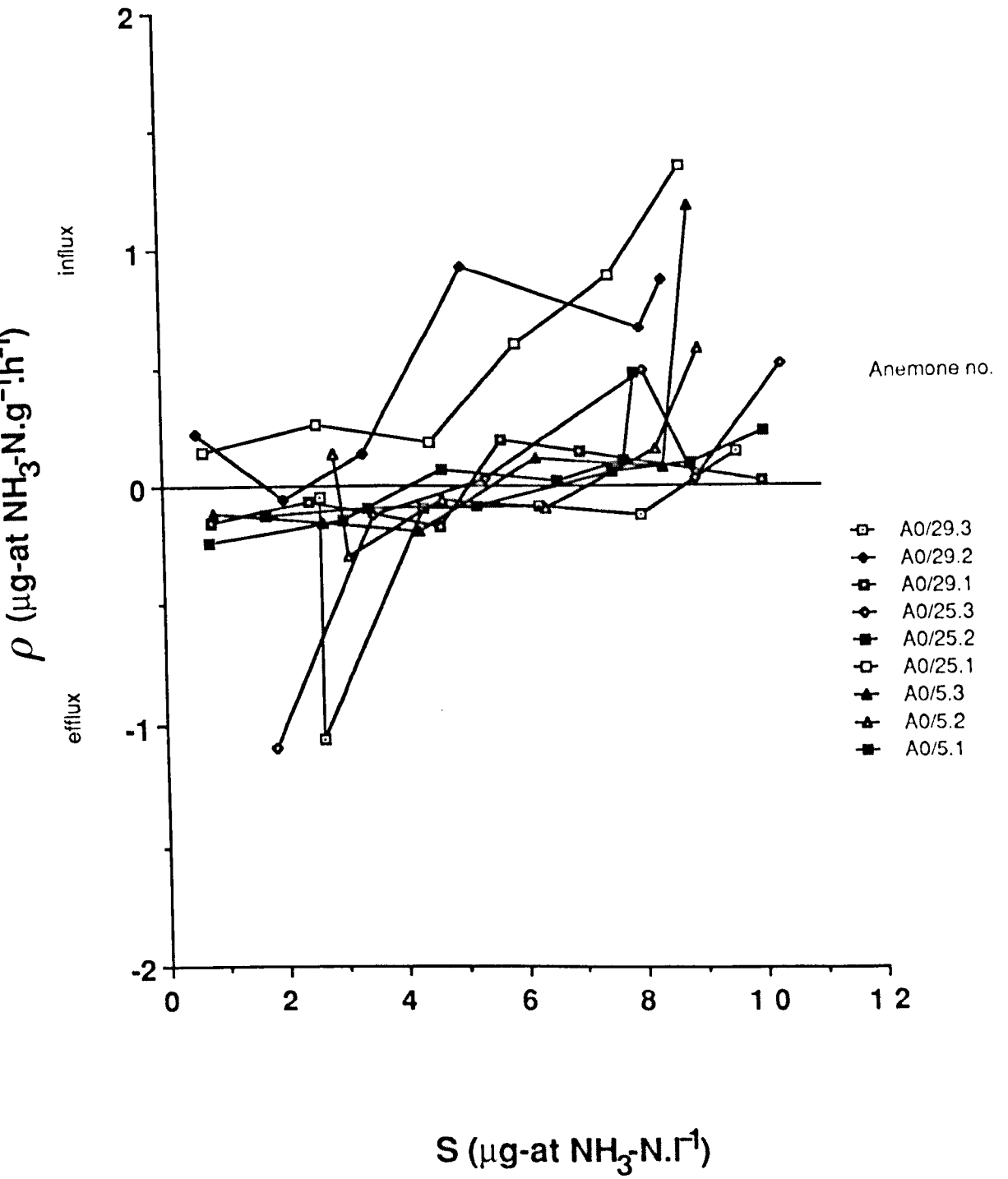

2
1
0
-1
-2
0
2
4
6
8
10
12
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
A0/29.3
A0/29.2
A0/29.1
A0/25.3
A0/25.2
A0/25.1
A0/5.3
A0/5.2
A0/5.1

Figure 4.8 *Anemonia viridis*: ammonia flux rates at different incubation ammonia concentrations for six starved symbiotic anemones exposed to a light level of 100 $\mu E.m^{-2}.s^{-1}$. Experiments took place after 12 hours of darkness, followed by two hours settling time at the experimental light level. Positive values of φ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water. The equations for linear regression lines fitted to the data are given in table 4.3.

Figure 4.8

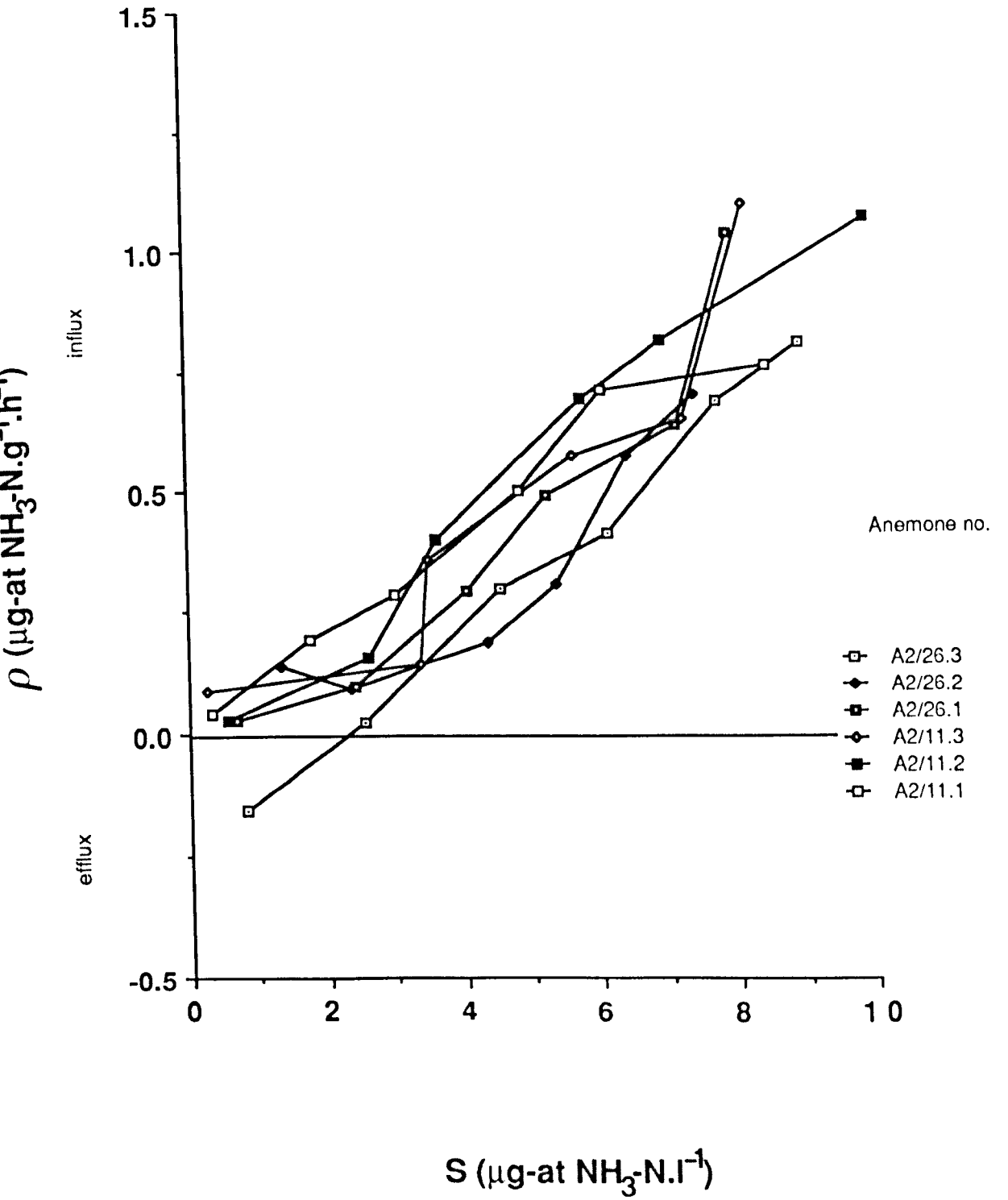

1.5
1.0
0.5
0.0
-0.5
0
2
4
6
8
10
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
A2/26.3
A2/26.2
A2/26.1
A2/11.3
A2/11.2
A2/11.1

Table 4.3 *Anemonia viridis*: equations of linear regression lines fitted to the ammonia flux rate/incubation concentration data of individual starved anemones, both symbiotic and aposymbiotic, exposed to a range of light intensities. The data for starved symbionts in darkness and at 100 $\mu E.m^{-2}.s^{-1}$ are shown in figures 4.7 and 4.8 respectively. All data is given in appendix II. Incubations were of one hour duration for symbiotic anemones, and of 1/2 hour duration for aposymbionts.

Anemone number	Light intensity ($\mu E.m^{-2}.s^{-1}$)	No. of points	Equation of regression line
A0/ 5.1	0 (symbionts)	6	y = 0.073x - 0.328
A0/ 5.2		6	y = 0.079x - 0.388
A0/ 5.3		6	y = 0.112x - 0.434
A0/25.1		6	y = 0.142x - 0.146
A0/25.2		6	y = 0.040x - 0.234
A0/25.3		6	y = 0.150x - 0.981
A0/29.1		6	y = 0.026x - 0.142
A0/29.2		6	y = 0.101x - 0.010
A0/29.3		6	y = 0.086x - 0.696
05/44.1	5 (symbionts)	6	y = 0.139x - 1.387
05/44.2		6	y = 0.176x - 1.882
05/44.3		6	y = 0.075x + 0.030
05/46.1		6	y = 0.098x + 0.004
05/46.2		6	y = 0.104x - 1.445
05/46.3		6	y = 0.092x + 0.158
10/42.2	10 (symbionts)	6	y =-0.014x + 0.484
10/42.3		6	y = 0.066x - 0.821
10/43.1		6	y = 0.124x + 0.211
10/43.2		6	y = 0.033x - 0.924
10/43.3		6	y = 0.096x - 0.755
20/39.1	20 (symbionts)	5	y = 0.121x - 0.214
20/39.2		6	y = 0.026x - 0.798
20/39.3		6	y = 0.157x + 0.024
20/41.1		6	y = 0.061x + 0.058
20/41.2		6	y = 0.078x + 0.149
20/41.3		6	y = 0.069x - 0.673
A1/ 7.1	50 (symbionts)	6	y = 0.100x - 0.103
A1/ 7.2		6	y = 0.043x + 0.119
A1/ 7.3		6	y = 0.080x - 0.035
A1/12.1		6	y = 0.082x + 0.017
A1/12.2		6	y = 0.116x - 0.097
A1/12.3		6	y = 0.051x + 0.200
A2/11.1	100 (symbionts)	6	y = 0.096x + 0.022
A2/11.2		6	y = 0.119x - 0.058
A2/11.3		6	y = 0.118x - 0.073
A2/26.1		6	y = 0.130x - 0.167
A2/26.2		6	y = 0.097x - 0.112
A2/26.3		6	y = 0.120x - 0.272
A4/ 9.1	300 (symbionts)	6	y = 0.139x - 0.113
A4/ 9.2		6	y = 0.122x - 0.043
A4/ 9.3		6	y = 0.091x - 0.008
A4/10.2		6	y = 0.139x - 0.281
A4/10.3		6	y = 0.131x - 0.158
C0/35.1	0 (aposymbionts)	6	y = 0.046x - 1.152
C0/35.2		6	y = 0.064x - 1.342
C0/35.3		6	y = 0.044x - 0.898
C0/40.1		6	y = 0.122x - 2.085
C0/40.2		6	y = 0.075x - 1.985
C0/40.3		6	y = 0.135x - 2.158

Table 4.4 *Anemonia viridis*: results of covariance analysis (Snedecor, 1966) carried out to compare slopes and elevations of the ammonia uptake rate vs. incubation concentration relationships for starved anemones under different light intensities. Results are given for comparisons within each light treatment (symbiotic and aposymbiotic anemones under similar light intensities were considered as separate treatments). Also shown are the common regression coefficients calculated for each treatment by pooling sums of squares and sums of products for individual anemones, and common elevations (calculated in the same way) for treatments where no significant differences in elevation could be shown.

* aposymbionts, 1/2 hour incubations
** aposymbionts, 1 hour incubations

Illumination ($\mu E.m^{-2}.s^{-1}$)	No. of anemones in comparison	Variance ratio (F) for slopes	Variance ratio (F) for elevations	Common regression coefficient	Common y-intercept
0	9	1.192, 8 and 36 d.f. N.S., P>0.25	5.933, 8 and 44 d.f. P<0.001	0.090	-
5	6	0.556, 5 and 24 d.f. N.S., P>0.50	35.621, 5 and 29 d.f. P<0.001	0.113	-
10	5	1.027, 4 and 20 d.f. N.S., P>0.25	25.688, 4 and 24 d.f. P<0.001	0.072	-
20	6	0.277, 5 and 23 d.f. N.S., P>0.75	6.501, 5 and 28 d.f. P<0.001	0.078	-
50	6	1.608, 5 and 24 d.f. N.S., P>0.10	0.732, 5 and 29 d.f. N.S., P>0.05	0.079	0.010
100	6	0.625, 5 and 24 d.f. N.S., P>0.50	4.034, 5 and 29 d.f. P<0.010	0.114	-
190 (for comparison)	6	1.573, 5 and 24 d.f. N.S., P>0.10	1.724, 5 and 29 d.f. N.S., P>0.10	0.132	0.038
300	5	0.852, 4 and 20 d.f. N.S., P>0.50	1.628, 4 and 24 d.f. N.S., P>0.10	0.123	-0.116
0*	6	2.148, 5 and 24 d.f. N.S., P>0.05	8.375, 5 and 29 d.f. P<0.001	0.071	-
190**	6	1.849, 5 and 24 d.f. N.S., P>0.10	9.043, 5 and 29 d.f. P<0.001	0.062	-
190*	6	0.510, 5 and 24 d.f. N.S., P>0.75	3.141, 5 and 29 d.f. P<0.025	0.071	-

creating two groups: symbionts at 0 to 50 $\mu E.m^{-2}.s^{-1}$ and all aposymbionts, and symbionts at 100 to 300 $\mu E.m^{-2}.s^{-1}$. To test this, the common regression coefficients for the two groups were compared in separate covariance analyses. In both cases there were no significant differences in the regression coefficients within the grouped treatments (table 4.5). Common regression coefficients for each of these two groups were then obtained as before, after pooling sums of squares and sums of products (Snedecor, 1966), to give values of 0.080 for the aposymbiotic and "low-light" anemones, and 0.123 for the "high-light" symbiotic anemones. Comparison of these common regression coefficients by covariance analysis confirmed that they were statistically different ($F= 13.571$, 1 and 332 d.f., $P<0.001$).

Because the elevations of regression lines within each treatment were generally statistically different, it was not possible to use covariance analysis to test for elevation differences between treatments. Despite this, it was clear from the data that there was a vertical difference in the positions of the regressions for aposymbionts and symbionts (compare figures 4.4 and 4.6), and it was possible that similar, if smaller, differences occurred between the symbiotic anemones in different treatments. To test for this, it was first necessary to replot the data from individual anemones to one of the two common regression coefficients calculated in the preceding paragraph. In this way, all "low-light" anemones had their regression lines replotted to a slope of 0.080, and "high-light" anemones to a slope of 0.123, which resulted in two groups of parallel lines. The elevation of the line for each anemone was then taken as the y-intercept value, and the mean elevation of the anemones from each treatment calculated. The mean values are given in table 4.6, and the treatment regression lines, plotted to corrected regression coefficient and mean y-intercept, are shown in figure 4.9. The

Table 4.5 *Anemonia viridis*: results of covariance analyses carried out to compare the common regression coefficients of starved anemone treatments (see table 4.4). All treatments were initially compared in a single analysis, and then two further analyses were carried out on treatments within the two groups formed by dividing the treatments into aposymbionts (irrespective of irradience) and symbionts at irradiences of 50 $\mu E.m^{-2}.s^{-1}$ and below, and symbionts at irradiences of 100 $\mu E.m^{-2}.s^{-1}$ and above. The common regression coefficients for treatments within the two groups were calculated by pooling all sums of squares and sums of products from the treatments compared, and are shown in the table.

Treatments compared (light intensity, in $\mu E.m^{-2}.s^{-1}$)	No. of anemones in treatment	Treatment common regression coefficient	Treatment common y-intercept	F ratio (slopes)	Between-treatment common regression coefficient
All treatments	-	-	-	2.054, 10 and 323 d.f. P<0.025	-
0*	6	0.071	various		
190**	6	0.062	various		
190*	6	0.071	various	0.760,	
0	9	0.090	various	7 and 241 d.f.,	
5	6	0.113	various	N.S.	0.080
10	5	0.072	various	P>0.50	
20	6	0.078	various		
50	6	0.079	0.010		
100	6	0.114	various	0.683,	
190	6	0.132	0.038	2 and 82 d.f.,	0.123
300	5	0.123	-0.116	N.S., P>0.50	

* aposymbionts, 1/2 hour incubations
** aposymbionts, 1 hour incubations

Table 4.6 *Anemonia viridis*: mean y-intercept values calculated for each starved anemone light treatment, after replotting the lines of individual anemones to the common regression coefficients given in table 4.5. The mean y-intercept and common regression coefficient given in the table were used to plot the regression lines for each treatment, shown in figure 4.9.

Irradiance ($\mu E.m^{-2}.s^{-1}$)	No. of anemones	Mean y-intercept	Common regression coefficient
0*	6	-1.557	0.080
190*	12	-1.708	0.080
all aposymbionts	18	-1.657	0.080
0	9	-0.318	0.080
5	6	-0.505	0.080
10	5	-0.495	0.080
20	6	-0.253	0.080
50	6	0.004	0.080
100	6	-0.153	0.123
190	6	0.079	0.123
300	5	-0.116	0.123

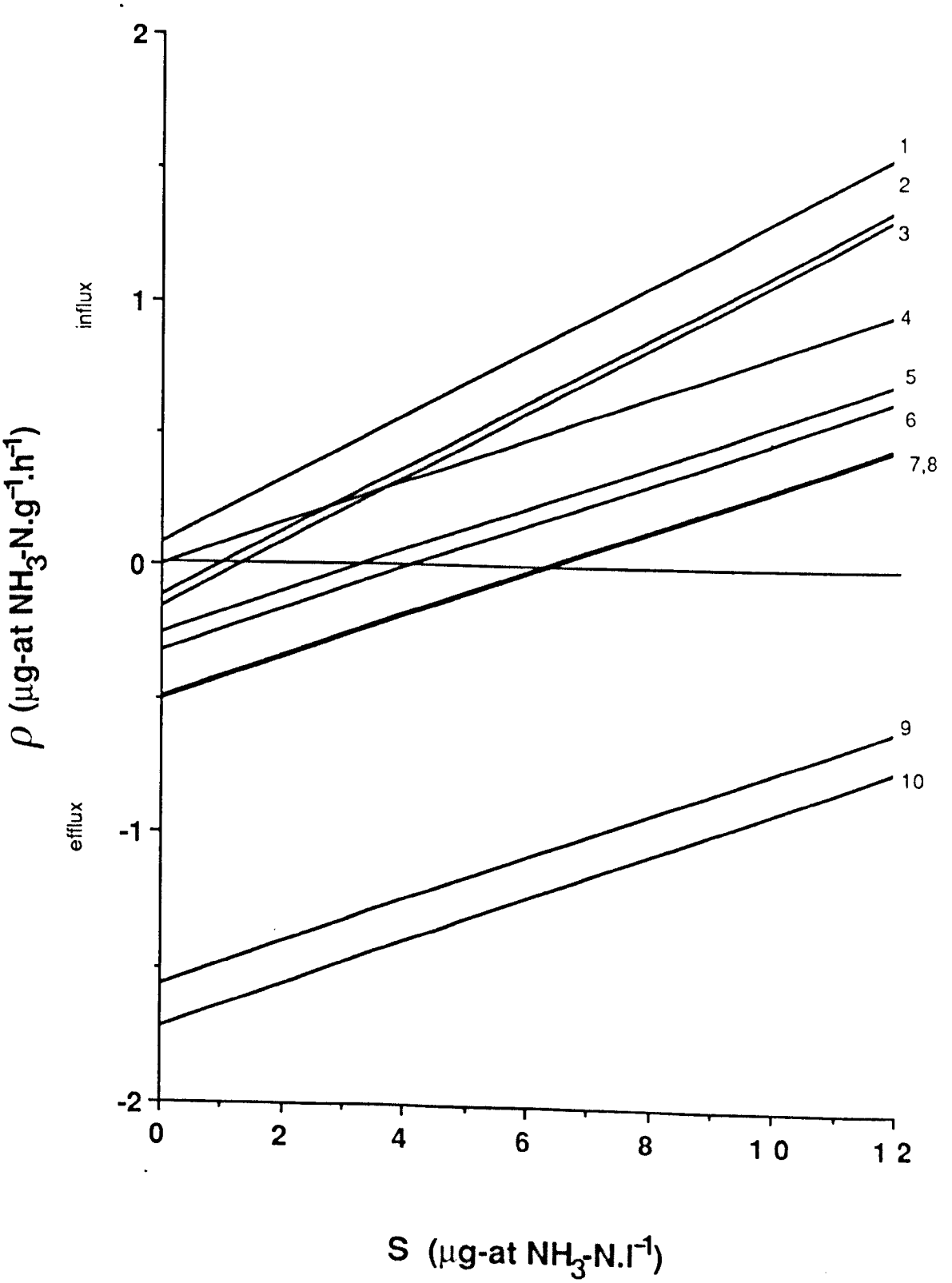
* = aposymbiotic anemones

Figure 4.9 *Anemonia viridis*: the relationship between ammonia flux rate and mean incubation concentration for starved symbiotic and aposymbiotic anemones at different light intensities. Lines representing groups of aposymbiotic anemones, and symbiotic anemones at irradiances of 50 $\mu E.m^{-2}.s^{-1}$ or less are drawn to a common regression coefficient of 0.080, whilst lines representing groups of symbiotic anemones at irradiances of 100 $\mu E.m^{-2}.s^{-1}$ or higher are drawn to a common regression coefficient of 0.123. The line for each treatment passes through the point $(\bar{x}, \bar{y})$, calculated from the pooled data from that treatment. Sample sizes for each light treatment ranged from 5 to 12 anemones (see table 4.6), and each anemone had uptake rates calculated at 6 different incubation ammonia concentrations. Positive values of ρ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water. The equations of the lines are given in table 4.6.

Key:

1 -	symbionts	at	190 $\mu E.m^{-2}.s^{-1}$	"High light" symbionts
2 -	"	"	300 $\mu E.m^{-2}.s^{-1}$	
3 -	"	"	100 $\mu E.m^{-2}.s^{-1}$	
4 -	"	"	50 $\mu E.m^{-2}.s^{-1}$	"Low light" symbionts
5 -	"	"	20 $\mu E.m^{-2}.s^{-1}$	
6 -	"	"	0 $\mu E.m^{-2}.s^{-1}$	
7 -	"	"	10 $\mu E.m^{-2}.s^{-1}$	
8 -	"	"	5 $\mu E.m^{-2}.s^{-1}$	
9 -	aposymbionts	at	0 $\mu E.m^{-2}.s^{-1}$	Aposymbionts
10 -	"	"	190 $\mu E.m^{-2}.s^{-1}$	

Figure 4.9


2
1
0
-1
-2
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
1
2
3
4
5
6
7,8
9
10
0
2
4
6
8
10
12
S (µg-at NH3-N.l-1)

distribution of the y-intercept values for anemones in a given treatment could not be assumed to be normal, because of the small sample sizes used. For this reason, the distributions of elevations of the lines from two treatments could not be compared with parametric statistics. It was possible, however, to compare the y-intercept values of different treatments by the use of a Wilcoxon two sample ranked test (Diem and Lentner, 1970; Bailey, 1981). In this test the y-intercept values of the two treatments to be compared were combined and ranked, and the sum of the ranks of each treatment compared to see if the rankings overlapped significantly. Table 4.7 shows examples of this test. Starting with the hypothesis that there was no difference in y-intercept distributions between any of the treatments, each treatment was tested against each of the other treatments with the same common regression coefficient. The results of tests on treatments with common regression coefficient 0.080 (the low light group) are given in table 4.8a, whilst those of tests on treatments with common regression coefficient 0.123 (the high light group) are given in table 4.8b. The anemones from the low light group can be subdivided into two smaller groups from the results of these tests, aposymbiotic anemones having significantly lower elevations than symbiotic anemones, regardless of light intensity. Only one comparison within the symbiotic anemone treatments from this group showed any sign of significant difference, and that was between the treatments in darkness and at 50 $\mu E.m^{-2}.s^{-1}$. In this comparison, the y-intercept values of the anemones at 50 $\mu E.m^{-2}.s^{-1}$ were higher than those of the anemones in darkness, although there is only significance at the $P=0.10$ level. From comparisons between the treatments with regression coefficient 0.123 it appears that the anemones at 190 $\mu E.m^{-2}.s^{-1}$ have significantly higher elevations than the anemones at 100 or 300 $\mu E.m^{-2}.s^{-1}$.

Table 4.7 *Anemonia viridis*: examples of rankings in Wilcoxon two-sample comparisons between the y-intercepts of different starved anemone treatments. a) Comparison between aposymbionts at 190 $\mu E.m^{-2}.s^{-1}$ and symbionts at 20 $\mu E.m^{-2}.s^{-1}$. b) Comparison of symbionts at 10 and 20 $\mu E.m^{-2}.s^{-1}$. In the first example, the sum of the ranks for aposymbionts at 190 $\mu E.m^{-2}.s^{-1}$ was 57, which, with six values in each treatment, shows a significant difference in the distribution of y-intercept values of these two treatments ($P<0.01$). In the second example, the sum of the ranks for symbionts at 10 $\mu E.m^{-2}.s^{-1}$ was 33, which shows no significant difference between y-intercept value distributions, with 6 and 5 values ($P>0.1$) (Diem and Lentner, 1970).

Table 4.7

a)

Rank	y-intercept values	
	Aposymbionts at 190 $\mu E.m^{-2}.s^{-1}$	Symbionts at 20 $\mu E.m^{-2}.s^{-1}$
1		0.368
2		0.136
3		-0.006
4		-0.031
5		-0.745
6		-1.240
7	-1.386	
8	-1.521	
9	-1.635	
10	-1.760	
11	-1.827	
12	-1.857	

b)

Rank	y-intercept values	
	Symbionts at 10 $\mu E.m^{-2}.s^{-1}$	Symbionts at 20 $\mu E.m^{-2}.s^{-1}$
1	0.398	
2		0.368
3		0.136
4		-0.006
5		-0.031
6	-0.078	
7	-0.650	
8		-0.745
9	-0.920	
10	-1.223	
11		-1.240

Table 4.8 *Anemonia viridis*: results of Wilcoxon two-sample tests to compare the y-intercept values of anemones from starved anemone treatments with statistically similar regression coefficients. a) Comparisons between treatments with common regression coefficient 0.080 (aposymbionts, and symbionts at irradiences of 50 $\mu E.m^{-2}.s^{-1}$ or less). b) Comparisons between treatments with common regression coefficient 0.123 (symbionts at irradiences of 100 $\mu E.m^{-2}.s^{-1}$ or more). Results showing differences in the distribution of y-intercept values are given with significance level.

N.S.= no significant difference.

a)

	0*	190**	190*	0	5	10	20	50
0*		NS	NS	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
190**			NS	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
190*				P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
0					NS	NS	NS	P<0.1
5						NS	NS	NS
10							NS	NS
50								NS

* = aposymbionts (1/2 hour incubations)
** = aposymbionts (1 hour incubations)

b)

	100	190	300
100		P<0.01	NS
190			P<0.02

The different light treatments could now be divided into groups of treatments, on the basis of the elevations and slopes of their φ vs. S relationships. Treatments within a group had regression coefficients and distributions of elevations that could not be statistically distinguished from one another. The three groups obtained in this way were:

1) Aposymbionts, at any light intensity (common regression coefficient= 0.080, mean y-intercept= -1.658).
2) Symbionts at irradiances of 50 $\mu E.m^{-2}.s^{-1}$ or less (common regression coefficient= 0.080, mean y-intercept= -0.308).
3) Symbionts at irradiances of 100 $\mu E.m^{-2}.s^{-1}$ or more (common regression coefficient= 0.123, mean y-intercept= -0.060).

The results for the 24 hour full light cycle experiments, using six symbiotic and three aposymbiotic anemones, are shown in figure 4.10a. The symbiotic anemones each demonstrated lower uptake rates during the hours of darkness, even to the point of ammonia efflux in some individuals, returning to higher rates of uptake when the lights were turned on again. The aposymbiotic anemones showed net efflux during the day and the night, with very little difference in rates between the two periods. Figure 4.10b represents the mean flux rates for the two groups of anemones, and shows more clearly the patterns of flux exhibited by the two groups of anemones over the full light cycle.

The approximate mean nutrient concentrations for each incubation were 4 and 5 $\mu g\text{-at } NH_3\text{-N}.l^{-1}$ for the symbiotic and aposymbiotic anemones respectively. The predicted flux rates (from figure 4.9) are roughly 0.5 and 0.0 $\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$ for symbiotic anemones in light and dark respectively, and -1.3 and -1.2 $\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$ for aposymbionts under the same light regime. The predictions are close to

Figure 4.10 *Anemonia viridis*: ammonia flux rates of starved aposymbiotic and symbiotic anemones measured during a full aquarium light cycle (12 hours light: 12 hours dark). a) Individual data from six symbiotic and three aposymbiotic anemones. b) mean uptake rates calculated for symbiotic and aposymbiotic anemones. Irradiance during light periods was 190 $\mu E.m^{-2}.s^{-1}$. Uptake rates for symbiotic anemones were calculated from repeated 1-hour incubations with mean concentrations of approximately 4 μg-at NH_3-$N.l^{-1}$, whilst rates for aposymbionts were calculated from repeated 1/2-hour incubations with mean concentrations of approximately 5 μg-at NH_3-$N.l^{-1}$. Positive values of φ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water.

Figure 4.10a

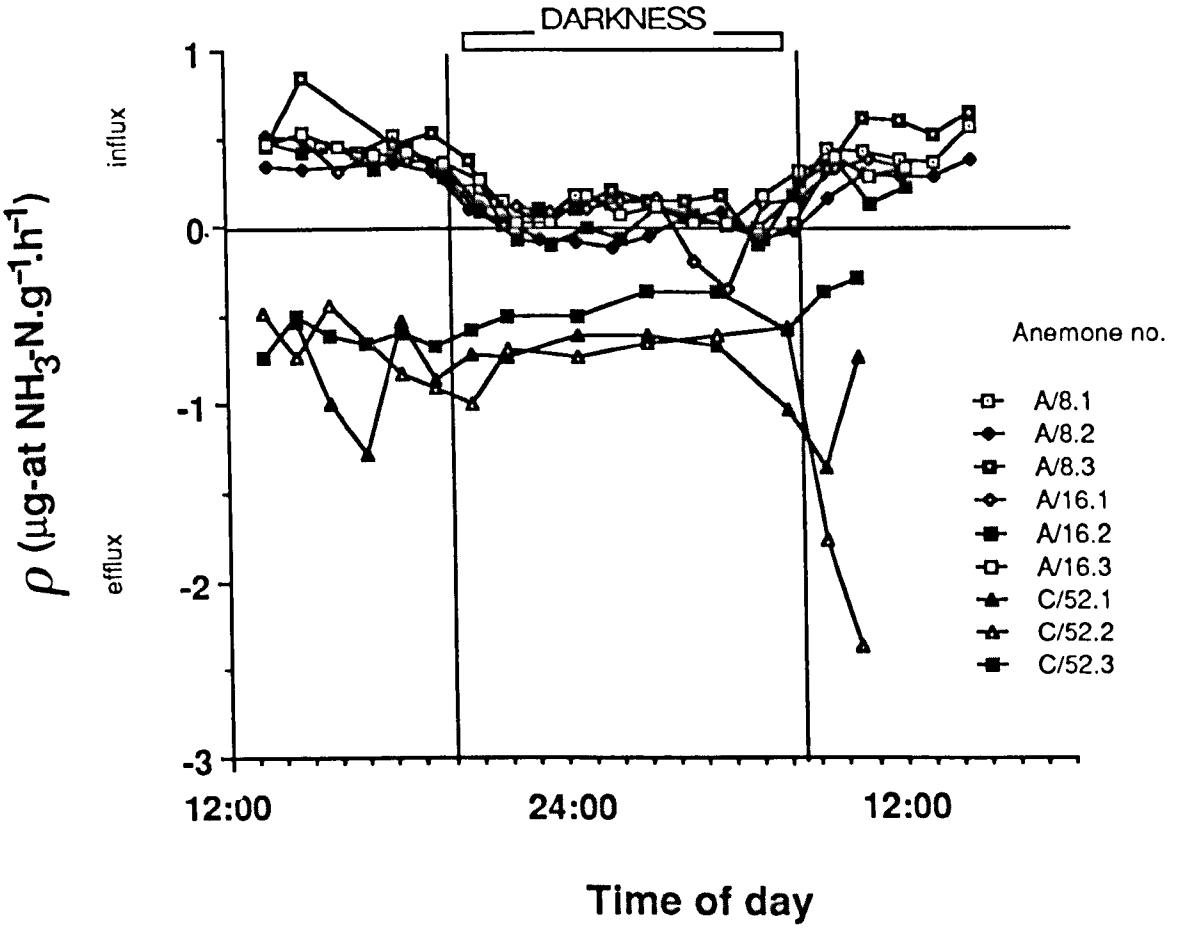
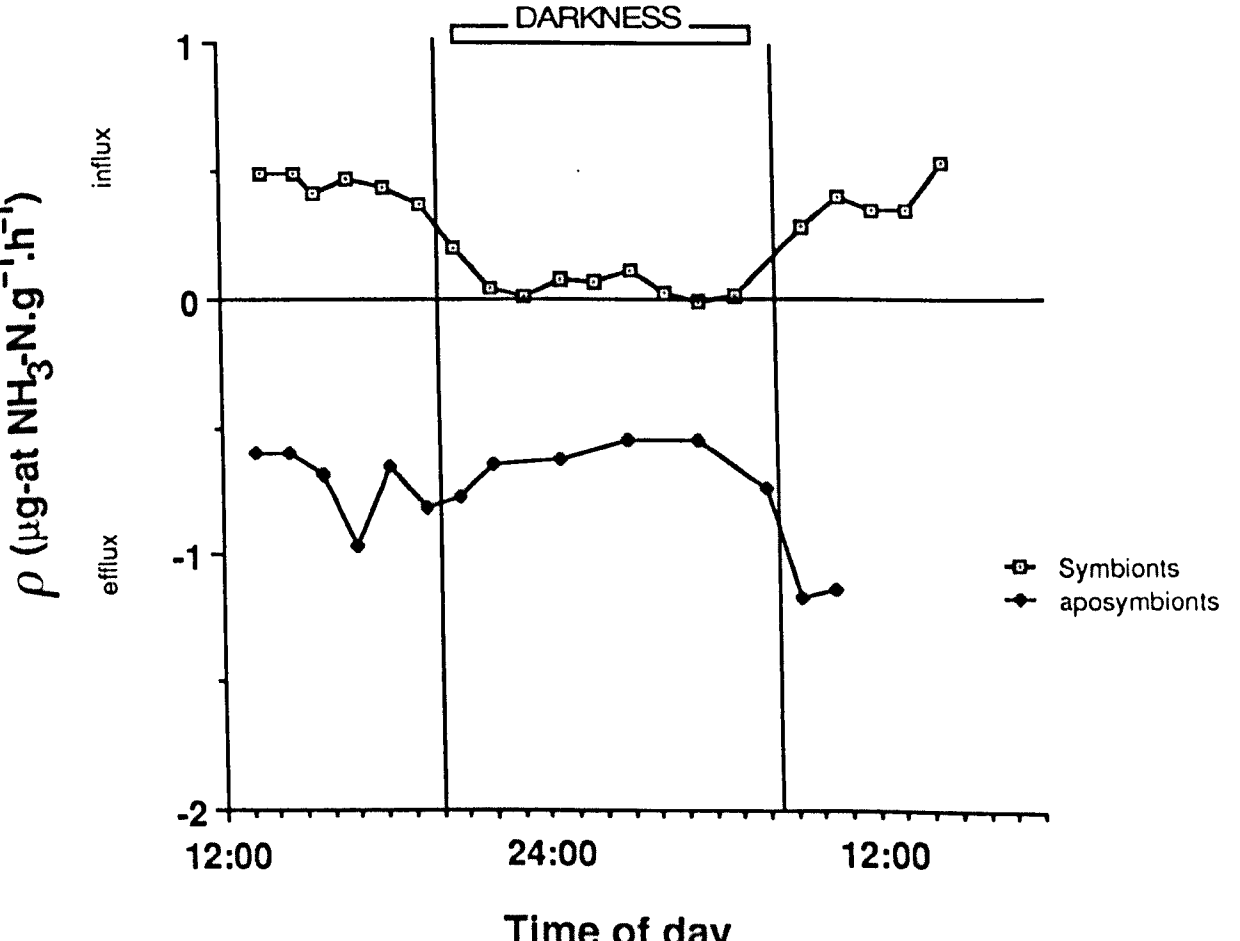


Figure 4.10b



the observed mean day and night-time flux rates for symbiotic anemones, but over-estimate the mean efflux rates observed in the aposymbionts. The over-estimation is possibly due to the greater variability in the rate/concentration relationships for aposymbiotic anemones, which would make predictions less reliable, particularly in view of the smaller sample size in this group.

Whilst the symbiotic anemones showed higher uptake rates during the day than during the night, there was no abrupt change in rate following the onset of darkness, or when the lights were switched on again in the morning (figure 4.10b). The change from one rate to the other took one to two hours.

4.5 Discussion

4.5.1 DIN flux in symbiotic and aposymbiotic *A. viridis*

The results of this set of experiments clearly show that symbiotic *Anemonia viridis* have the ability to take up ammonia from sea water. However, when symbiotic anemones are incubated at low ambient DIN concentrations in darkness the anemones show a net loss of ammonia. Aposymbiotic individuals also release ammonia into the medium, and at much faster rates than those of symbionts in darkness.

The use of aposymbionts provides data on the nitrogen production rates of the animal cells, and hence the magnitude of the algal-promoted contribution to the association's total nitrogen budget can be assessed. This sort of comparison has some drawbacks, since a starved symbiont will probably have a higher rate of nitrogen turnover, resulting from the nitrogen uptake by its algae. The only alternative method which has been used has been the comparison of related symbiotic and non-symbiotic species (e.g. Kawaguti, 1953;

Muscatine and D'Elia, 1978), and this is even less satisfactory, because of likely interspecific differences in nitrogen turnover rates. The contribution to the nitrogen supply of the host from the algal symbionts will be quantified in Chapter 7, using the aposymbiont as a model for nitrogen turnover in the animal cell.

4.5.2 Preferential uptake of ammonia

Whilst ammonia was taken up by symbiotic *Anemonia* under most conditions, there was no sign of similar uptake of nitrate, since incubations of anemones with nitrate in the water resulted in no apparent change in nitrate concentrations.

These results agree with the majority of studies that have investigated both ammonia and nitrate flux in algal-coelenterate symbioses (see Chapter 1). Work on microalgae has shown that if ammonia and nitrate are presented simultaneously to algal cells, nitrate uptake is usually either inhibited completely or occurs at a diminished rate, compared with the rate of uptake of nitrate supplied alone (Syrett, 1962; 1981). Various mechanisms for such preferential uptake in algae have been suggested. These have included the suppression of the enzyme responsible for nitrate assimilation (nitrate reductase) or the membrane-bound nitrate uptake system (Falkowski, 1975a; 1975b), either by ammonia (Eppley, Coatsworth and Solórzano, 1969; Eppley and Rogers, 1970; Tischner and Lorenzen, 1979; Hipkin *et al.*, 1980) or by the build up of ammonia assimilation products (Syrett, 1962; Thacker and Syrett, 1972). The concentrations of ammonia normally required to inhibit nitrate uptake are low, 0.2-1.0 μg-at NH_3-$N.l^{-1}$ being the standard threshold above which nitrate uptake is strongly affected (e.g. MacIsaac and Dugdale, 1969; McCarthy *et al.*, 1977; Garside, 1981). These ammonia concentrations, whilst not

exceeded in the incubation water for the nitrate uptake experiments in this study, are likely to be far lower than the concentration of ammonia in the animal cell. Intracellular ammonia concentrations have been estimated at 5 to 50 µg-at NH_3-N.l^{-1} in the corals *Acropora acuminata* and *Goniastrea australensis* (Crossland and Barnes, 1977), and 20 to 30 µg-at NH_3-N.l^{-1} in *Anemonia viridis* (this study, see section 4.5.3). If the DIN uptake is directly mediated by the zooxanthellae, their ability to utilize nitrate may therefore be impaired by the animal production of ammonia.

Nitrate uptake by similar symbioses has, however, been reported by other investigators (Franzisket, 1973; 1974; D'Elia and Webb, 1977; Cates and McLaughlin, 1979; Wilkerson and Trench, 1986), and so it is possible that the intracellular ammonia pools are smaller in these associations, or that their zooxanthellae are less sensitive to ammonia inhibition of nitrate uptake. The former condition seems unlikely because the animal cells might be expected to show similar rates of nitrogen metabolism and ammonia production to those of species not able to take up nitrate. The second possibility could have been caused by nitrogen deficiency of the algae, or some strain-specific loss of sensitivity of the zooxanthellar nitrate uptake system to inhibition by ammonia. Algal nitrogen deficiency has long been reported as a factor in the loss of ammonia inhibition of nitrate uptake in phytoplankton (Urhan, 1932; Syrett, 1981), nitrogen-starved algae also showing higher rates of uptake of nitrate and ammonia separately than nitrogen-replete algae. D'Elia *et al.* (1983), working on freshly-isolated zooxanthellae from a variety of coelenterate and molluscan hosts, found that nitrate uptake was not suppressed in the presence of ammonia, and also that zooxanthellae isolated from some Australian corals were not able to take up nitrate at all, even in the absence of ammonia. Freshly isolated zooxanthellae from the anemone

Aiptasia pulchella have also been found to be unable to utilize nitrate, and this feature extends to the intact association (Wilkerson and Muscatine, 1984). It is therefore possible that zooxanthellae in symbiosis with coelenterates are generally nitrogen-deficient and so are in a condition to take up nitrate and ammonia simultaneously, but that not all strains of zooxanthellae have the capability to utilize nitrate when it is made available. The question of the nutrient status of endosymbionts is one that has not yet been fully addressed, because of the problems of direct observation of their metabolism *in situ*. That similar questions about natural phytoplankton populations, which are more readily studied, have yet to be answered (Elrifi and Turpin, 1987) may be an indication of the magnitude of the problem facing researchers into symbiotic relationships.

4.5.3 The effects of concentration on ammonia flux

From figure 4.9 it is apparent that for all experimental anemones, regardless of the light level or presence or absence of algae, the flux rate of ammonia became more positive as the concentration of ammonia in the incubation medium was increased. For symbiotic anemones this generally meant that net uptake increased as ammonia availability increased, whilst for aposymbionts it indicated a decrease in efflux with increasing incubation concentration. The relationship was linear, and similar linear relationships have been shown to occur in several symbiotic associations (Burris, 1983; Wilkerson and Muscatine, 1984; Wilkerson and Trench, 1986). This is in contrast with studies on algae alone (both isolated endosymbionts and free-living phytoplankton) and a number of nutrient uptake studies on intact symbioses, where the graphical representation of the concentration-uptake relationship tends to follow a rectangular

hyperbola (e.g. Dugdale, 1967; D'Elia, 1977; Webb and Wiebe, 1978; D'Elia *et al.*, 1983; see section 6.2).

The linearity of the relationship for symbiotic *A. viridis* may represent the initial, linear portion of a typical rectangular hyperbola, but this is made less likely by the results of the high concentration uptake experiments, where the linear relationship held for mean incubation concentrations up to 30 µg-at NH_3-N.l^{-1}. This is much higher than for other symbioses that achieve saturation, most of which have half-saturation constants (the concentration at which uptake proceeds at half the maximal rate) of less than 1 µg-at NH_3-N.l^{-1}. A second explanation advanced for the lack of any tendency towards saturation is that diffusion processes are masking the kinetics of the carrier or assimilatory mechanism involved in the nutrient uptake. Muscatine and D'Elia (1978) were able, after subtraction of a diffusion constant, to show a typical rectilinear hyperbolic relationship between uptake and concentration for reef corals. Before the correction for diffusion, the relationship had appeared close to linear. The kinetics of ammonia uptake by *A. viridis* will be discussed more fully in Chapter 7.

The change in efflux rate with incubation concentration shown by aposymbionts has not been previously demonstrated. If ammonia excretion is passive, achieved by production of ammonia in the animal cell followed by simple diffusion across the cell membrane along a concentration gradient (Baldwin, 1967), then a linear relationship is to be expected. The direction and rate of diffusion will depend on the difference between the intra- and extracellular ammonia concentrations, and the diffusion constant, k_D. This latter term is a measure of the rate at which the molecules of a particular solute penetrate a region of membrane, the spatial extent and chemical and physical properties of which are defined (Neame and Richards, 1972).

If the intracellular ammonia concentration remains constant, and k_D does not change, then the diffusion rate will change in proportion to the change in extracellular ammonia concentration. Hence, if the intracellular ammonia concentration is initially higher than that outside the cell, as extracellular (incubation) concentration is increased the concentration gradient across the membrane will decrease, and efflux rate will also decrease in a linear fashion.

Mangum *et al.* (1978) have suggested that ammonia excretion in some invertebrates is not simply due to diffusion, but involves a carrier-mediated movement out of the cells. This was demonstrated for different invertebrate species, all of which transported ammonia from the body cells into a transport fluid, then from the fluid into cells of the excretory epithelium, before finally losing the ammonia into the surrounding medium. Such an involved system is not necessary in the anthozoa, where most of the cells are in direct contact with sea water for most of the time, and so the passive diffusion model seems adequate to explain the pattern of ammonia loss seen.

The relative simplicity of the kinetics of ammonia loss by diffusion allows two further estimations from the data provided in the aposymbiont experiments. If diffusion rate is directly proportional to external concentration, then the concentration at which net efflux rate is zero should be that at which extracellular and intracellular concentrations of ammonia are equal. From the results (see table 4.6), it is possible to estimate that the intracellular concentration of ammonia in starved aposymbiotic *A. viridis* was 20.72 $\pm$ 2.74 µg-at NH_3-N.l^{-1} ($\bar{x} \pm$ S.D., n=18). This lies within the range of 5-50 µg-at NH_3-N.l^{-1} given by Crossland and Barnes (1977). Similarly, if the rate of production of ammonia is constant, then the estimate of maximum ammonia efflux, when external concentration equals zero, will be the closest estimate possible of the ammonia production rate of the

animal cell. By extrapolation of the replotted regression lines for the 18 aposymbionts tested (B= 0.080), this gives a production rate of 1.657 $\pm$ 0.220 µg-at NH_3-N.g^{-1}.h^{-1} ($\bar{x}$ $\pm$ S.D., n=18). Such estimations are clearly not as satisfactory as direct measurements, and would be subject to error if, for example, the anemone's excretion rate changed during the experiment, due, for example, to changing metabolic rate. This has been found for phosphorus excretion in zooplankton, oysters and the non-symbiotic anemone *Sagartia sp.* (Satomy and Pomeroy, 1965), where excretion rate was highly correlated with respiration rate.

4.5.4 The effects of light intensity on ammonia flux

Figure 4.9 shows that the relationship between φ and S in symbiotic *A. viridis* is affected by the intensity of available photosynthetically active radiation. Although the linearity of the relationship does not change, the slopes and elevations of the lines are light-dependent.

In darkness, and at light levels up to and including 50 µE.m^{-2}.s^{-1}, illumination has no demonstrable effect on the regression coefficient of the relationship, but the elevation as measured by y-intercept increases until, at 50 µE.m^{-2}.s^{-1}, it is almost 0 µg-at NH_3-N.g^{-1}.h^{-1}. The elevation increase is not even from 0 to 50 µE.m^{-2}.s^{-1}, and the 0 µE.m^{-2}.s^{-1} line is higher than those for 5 and 10 µE.m^{-2}.s^{-1}. This is probably due to the large amount of variation in the elevation of the rate/concentration relationship shown by anemones at low light levels, which, in turn, could be because of a natural variability in the sensitivity of individual associations to low light, both in physiology and behaviour.

The major implication for the association's nitrogen balance is that at light levels below 50 µE.m^{-2}.s^{-1} there is a net loss of

ammonia-N at low ambient ammonia concentrations. Any increase in the elevation of the φ vs. S relationship would result in a depression of the ambient ammonia concentration below which ammonia loss occurred, and above which ammonia uptake took place. This would be particularly important to symbiotic *A. viridis* that were shaded, or on very dull days, if environmental levels of ammonia were low (see Chapters 3 and 7).

At light levels of 100 $\mu E.m^{-2}.s^{-1}$ or more the regression coefficient of the φ vs. S graph increases. The elevation (although not directly comparable when slopes differ) cannot exceed a y-intercept value of 0 $\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$, since it is not possible to have a net uptake from water containing no ammonia. For this reason, the y-intercept of the line for the 190 $\mu E.m^{-2}.s^{-1}$ light intensity (φ= 0.079 $\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$) is probably an artifact resulting from the grouping together of data with fairly large variance.

Aposymbiotic anemones showed some difference in rate/concentration relationships depending on the presence or absence of light, and this is probably linked with the anemones' behavioural response to light. Contraction in darkness and expansion in light is well-known in symbiotic anemones in general (e.g. Pearse, 1974b; Gladfelter, 1975; Sebens and DeReimer, 1977; Muller-Parker, 1984) and in *Anemonia* in particular (Dorsett, 1984; J.R. Turner, unpublished observations), and aposymbiotic individuals of symbiotic species maintain this response to light (Pearse, 1974b; personal observations). As aposymbionts tend to be less expanded in darkness, the smaller rate of ammonia loss under such conditions is possibly due to a reduction in surface area, with resulting decrease in the diffusion coefficient k_D (see section 4.5.3). However, k_D is normally represented by the slope of a graph such as that in figure 4.9, and there is no statistical difference between the slopes of the

aposymbionts regardless of light intensity. The slightly, and statistically insignificant, lower efflux rates for the aposymbionts in darkness may in part have been due to lower metabolic rates when the anemones were flaccid (and presumably expending less energy on maintenance of posture). A drop in the animal cell production rate of ammonia, rather than a change in k_D, might be a more likely explanation for the differences in efflux rate with light in aposymbionts. That there is a difference is clear from the 24-hour experiments, but since the differences in flux associated with expansion state are small it is not possible to determine the relative importance of change in surface area and possible changes in metabolic rate.

It is likely that the different rate/concentration relationships exhibited by symbiotic anemones under varied light intensities are due to changes in the rate of ammonia uptake by the zooxanthellae, since this is characteristic of microalgae (Syrett, 1981). Uptake is not totally dependent on light, however, since the symbiotic anemones had a lower rate of excretion in darkness than aposymbionts. This continuation of uptake in darkness, albeit at lower rates than in the light, may be a less extreme example of the apparant lack of light-dependence of DIN uptake in some other symbioses (Franzisket, 1973; 1974; Propp, 1981; Burris, 1983). DIN uptake at rates that were not affected by light intensity has been shown by Thacker and Syrett (1972) in nitrogen-starved algae, and so full, or partial, light-independence of such uptake by intact symbioses may be further evidence of some degree of nitrogen-starvation in the endosymbionts (see section 4.5.2).

Some of the studies on symbioses that have demonstrated reduced DIN uptake in darkness have only succeeded in doing so after lengthy dark incubation periods prior to the experiments (Muscatine and

D'Elia, 1978; Wilkerson and Muscatine, 1984; Wikerson and Trench, 1986). This is consistent with the view that nitrogen-depleted algae would be able to utilize DIN as long as storage products were available to provide a source of fixed carbon (Thacker and Syrett, 1972; Elrifi and Turpin, 1987). In a prolonged period of darkness, such storage products would become depleted, and DIN uptake would then cease. This might also have been the case in *A. viridis* under the experimental conditions used, because the anemones had been pretreated with twelve hours of darkness in the aquarium, followed by a further two hours settling period at the experimental light intensity before the experiment commenced. In their natural environment the time taken for ammonia uptake driven by storage products to cease, would be extremely important in the anemone's 24 hour nitrogen budget. The 24 hour experiments provide important information about the immediacy of light effects on ammonia flux. After the lights went out, the onset of a stable "dark" rate of flux took less than two hours in most individuals. This rate was then maintained throughout the hours of darkness with no apparent increase in efflux towards the end of the period that might be associated with continued depletion of carbon reserves. There might therefore be a short period at the start of the night when the flux rates are more positive than might be predicted by the light intensity experiments, but uptake during the rest of the darkness hours appears to occur at rates that agree better with predictions.

Factors affecting the flux of ammonia in *A. viridis*, and a possible mechanism explaining the kinetics observed here, will be discussed further in Chapter 7.

CHAPTER 5: DISSOLVED INORGANIC NITROGEN FLUX IN FED *Anemonia viridis*

5.1 Introduction

Anemonia viridis, in common with many other symbiotic coelenterate species, is well-adapted for heterotrophic feeding. The anemones have numerous long, active tentacles, react rapidly to feeding stimuli (Pantin and Pantin, 1943), possess toxins effective against fish and crustaceans (Möller and Beress, 1975), and were one of the first anthozoan species to be shown to have the capability of food digestion within the coelenteron (Jordan, 1907). It is to be expected, therefore, that the natural state of individuals of this species in the field is not one of starvation, and this is reinforced by observations of specimens with prey in their coelenterons (e.g. Mesnil, 1901; Möller, 1978; J.R. Turner, pers. comm.). Observations of *Anemonia* in Scottish waters did not provide abundant evidence of prey capture, but showed that it occurred (see section 3.5). Since the metabolic state of the anemone can potentially affect the dissolved inorganic nitrogen (DIN) flux, it follows that extrapolation to the field of DIN flux rates estimated from experiments using starved anemones, such as those in the previous chapter, might be inappropriate.

The aim of this set of experiments was therefore to investigate the effects of previous heterotrophic feeding on the DIN flux rates of both the animal component of the symbiosis and the entire association, and to compare these rates with those shown by starved anemones. As in the experiments with starved anemones, the ultimate aims were to incorporate results into a model of DIN flux in the association, and to allow prediction of flux rates for anemones in the field under

measured conditions of light and DIN concentration. Because there were no quantitative data on rates of prey capture, assimilation efficiency, and corresponding metabolic state for *A. viridis* in the field, the predictions based on data from the following set of experiments were intended to be applicable to anemones arbitrarily categorized as "fed", rather than anemones with a specific nutritional history.

5.2 Methods

Experiments were carried out with the incubation apparatus and light hood described in section 4.3.2. Ammonia uptake rate versus mean incubation concentration relationships were calculated, as in section 4.3.2, for three anemones simultaneously and, as before, six initial ammonia concentrations (0, 2, 4, 6, 8 and 10 μg-at NH_3-N.l^{-1}) were tested during a twelve-hour experimental "day". Experiments were repeated to give larger sample sizes, and anemones that did not settle during the two hour period prior to the first incubation were replaced or excluded. Experiments were conducted under a range of light intensities, which were obtained in the same way as those used in section 4.4.2. Anemones were tested at light levels of 300, 190, 100 and 50 μE.m^{-2}.s^{-1} and in darkness.

As in the previous chapter, it was necessary to investigate the ammonia flux rate of the animal tissue alone, in order to assess the effects of zooxanthellae on the flux, and so aposymbiotic anemones were tested as well as symbionts. Whilst the symbiotic anemones were tested at all of the light intensities mentioned above, aposymbionts were only tested in darkness, since no statistical difference had been found between the flux rates in light and dark in earlier experiments on starved aposymbionts (see section 4.4.3).

All anemones used had been regularly fed with squid mantle tissue (see section 2.2.1), and experiments were carried out three days after last feeding. The delay between feeding and experimental use was necessary to avoid undigested food remaining in the coelenteron, since this may have been egested into the incubation chamber, possibly inducing bacterial activity. It was also hoped that delaying experimental use after feeding would avoid effects associated with specific dynamic action (SDA), the elevated respiration rate often shown by animals after feeding, and often accompanied by elevated nitrogen excretion rates (e.g. Wilhelmj and Bollman, 1928).

No attempt was made in this study to control the nitrogen content of meals fed to the anemones, because of the complications of matching meal size to anemone weight, and the variability in anemones feeding efficiencies. The anemones were fed to satiation on squid mantle tissue twice a week, and had been kept on the feeding regime for a minimum of four weeks prior to experimental use.

As in the previous chapter, 24 hour experiments were carried out to observe the fluxes of ammonia in anemones under a "normal" aquarium lighting regime (see section 4.4.2). The anemones were tested in the incubation apparatus, with light intensities of 190 $\mu E.m^{-2}.s^{-1}$ during the 12 hour "day" and darkness during the 12 hour "night". Each incubation started at the same ammonia concentration, 4 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$, and uptake rates were calculated as described in section 4.3.2. Because of time constraints, only symbiotic anemones were used in these experiments.

5.3 Results

As with the starved anemones tested in the previous chapter, results of incubations at all light intensities showed that ammonia

flux rates became more positive at higher mean incubation concentrations. Figures 5.1 and 5.2 show sets of results for fed symbiotic anemones at a light intensity of 100 $\mu E.m^{-2}.s^{-1}$ and aposymbionts in darkness respectively, and the data for all experiments are given in appendix III. The data suggested that the relationship between φ and S was linear for all of the anemones tested, and so least squares regression lines were fitted to the data from each anemone. The equations for the regression lines are given in table 5.1.

The regression lines for the anemones within each light treatment were compared by covariance analysis, and the results are given in table 5.2. There were no significant differences between slopes within any treatment, but the elevations of the regression lines belonging to each treatment were statistically different. These results are similar to those for the starved anemones, which also showed no differences in slopes, but generally had different elevations for lines within the different treatments. As in Chapter 4, common regression coefficients were calculated for each of the treatments, and these are also shown in table 5.2.

To determine the effects of light intensity on the φ vs. S relationships, the common regression coefficients calculated above for the six treatments (symbionts at five light intensities and aposymbionts in darkness) were compared. This was done in a single covariance analysis, to test the null hypothesis that light had no effect on the regression coefficients of the φ vs. S relationships. The analysis showed that there were significant differences in the slopes (F_{slopes}= 5.340, 5 and 162 d.f., P<0.001), proving that, as for starved anemones, the slope of the relationship is light-dependent.

The results of the previous chapter showed that the starved

Figure 5.1 *Anemonia viridis*: weight-specific hourly ammonia flux rates at different incubation ammonia concentrations for six fed symbiotic anemones at an irradience of 100 $\mu E.m^{-2}.s^{-1}$. Positive values of φ indicate net uptake of ammonia by the symbiosis, negative values indicate net loss of ammonia to the incubation water. The equations for linear regression lines fitted to the data for each anemone are given in table 5.1.

Figure 5.1

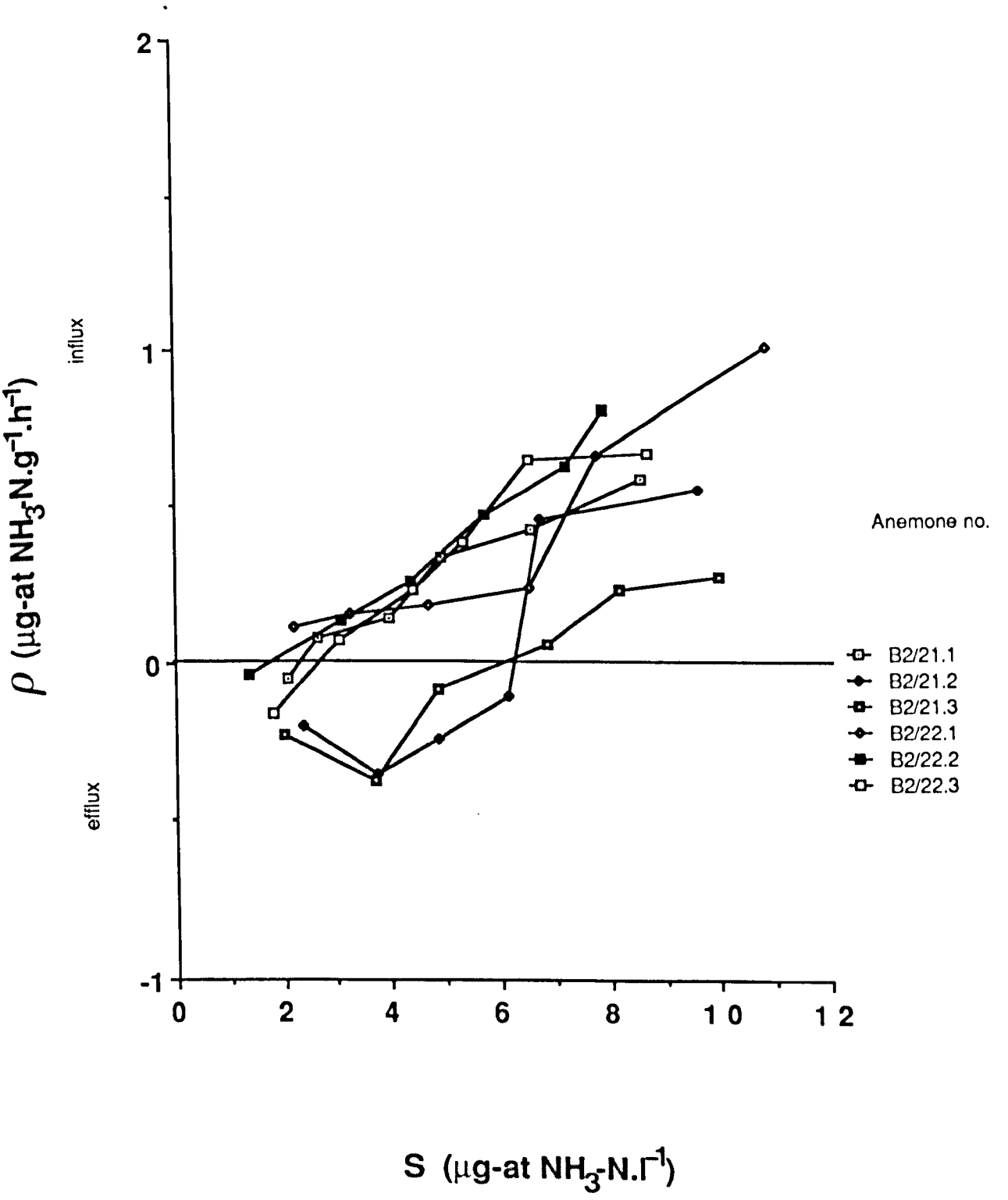

2
1
0
-1
0
2
4
6
8
10
12
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
S (µg-at NH3-N.l-1)
Anemone no.
B2/21.1
B2/21.2
B2/21.3
B2/22.1
B2/22.2
B2/22.3

Figure 5.2 *Anemonia viridis*: weight-specific hourly ammonia flux rates at different incubation ammonia concentrations for three fed aposymbiotic anemones in darkness. Negative values of φ indicate net loss of ammonia to the incubation water. The equations for linear regression lines fitted to the data for each anemone are given in table 5.1.

Figure 5.2

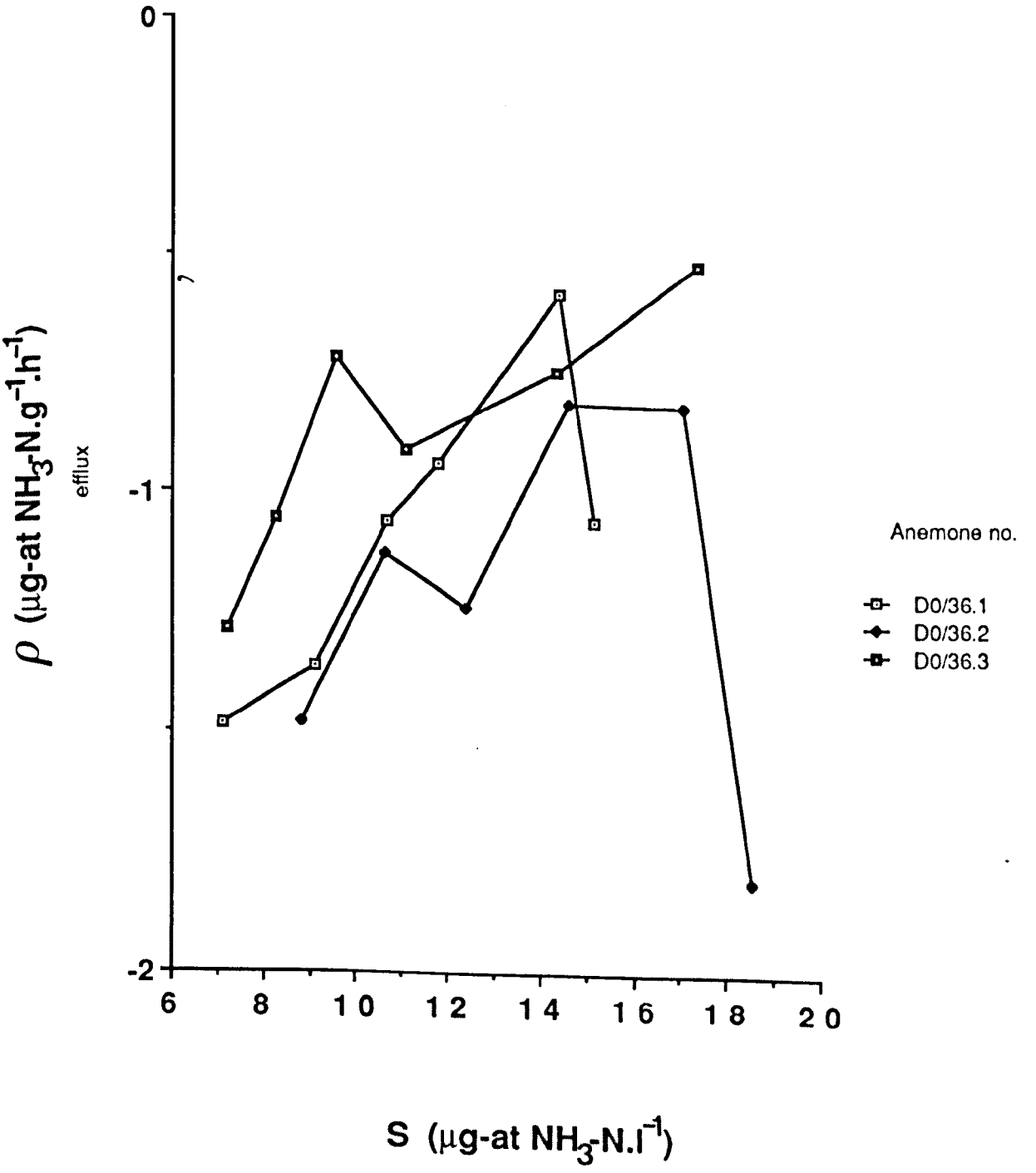

0
-1
-2
6
8
10
12
14
16
18
20
ρ (µg-at NH3-N.g-1.h-1)
efflux
S (µg-at NH3-N.l-1)
Anemone no.
D0/36.1
D0/36.2
D0/36.3

Table 5.1 *Anemonia viridis*: equations of linear regression lines fitted to the ammonia flux rate/ incubation concentration data of individual fed symbiotic and aposymbiotic anemones exposed to a range of light intensities. The data for symbionts at 100 $\mu E.m^{-2}.s^{-1}$ and aposymbionts in darkness are shown in figures 5.1 and 5.2 respectively, and all data are given in appendix III.

Type of anemone	Light intensity ($\mu E.m^{-2}.s^{-1}$)	No. of points	Equation of regression line
Fed symbionts	0	6	y = 0.020x - 0.765
		6	y = 0.028x - 0.967
		6	y = 0.051x - 0.204
		6	y = 0.074x - 0.448
		6	y = 0.046x - 0.782
Fed symbionts	50	6	y = 0.066x - 0.070
		6	y = 0.088x - 0.979
		6	y = 0.073x - 0.933
		7	y = 0.105x - 0.031
		7	y = 0.078x - 0.020
		7	y = 0.066x - 0.422
Fed symbionts	100	6	y = 0.095x - 0.202
		6	y = 0.129x - 0.705
		6	y = 0.080x - 0.498
		6	y = 0.106x - 0.234
		6	y = 0.125x - 0.243
		6	y = 0.127x - 0.328
Fed symbionts	190	6	y = 0.113x - 0.449
		6	y = 0.148x - 0.236
		6	y = 0.164x - 0.446
		6	y = 0.070x - 0.430
		6	y = 0.119x - 0.864
		6	y = 0.127x - 0.308
		6	y = 0.109x - 0.249
		6	y = 0.080x - 0.089
Fed symbionts	300	6	y = 0.096x - 0.209
		6	y = 0.069x + 0.061
		6	y = 0.092x + 0.046
		6	y = 0.102x - 0.471
		6	y = 0.101x - 0.177
Fed aposymbionts	0	6	y = 0.084x - 2.042
		6	y =-0.001x - 1.204
		6	y = 0.060x - 1.555

Table 5.2 *Anemonia viridis*: results of covariance analysis carried out to compare slopes and elevations of the ammonia uptake rate vs. incubation concentration relationships for fed anemones at different light intensities. Results are given for comparisons within each light treatment. Also shown are the common regression coefficients calculated for each treatment by pooling sums of squares and sums of products for individual anemones.

Table 5.2

Illumination ($\mu E.m^{-2}.s^{-1}$)	No. of anemones in comparison	Variance ratio (F) for slopes	Variance ratio (F) for elevations	Common regression coefficient	Common y-intercept
0	5	0.311, 4 and 20 d.f. N.S., P>0.75	16.661, 4 and 24 d.f. P<0.001	0.044	-
50	6	0.494, 5 and 27 d.f. N.S., P>0.75	57.706, 5 and 32 d.f. P<0.001	0.079	-
100	6	0.916, 5 and 24 d.f. N.S., P>0.25	24.063, 5 and 29 d.f. P<0.001	0.108	-
190	8	0.408, 7 and 32 d.f. N.S., P>0.75	7.208, 7 and 39 d.f. P<0.001	0.114	-
300	5	0.568, 4 and 20 d.f. N.S., P>0.50	12.582, 4 and 24 d.f. P<0.001	0.091	-
0*	3	1.393, 2 and 12 d.f. N.S., P>0.25	17.889, 2 and 14 d.f. P<0.001	0.044	-

* = aposymbionts

anemones tested could be divided into two groups, on the basis of their regression coefficient: symbionts in low light (50 $\mu E.m^{-2}.s^{-1}$ and below) and aposymbionts, and symbionts in high light (100 $\mu E.m^{-2}.s^{-1}$ and above). The regression coefficients for fed anemones were similarly grouped, to see if they could be divided in the same way. A covariance analysis was carried out to compare the common regression coefficients of the three low light treatments (symbionts and aposymbionts in darkness and symbionts at 50 $\mu E.m^{-2}.s^{-1}$), and a second was used to compare the common regression coefficients of the three high light treatments. The results, given in table 5.3, show that the treatments could be divided in the same way as the starved anemone treatments, with no significant difference being found in either comparison. Between-treatment common regression coefficients were calculated for both groups of treatments after pooling sums of squares and sums of products, giving values of 0.058 for the low light group and 0.105 for the high light group. These regression coefficients were compared by a further covariance analysis, which confirmed that they were statistically different ($F= 19.146$, 1 and 166 d.f., $P<0.001$).

As with the starved anemones, it was clear from graphs of the φ vs. S relationships for individual fed anemones that the elevations of the regression lines were different for symbionts and aposymbionts (see figures 5.1 and 5.2). In order to check for similar, smaller differences within the symbiotic anemones treatments, elevations were statistically compared by the proceedure described in section 4.4.3. To do this, the regression lines for individual anemones were replotted to the common regression coefficients calculated for their light group (i.e. $B= 0.058$ for low light anemones, $B= 0.105$ for high light anemones). Mean y-intercept values were then calculated for each treatment. These values are given in table 5.4, and lines plotted to

Table 5.3 *Anemonia viridis*: results of covariance analyses carried out to compare the common regression coefficients of fed anemone treatments (see table 5.2). Common regression coefficients, calculated by pooling the sums of squares and sums of products for groups of treatments with no significant difference in slopes, are also shown.

Table 5.3

Treatments compared (light intensity, in $\mu E.m^{-2}.s^{-1}$)	No. of anemones in treatment	Treatment common regression coefficient	Treatment common y-intercept	F ratio (slopes)	Between-treatment common regression coefficient
All treatments	-	-	-	5.340 5 and 162 d.f., P<0.001	-
0* 0 50	3 5 6	0.044 0.044 0.079	various various various	2.245 2 and 70 d.f., N.S., P>0.1	0.058
100 190 300	6 8 5	0.108 0.114 0.091	various various various	1.000, 2 and 92 d.f., N.S., P>0.25	0.105

* = aposymbionts

Table 5.4 *Anemonia viridis*: mean y-intercept values calculated for each fed anemone light treatment, after replotting the lines of individual anemones to the common regression coefficients given in table 5.3. The mean y-intercept and common regression coefficient given in the table were used to plot the regression lines for each treatment, shown in figure 5.3.

Irradiance ($\mu E.m^{-2}.s^{-1}$)	No. of anemones	Mean y-intercept	Common regression coefficient
0*	3	-1.761	0.058
0	5	-0.753	0.058
50	6	-0.283	0.058
100	6	-0.342	0.105
190	8	-0.338	0.105
300	5	-0.217	0.105

* = aposymbiotic anemones

the group regression coefficient and the mean y-intercept for each treatment are shown in figure 5.3. The y-intercept values of each treatment were then ranked, and comparisons of the rankings were carried out by Wilcoxon two-sample test (Diem and Lentner, 1970; Bailey, 1981; see table 4.7). Comparisons were only possible between treatments with similar regression coefficients. The elevations for symbiotic anemones in darkness and 50 $\mu E.m^{-2}.s^{-1}$ (corresponding to the "low light" group) were compared, with no significant differences being found, and the elevations for anemones at 100, 190 and 300 $\mu E.m^{-2}.s^{-1}$ (the "high light" group) were compared, in a series of tests, again with no differences being found.

The "low light" group, consisting of all treatments with a regression coefficient of 0.058, could therefore be subdivided into two smaller groups of treatments in which elevations were similar: aposymbionts and symbionts in darkness and at 50 $\mu E.m^{-2}.s^{-1}$. The "high light" group of treatments could not be subdivided on the basis of elevations, and so consisted of all treatments with a regression coefficient of 0.105, all of which had similar elevations.

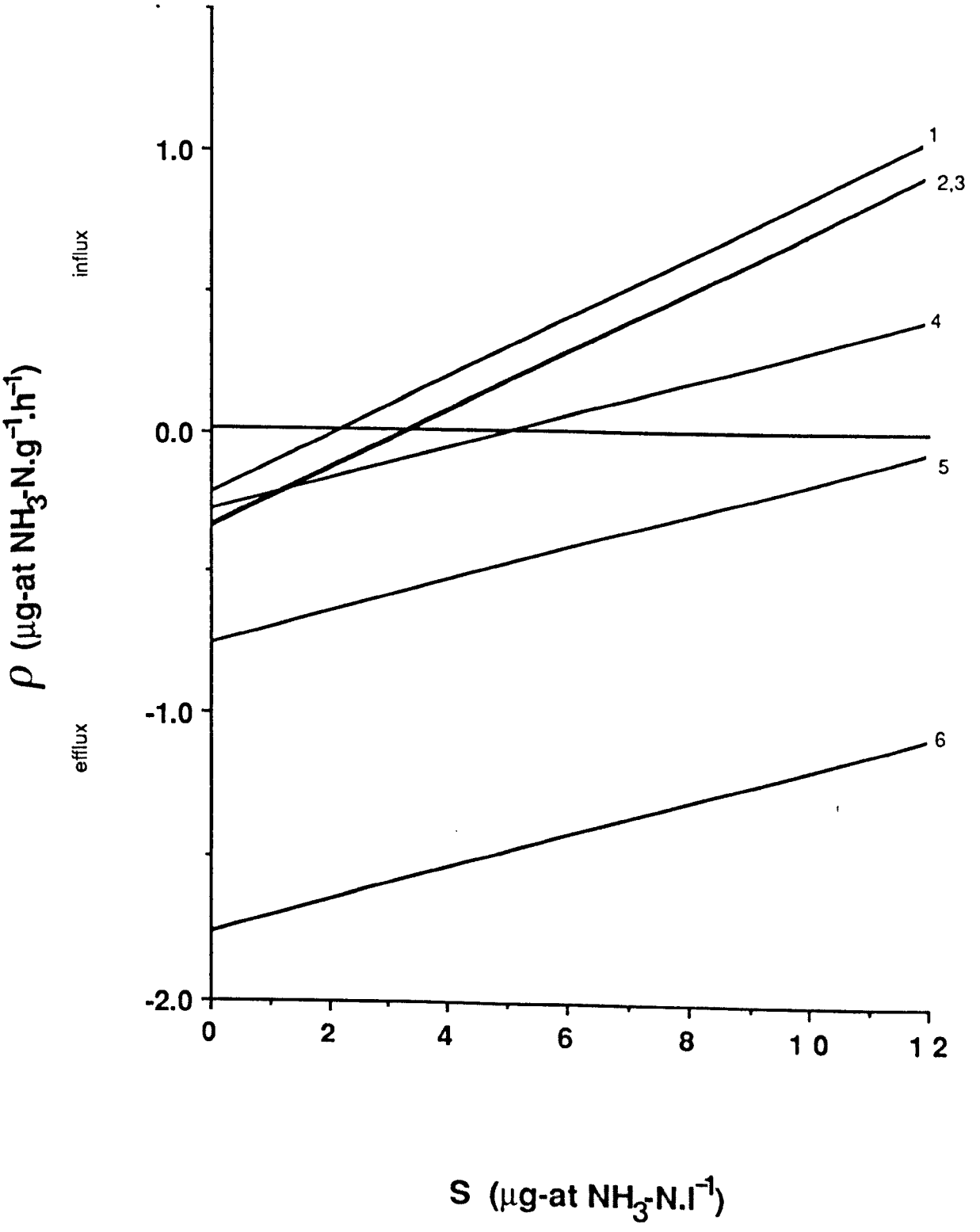
Comparison of figures 4.9 and 5.3 shows that the effects of different light intensities in fed *A. viridis* followed a similar pattern to those in starved *A. viridis*, the treatments dividing into three groups (aposymbionts, low light symbionts, and high light symbionts) under both nutritional regimes. Further covariance analyses were therefore carried out to compare the slopes of the φ vs. S relationships for fed and starved anemones under similar lighting conditions. Because the aposymbionts and low light symbionts within each feeding state had similar regression coefficients, only two covariance analyses were carried out, one comparing the common regression coefficients of all aposymbiont and low light symbiont treatments (given in tables 4.4 and 5.2), and the other comparing the

Figure 5.3 *Anemonia viridis*: the relationship between ammonia flux rate and mean incubation concentration for fed symbiotic and aposymbiotic anemones at different light intensities. Lines representing anemone treatments at irradiences of 50 $\mu E.m^{-2}.s^{-1}$ or less are drawn to a common regression coefficient of 0.058, whilst lines representing treatments at higher irradiences are drawn to a common regression coefficient of 0.105. The line for each treatment passes through the point $(\bar{x}, \bar{y})$, calculated from the pooled data from that treatment. Positive values of φ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water. The equations of the lines are given in table 5.4.

Key:

1 -	Symbionts at	300 $\mu E.m^{-2}.s^{-1}$	"High Light" Symbionts
2 -	" "	190 $\mu E.m^{-2}.s^{-1}$	
3 -	" "	100 $\mu E.m^{-2}.s^{-1}$	
4 -	" "	50 $\mu E.m^{-2}.s^{-1}$	"Low Light" Symbionts
5 -	" "	0 $\mu E.m^{-2}.s^{-1}$	
6 -	Aposymbionts at	0 $\mu E.m^{-2}.s^{-1}$	Aposymbionts

Figure 5.3


1.0
0.0
-1.0
-2.0
influx
efflux
ρ (µg-at NH3-N.g-1.h-1)
0
2
4
6
8
10
12
S (µg-at NH3-N.l-1)
1
2,3
4
5
6

common regression coefficients of all high light symbiont treatments (also given in tables 4.4 and 5.2). There were no significant differences in either comparison ($F_{\text{low light slopes}}$= 1.385, 10 and 311 d.f., P>0.1 NS; $F_{\text{high light slopes}}$= 1.345, 5 and 174 d.f., P>0.25 NS), and so the null hypothesis, that feeding state did not affect the regression coefficient of the φ vs. S relationship, was proved. A common regression coefficient was calculated for all anemones in low light (and also starved aposymbionts in 190 $\mu E.m^{-2}.s^{-1}$), giving a value of B= 0.075, and the same was done for high light anemones to give a value of B= 0.114.

Elevations of the φ vs. S relationships for starved and fed anemones were compared after replotting the lines for individual anemones to the regression coefficients calculated above for all anemones under low light (i.e. B= 0.075) and high light (i.e. B= 0.114). Wilcoxon two-sample tests were then carried out as previously described, to check for statistical differences in elevation due to feeding state amongst aposymbionts, low light symbionts, and high light symbionts. The results of tests are given in table 5.5. Comparison of starved and fed aposymbionts was not possible because of the small number of fed individuals successfully tested.

Few of the low light comparisons showed any significant difference in y-intercept value between fed and starved symbiotic anemones (table 5.5b). The only difference significant at higher than the 95% level was between fed anemones in darkness and starved anemones at 50 $\mu E.m^{-2}.s^{-1}$, the highest light intensity within the low light grouping. Despite this, the mean y-intercept values were consistantly lower for fed anemones than for starved anemones under the same light intensity.

Table 5.5c shows that in nearly all cases, the elevations of the φ vs. S regression lines for fed symbionts were significantly different

Table 5.5 *Anemonia viridis*: results of Wilcoxon two-sample tests comparing the y-intercept values of anemones from fed and starved treatments. a) Aposymbiotic anemones. b) Low light symbiotic anemones. c) High light symbiotic anemones. Results showing differences in the distribution of y-intercept values are given with significance level. N.S.= no significant difference.

a) Comparison of starved and fed aposymbionts:

Not possible, because of small sample size for fed aposymbionts.

b) Comparison of starved and fed "low light" symbionts:

	Fed, darkness	Fed, 50 $\mu E.m^{-2}.s^{-1}$
Starved, darkness	P<0.1	NS
" , 5 $\mu E.m^{-2}.s^{-1}$	NS	NS
" , 10 $\mu E.m^{-2}.s^{-1}$	NS	NS
" , 20 $\mu E.m^{-2}.s^{-1}$	P<0.1	NS
" , 50 $\mu E.m^{-2}.s^{-1}$	P<0.01	NS

c) Comparison of starved and fed "high light" symbionts:

	Fed, 100 $\mu E.m^{-2}.s^{-1}$	Fed, 190 $\mu E.m^{-2}.s^{-1}$	Fed, 300 $\mu E.m^{-2}.s^{-1}$
Starved 100 $\mu E.m^{-2}.s^{-1}$	P<0.01	P<0.01	NS
Starved 190 $\mu E.m^{-2}.s^{-1}$	P<0.01	P<0.01	P<0.01
Starved 300 $\mu E.m^{-2}.s^{-1}$	P<0.01	P<0.01	P<0.1

from those for starved anemones. From the mean y-intercept values (tables 4.6 and 5.4), it can be seen that this difference was due to the elevations for fed anemones being lower than those for starved anemones. The exception was the comparison of starved anemones at 100 $\mu E.m^{-2}.s^{-1}$ and fed anemones at 300 $\mu E.m^{-2}.s^{-1}$, which nevertheless showed a tendency towards lower elevations in fed anemones that the test was not sensitive enough to detect.

From the statistical tests it therefore appeared that the nutritional history of anemones had no effect on the slopes of φ vs. S regression lines, or on the elevations of the lines in symbionts exposed to light levels below 100 $\mu E.m^{-2}.s^{-1}$, but did affect the elevations of the lines for symbiotic anemones at higher light intensities.

The results of the 24 hour experiments are shown in figure 5.4. The anemones generally exhibited uniform uptake during the light periods, with most values of φ being between 0.1 and 0.4 $\mu g\text{-at } NH_3\text{-}N.g^{-1}.h^{-1}$. During the hours of darkness the results became more variable, but showed a strong tendancy towards efflux of ammonia at rates that exceeded rates of ammonia uptake in the light. The anemones therefore lose more ammonia during the night than they gain during the day, and so must show a net loss of ammonia-nitrogen over 24 hours. Comparison with figure 4.10 shows that this was not the case in starved symbiotic anemones, which tended to show very little flux of ammonia during the "night". It is also apparent from the comparison that daytime uptake rates of fed anemones tended to be slightly lower than those of starved symbionts, although there was some overlap in uptake values for starved and fed individuals.

With an initial incubation concentration of 4 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$, predicted flux rates for fed anemones (from figure 5.3) are

Figure 5.4 *Anemonia viridis*: ammonia flux rates of four fed symbiotic anemones measured during a full aquarium light cycle (12 hours light: 12 hours dark). a) Individual data from four anemones. b) mean uptake rates calculated for each incubation period. Irradiance during light periods was 190 $\mu E.m^{-2}.s^{-1}$. Uptake rates were calculated from repeated 1-hour incubations with mean concentrations of approximately 4 μg-at NH_3-$N.l^{-1}$. Positive values of ρ indicate net ammonia uptake by the symbiosis, negative values indicate net loss to the incubation water.

Figure 5.4a

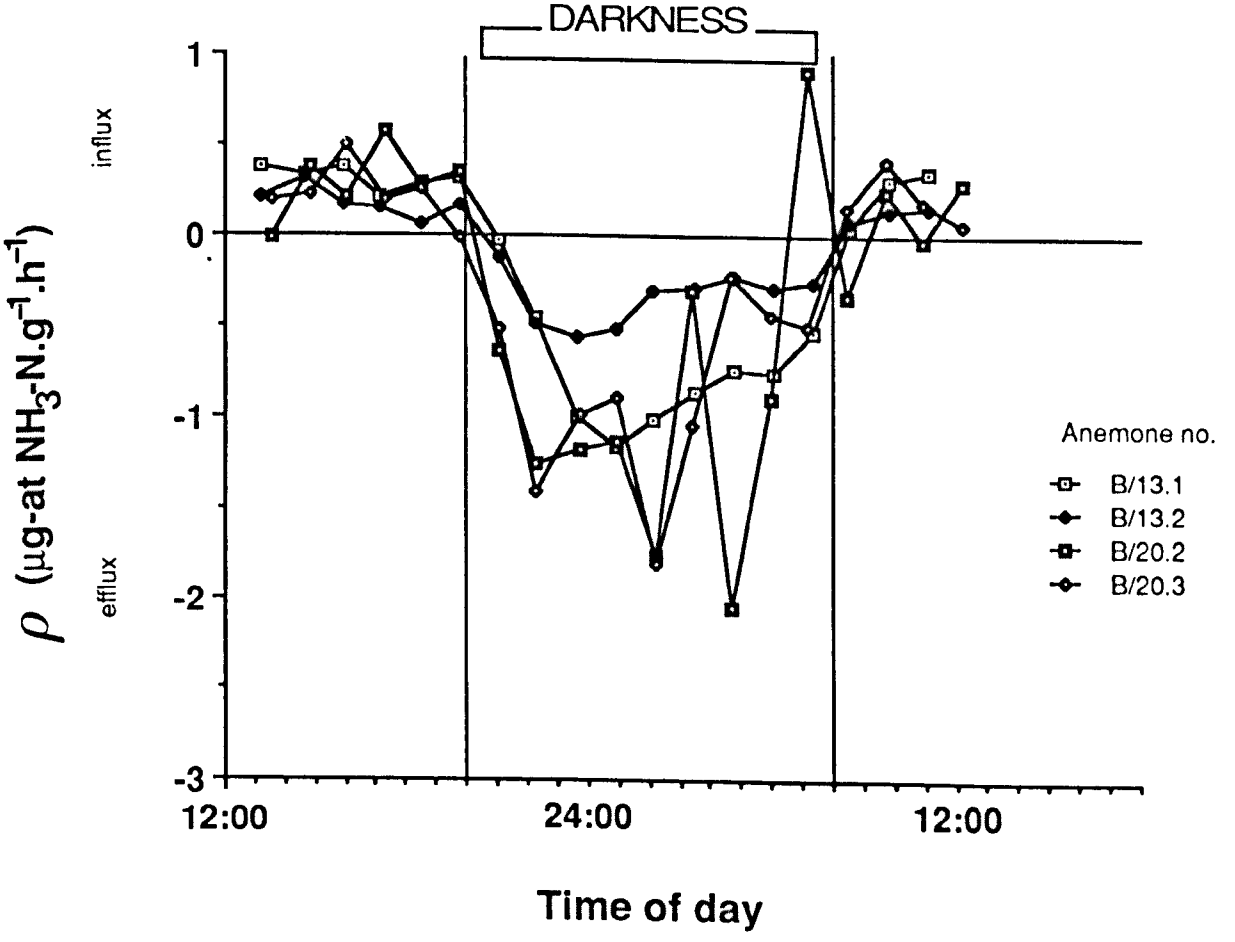
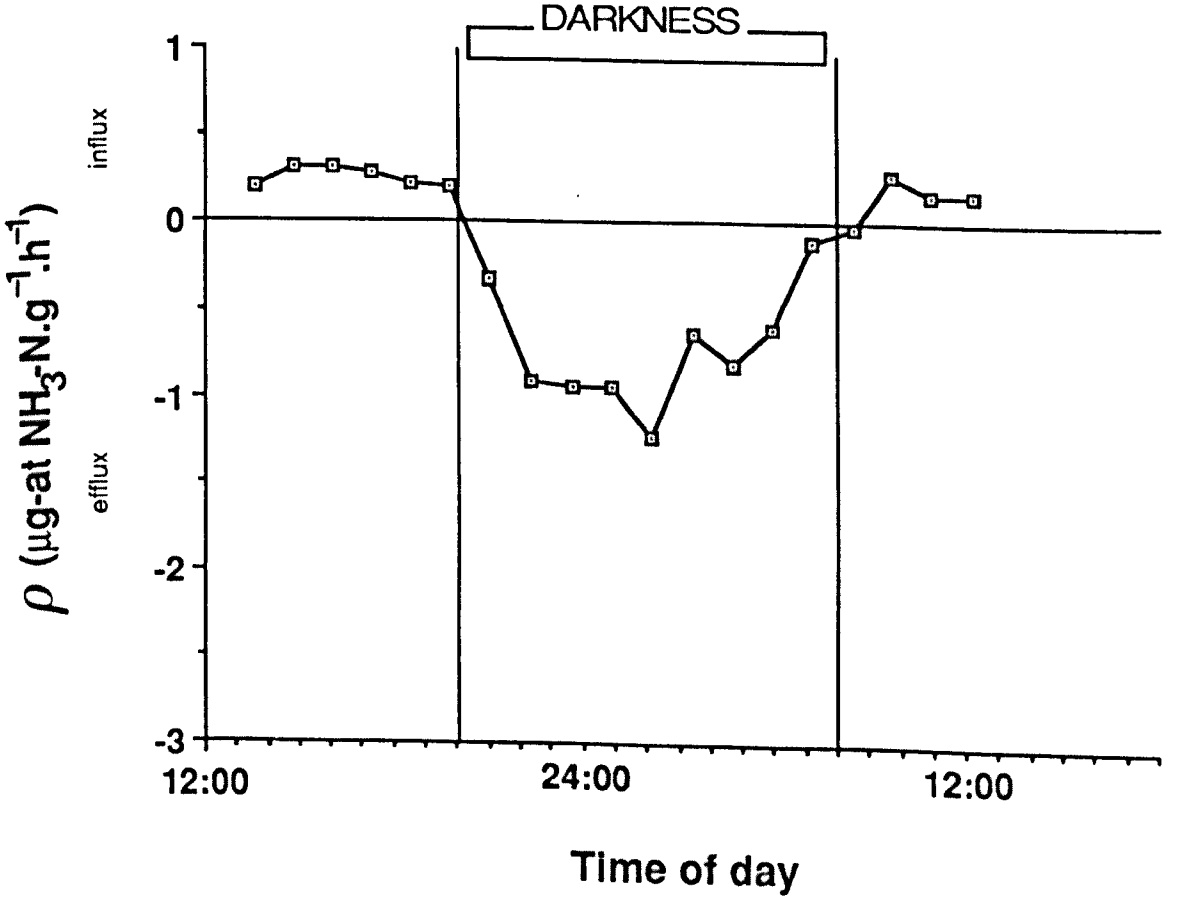


Figure 5.4b



0.082 µg-at NH_3-N.g^{-1}.h^{-1} during the light period, and -0.521 µg-at NH_3-N.g^{-1}.h^{-1} during the darkness hours. These predictions tend to under-estimate both the average daytime uptake rate, of approximately 0.2 µg-at NH_3-N.g^{-1}.h^{-1}, and the average night-time excretion rate of -0.7 µg-at NH_3-N.g^{-1}.h^{-1}. However, both the data on which the predictions are based and the data in the 24 hour experiment included a high degree of variation, and a better agreement would have been expected if larger sample sizes had been available.

5.4 Discussion

In 1978 C.F. D'Elia, writing on the design of experiments aimed at investigating nutrient flux in corals, numbered nutritional state of the organism amongst factors likely to affect flux rates, because "changes in the concentrations of nutrients in internal pools may affect concentration gradients and in turn, nutrient fluxes". Despite this recognition, very few studies on nutrient flux in algal-coelenterate symbioses have attempted to control the nutritional state of the experimental organism, much less investigate the effects of nutritional state on flux rates. The majority of studies have used freshly-collected specimens which have been used immediately after removal from their environment (e.g. Muscatine and D'Elia, 1978; Muscatine and Marian, 1982), held for several days in tanks prior to experimental use (e.g. Propp, 1981; Wilkerson and Trench, 1986), or, commonly, a mixture of both (e.g. D'Elia, 1977; D'Elia and Webb, 1977; Burris, 1983). This presents no problems if the associations are assumed to be feeding at a constant rate, or not at all, but results are more likely to be unrepresentative, given the characteristically small sample sizes, if feeding is variable in frequency or meal size.

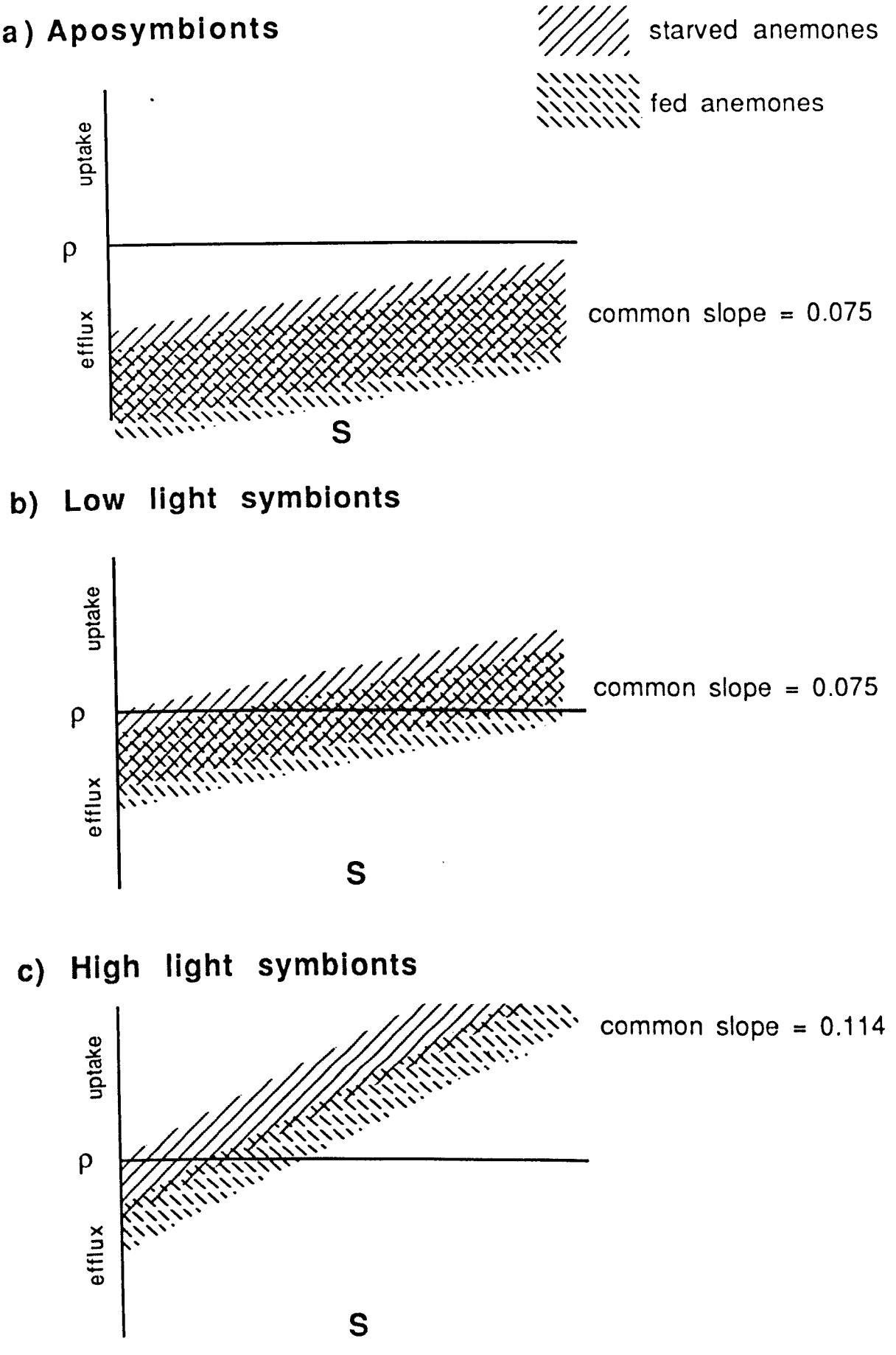
Szmant-Froelich and Pilson (1977; 1984) compared nitrogen flux in

symbiotic and aposymbiotic specimens of the temperate scleractinian coral *Astrangia danae*. These were starved or fed at various frequencies, and nitrogen excretion rates monitored. In almost all cases, fed symbionts were found to excrete nitrogen faster than starved symbionts, and the same was found for aposymbionts (which tended to have higher excretion rates than symbionts under similar feeding regimes). Whilst differences in excretion rates due to feeding were relatively consistent when rates were tested daily for over two weeks (Szmant-Froelich and Pilson, 1977), they were more marked when rates were tested several times during a 24 hour light cycle two days after last feeding (Szmant-Froelich and Pilson, 1984). This latter observation may be associated with the immediate elevation in N excretion and oxygen consumption rates immediately after feeding which was observed in the same study, although both rates "returned to their pre-feeding levels approximately 48 hours after feeding" (Szmant-Froelich and Pilson, 1984).

Comparison of flux data for *Anemonia viridis* in the fed and starved (see Chapter 4) state does not consistently show such clear differences (figure 5.5). Unlike the work on *Astrangia danae*, where incubations had an unspecified, but presumably consistent starting concentration, this study has considered the effects of feeding on the relationship between flux rate and incubation concentration. There are therefore two parameters that may be dependant upon nutritional state: the slope and the elevation of the relationship. No differences were found in slopes that could be ascribed to feeding, both fed and starved anemones under similar light intensities having similar regression coefficients. Similarly, it was generally not possible to show statistical differences in the elevations of the ρ vs. S relationships in comparisons of starved and fed aposymbionts, or

Figure 5.5 *Anemonia viridis*: comparison of the relationship between φ and S for starved and fed anemones. a) aposymbionts (all light intensities). b) Symbionts at light intensities of 50 $\mu E.m^{-2}.s^{-1}$ and below. c) Symbionts at light intensities of 100 $\mu E.m^{-2}.s^{-1}$ and above. Cross-hatching indicates the region of the graph in which the regression line for an individual anemone from the specified feeding class would be expected to lie. Where there is extensive overlap in the regions for fed and starved anemones at a similar light intensity, feeding state can not be shown to affect the φ vs. S relationship. This is the case in figures 5.5a and 5.5b, but in figure 5.5c the overlap is sufficiently small for the difference in elevations between starved and fed anemones to be significant.

Figure 5.5


a) Aposymbionts
starved anemones
fed anemones
uptake
ρ
efflux
common slope = 0.075
S
b) Low light symbionts
uptake
ρ
efflux
common slope = 0.075
S
c) High light symbionts
uptake
ρ
efflux
common slope = 0.114
S

symbiotic anemones in low light conditions (50 $\mu E.m^{-2}.s^{-1}$ or below). It is felt, however, that this was due to the relative insensitivity of the Wilcoxon two sample test when used on small sample sizes, as it was invariably the trend for fed anemones to have more negative y-intercept values than starved anemones (non-significant results were often obtained by the incursion of a single value from one treatment into the ranks of the other treatment in the comparison). The y-intercepts of fed and starved anemones in high light conditions (100 $\mu E.m^{-2}.s^{-1}$ or above) showed less variation and comparisons did not suffer from isolated overlapping of values. The Wilcoxon tests therefore showed significant differences in the elevations for fed and starved "high light" anemones, giving clear evidence that feeding depressed the φ vs. S regression line. Visual comparison of the full light cycle data given in figures 4.10 and 5.4 reinforces the conclusion that under identical conditions of lighting, incubation ammonia concentration and symbiotic state, fed anemones show lower rates of uptake and higher rates of efflux than starved anemones.

If it is assumed that the observed (net) flux in symbiotic anemones is the result of two antagonistic processes, the metabolic production of ammonia by the animal cell and the uptake of ammonia by the algal cell (see Chapters 6 and 7), then the lowered elevation of the φ vs. S regression line in fed anemones relative to starved anemones may be due to two factors: decreased uptake activity by the algal cells or increased animal ammonia production.

A reduced capacity to take up ammonia in the algal population could result from a reduction in algal density in fed anemones, or by a reduction in the photosynthetic activity of the algal population (assuming photo-linking of the ammonia uptake mechanism). Algal densities appear to be affected by feeding regime in different ways in different host species. No differences in algal densities were found

in starved and fed *Plesiastra urvillei* (Kevin and Hudson, 1979), starved *Astrangia danae* and *Aiptasia pallida* showed reduced densities when compared to fed specimens (Szmant-Froelich and Pilson, 1980; Clayton and Lasker, 1984), but starved *Aiptasia pulchella* had higher algal densities than fed individuals (Muller-Parker, 1985). *Anemonia viridis* show increased algal densities when starved in light (Tytler, 1981), and so it is possible that starved anemones are more able to take up ammonia than those fed regularly. Chlorophyll content of zooxanthellae in starved *Aiptasia pulchella* was higher than in fed anemones (Muller-Parker, 1985), although no difference was found in *A. pallida* (Clayton and Lasker, 1984). A similarly increased chlorophyll content could augment higher algal densities in *Anemonia viridis* to give more potential for ammonia uptake in starved individuals.

Changes in the ability of the algal population to take up ammonia could not have been the sole reason for lower elevations of the ρ vs. S relationship in fed anemones, because aposymbiotic individuals also showed some effects of feeding. There must therefore have been an increase in the animal production of ammonia in fed anemones. Large increases in ammonia excretion due to amino acid deamination after feeding have long been known in vertebrates (e.g. Wilhelmj and Bollman, 1928), and were clearly shown by Szmant-Froelich and Pilson (1984) in *Astrangia danae*. The classification of such post-prandial excretion rates as "increased" relies upon some comparison with "background" or pre-prandial excretion rates. These "background" excretion rates are the result of constant nitrogen turnover in the animal cell, where, despite some recycling of nitrogenous products, there is always some loss of nitrogen from the metabolic pathways. It is therefore possible to categorize an animal as "nitrogen-starved" as soon as the post-prandial nitrogen excretion rates have returned to

"background" levels, since, unless there is a further input of amino acids, the animal is suffering an irreversible depletion of its nitrogen reserves. What has not been shown is whether or not the background nitrogen excretion rate is relatively constant, regardless of the duration of "starvation". Prolonged starvation would result eventually in all lipid and carbohydrate energy reserves becoming depleted, at which point the animal will catabolize structural proteins for energy, leading to increased deamination and nitrogen excretion before the animal ultimately dies. The animal may have the ability to ameliorate the effects of nitrogen starvation through more efficient recycling of nitrogenous metabolic end products, possibly by increasing the activity of the enzymes responsible for this. In this case the "background" nitrogen excretion rate may slowly fall, although ultimately, without feeding, the excretion rate must rise, as explained above. There is some difficulty, therefore, in defining exactly when the "fed" anemones in this study became "starved". With regular feeding it is conceivable that the background low level of excretion is never reached, because the anemone is fed again whilst still showing signs of post-prandial elevated deamination rates. It was hoped that this would be avoided in these experiments by assuming that the elevated deamination rates would last for a maximum of 3 days. Preliminary experiments with fed anemones, using the methods described in section 4.2.2, had suggested that 72 hours would be an adequate period, but this methodology gave very variable results (see section 4.2.3). It is therefore likely that some residual signs of elevated deamination rates might be seen in some individuals after this time. It was therefore not clear if the animal basal ammonia production rate actually differed between "fed" and long-term starved individuals, once any residual post-prandial deamination effect is discounted.

The anemones in this study may therefore have had S vs. φ relationships with lower elevations when fed because of:

1) a reduced algal capacity to take up ammonia due to lower algal densities and/or less chlorophyll *a* per algal cell (symbionts only)

2) residual post-prandial deamination, resulting from testing too soon after feeding

3) starved anemones showing higher rates of animal nitrogen recycling activity.

Any of these factors could work alone, or in conjunction with the others, to produce the results given in the previous section.

It still appears from these results that fed symbiotic *Anemonia viridis* at low ambient ammonia concentrations lose less nitrogen to the environment than aposymbionts, although the proportion of total ammonia production retained may be less than in starved symbionts (see Chapter 7). Over 24 hours it is notable that, whilst starved symbiotic *A. viridis* show a net accumulation of DIN, fed symbionts show a net loss.

Chapter 7 will discuss the relative importance of DIN uptake in *A. viridis* of the two nutritional categories covered in this study. It is important to note that any future work on the contribution of DIN uptake to the N requirements of algal-coelenterate symbioses should take into account the nutritional status of both the experimental animals and animals in the field, if extrapolation from the laboratory environment is intended. Future work should include further investigation of the magnitude of ammonia fluxes during the period immediately after feeding, since discounting these might result in serious errors in 24 hour nitrogen budgets if feeding occurs. To

complement this, more information is also required about feeding rates, assimilation rates, and biochemical composition of prey in the field.

CHAPTER 6: AMMONIA UPTAKE AND INCORPORATION IN ZOOXANTHELLAE

6.1 Introduction

It is generally accepted that the uptake of DIN by algal-coelenterate symbioses is due to the assimilation of ammonia, nitrate and nitrite by the symbionts (see Muscatine (1980) for review). This ability has been demonstrated by a number of studies using zooxanthellae isolated from various coelenterate and molluscan hosts (e.g. McLaughlin and Zahl, 1966; Taylor, 1978b; Muscatine *et al.*, 1979; Muscatine and Marian, 1982; Wilkerson and Trench, 1986). Very few studies, however, have attempted to go beyond this and investigate the kinetics of uptake by zooxanthellae (D'Elia *et al.*, 1983). Such investigations, when combined with knowledge of the availability of ammonia in the animal cell, potentially allow better understanding of the mechanism that controls DIN uptake in the intact association. The first aim of the work in this section was therefore to investigate the kinetics of ammonia uptake by zooxanthellae isolated from *Anemonia viridis*.

In the previous chapters it was shown that the uptake of DIN by the *Anemonia viridis* symbiosis is photodependent, and that it does not occur in aposymbiotic individuals. These observations suggest that the zooxanthellae are the primary site of ammonia uptake. The possibility remains, however, that the presence of zooxanthellae in some way stimulates an animal-tissue based uptake mechanism, such as host glutamine synthetase or glutamate dehydrogenase as suggested by Rees (1987) for the zoochlorellae in the *Hydra* symbiosis. The second aim of this part of the study was therefore to test whether the ammonia taken up passes directly to the zooxanthellae or to the animal host.

6.2 The kinetics of ammonia uptake by freshly isolated zooxanthellae

6.2.1 Introduction

The simplest available way to investigate the uptake of ammonia by isolated zooxanthellae was to use the methods of the previous two chapters, based upon the measurement of rates of depletion of ammonia from the incubation media. Since it has been shown that cultured endosymbionts may differ markedly from freshly isolated ones in their ability to assimilate dissolved inorganic nitrogen (D'Elia *et al.*, 1983; Domotor and D'Elia, 1984), the zooxanthellae in this study were used immediately after isolation from the host.

6.2.2 Methods

Zooxanthellae from the tentacles of two large, starved *A. viridis* were isolated by the procedure described in section 2.3.2. The washed zooxanthella pellet so obtained was suspended in 10 ml of filtered sea water (5 μm pore size membrane filtered) and then divided into two equal aliquots. These were each made up to 180 ml with filtered sea water, to give chlorophyll *a* concentrations ranging from 0.3 to 0.8 μg $chl_a.ml^{-1}$. These suspensions were placed in two of the temperature-controlled incubation chambers in the apparatus used for whole anemone incubations (see section 4.3.2). The third chamber contained 180 ml of filtered sea water, as a control. The water in the chambers was maintained at 10°C, and was circulated by the magnetic stirrers to keep the zooxanthellae in suspension.

At the start of the experiment a small volume of concentrated ammonium sulphate solution was injected into each of the chambers, to raise the ammonia concentrations to between 40 and 50 μg-at NH_3-$N.l^{-1}$.

These relatively high concentrations were used to ensure that the algae would be exposed to levels of ammonia sufficient to saturate the uptake mechanism (see section 6.2.3). 5 ml water samples were immediately taken for ammonia analysis by the method described in section 2.5.1.2, and samples of the same volume were withdrawn from the chambers at five or ten minute intervals for the following two hours. The water samples, free of particulate matter, were obtained by drawing 6 ml of the incubation suspension into a plastic syringe, and expelling 5 ml of this sample into a 2-dram glass vial through a 5 μm pore size membrane filter mounted in a Swinnex filter holder (Whatman Ltd., UK). The remainder of the sample solution was returned to the incubation chamber, so that the volume of the incubation was reduced by 5 ml with each sample, whilst the zooxanthella concentration remained constant. To prevent sample contamination with ammonia from the filters (c.f. Marvin *et al.*, 1972), 50 ml of ammonia-free sea water was flushed through each prior to use. The filters were used for one sample each and then discarded.

At the end of the incubation, a 5 ml unfiltered sample was taken from each experimental chamber for chlorophyll *a* determination, following the method given in section 2.4.2.2.

Depletion curves were obtained for zooxanthellae in light (190 $\mu E.m^{-2}.s^{-1}$) and darkness. For the darkness depletions, the zooxanthella separation was carried out under low intensity red light immediately following the anemones' 12 hour dark period, to avoid pre-experimental exposure of the algae to photosynthetically active radiation.

6.2.3 Results

In all illuminated incubations containing zooxanthellae, ammonia concentrations were reduced from above 40 µg-at NH_3-N.l^{-1} to almost undetectable levels within 120 minutes. Control incubations showed no change in ammonia concentration. Figure 6.1 shows the time course of depletion in the incubations of one experiment. Uptake was initially rapid, as shown by the steep curve for the first 20 minutes, then continued at a steady rate, before gradually slowing as incubation concentrations dropped below 10 µg-at NH_3-N.l^{-1}.

In order to investigate the kinetics of uptake, graphs of uptake rate (φ) against mean incubation concentration (S) were constructed. To obtain values of φ and S, the eye-fitted curves of the depletion time course graphs (see figure 6.1) were divided into five or ten minute intervals. For each interval, uptake rate was calculated by equation (1) from section 4.3.2:

$$\varphi = \Delta S \times v \times t^{-1} \times w^{-1} \qquad (1)$$

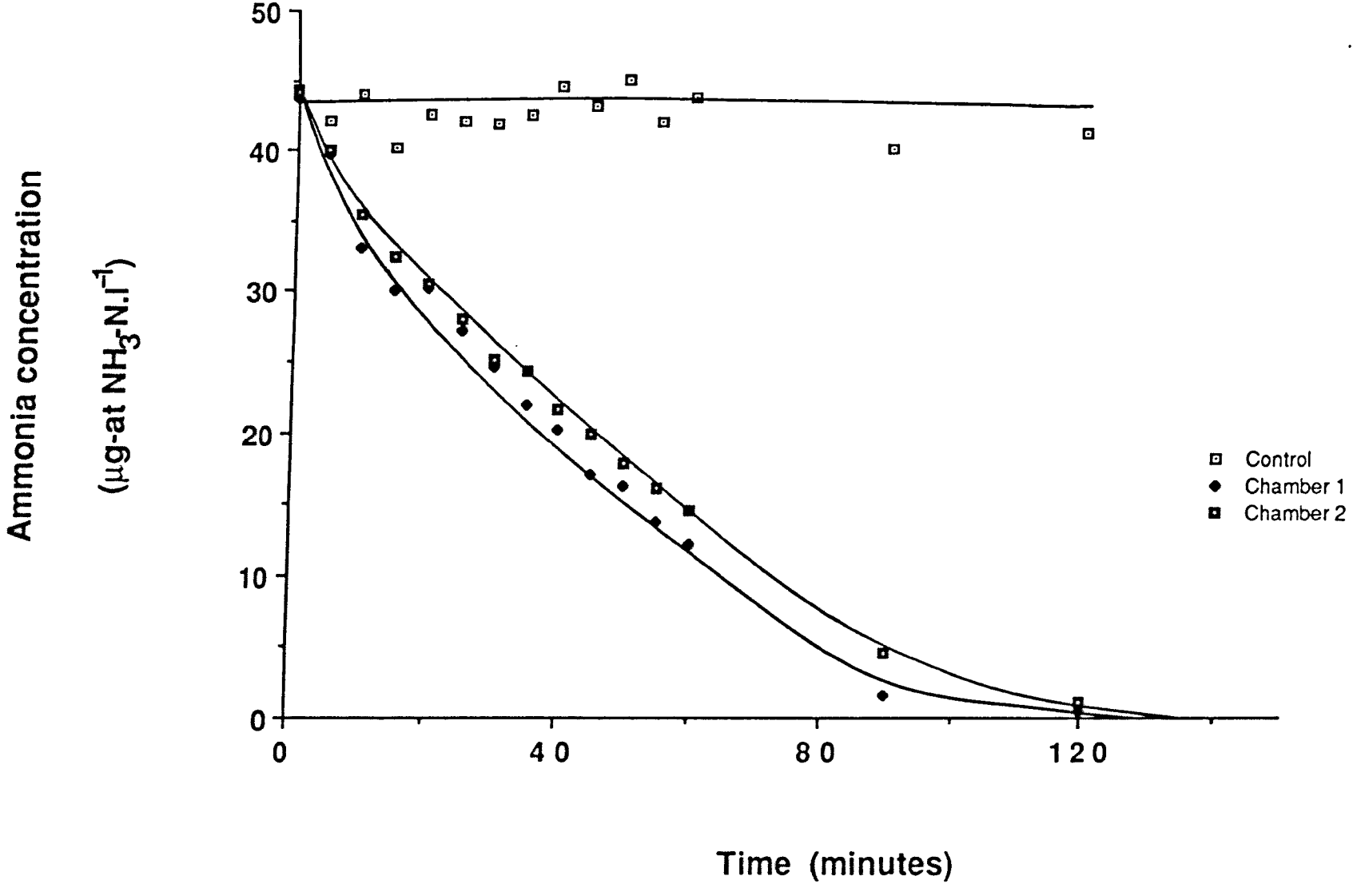
The symbols are as given in section 4.3.2, except that ΔS, the difference in ammonia concentration over the interval, is read from the eye-fitted curve, and w is the mass of chlorophyll *a*, in µg. When rearranged to:

$$\varphi = \frac{\Delta S}{t} \times \frac{v}{w} \qquad (2)$$

The term v/w represents the inverse of the concentration of the biomass in the chamber. Since this was assumed not to change, it was not necessary to calculate v or w^{-1} for each interval, and the term can be changed to chlorophyll *a* concentration^{-1}. Equation (2) then

Figure 6.1 Zooxanthellae: typical time-course for ammonia depletion in light by zooxanthellae freshly isolated from *Anemonia viridis*. The two experimental incubations shown each had chlorophyll *a* concentrations of approximately 1.1 μg Chl*a*.ml^{-1}. Light level was 190 μE.m^{-2}.s^{-1}. The control chamber contained membrane filtered seawater. Curves fitted by eye.

Figure 6.1


Ammonia concentration
(µg-at NH_3-N.l^{-1})
50
40
30
20
10
0
0
40
80
120
Time (minutes)
Control
Chamber 1
Chamber 2

simplifies to:

$$\varphi = \Delta S \times t^{-1} \times c^{-1} \qquad (3)$$

where c = concentration of chlorophyll *a* measured at the end of the incubation ($\mu g\ chl a.l^{-1}$).

Figure 6.2 shows a typical graph of φ vs. S, calculated from figure 6.1. In all cases, the curve obtained for the φ vs. S relationship was a modified rectangular hyperbola, with an extra steepening of the curve above incubation concentrations of 20 to 30 μg-at NH_3-$N.l^{-1}$. This steepening of the curve corresponds to the high rates of uptake seen in the first 10 to 20 minutes of every depletion experiment. Such unexpectedly high rates occurred even in preliminary experiments where the starting concentration was 10 μg-at NH_3-$N.l^{-1}$, and when the zooxanthellae used to provide figure 6.1 were incubated under the same conditions for a second time. This phenomenon has not been reported in other studies on isolated zooxanthellae or free-living phytoplankton, and may be an artifact of the experimental design.

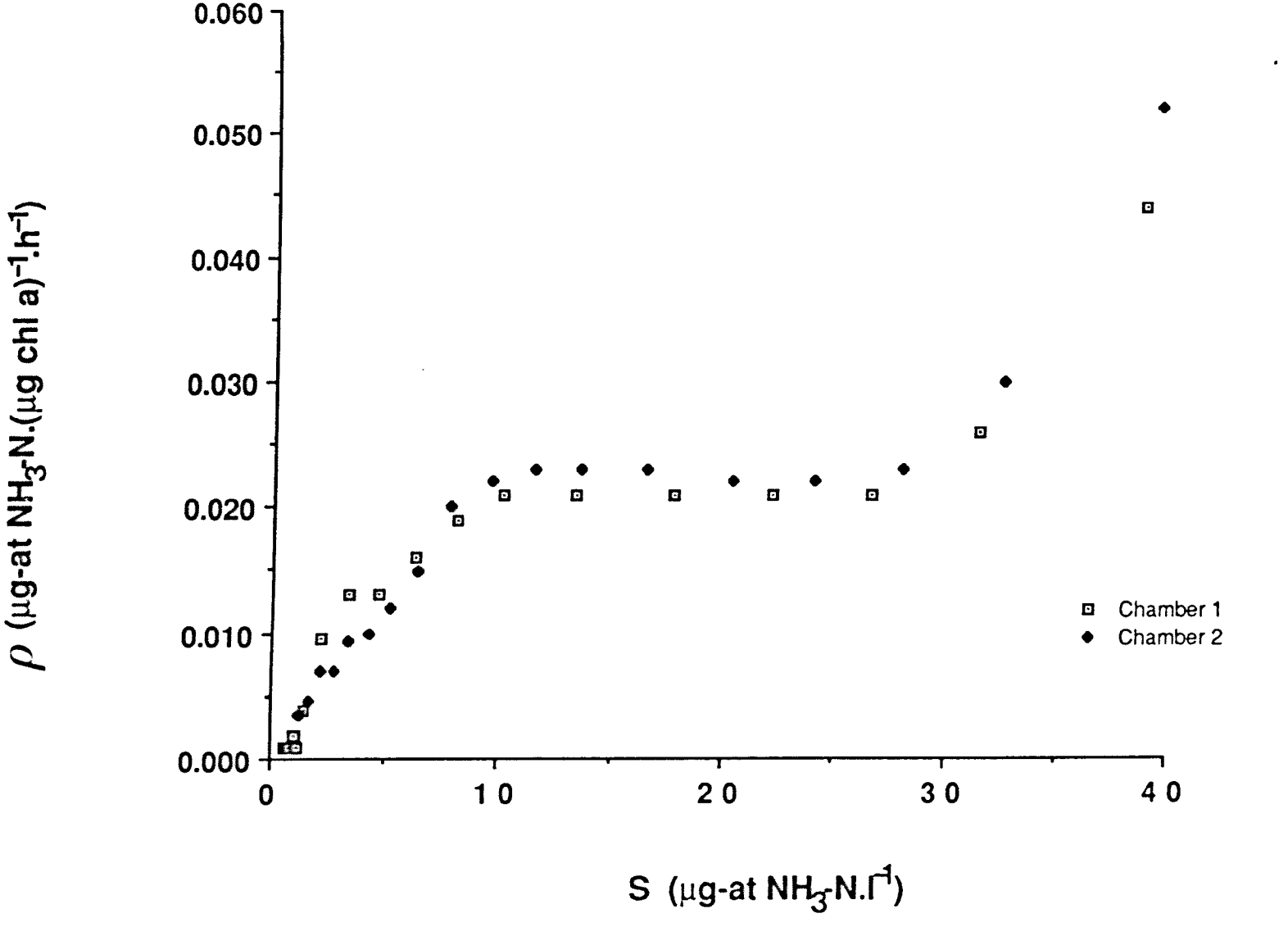
At values of S below 20-25 μg-at NH_3-$N.l^{-1}$ the φ vs. S relationship appeared to approximate to a rectangular hyperbola, characteristic of Michaelis-Menten uptake kinetics. The equation of the Michaelis-Menten relationship is:

$$V = \frac{V_{max}.S}{S + K_s} \qquad (4)$$

Where V = uptake rate, and is analogous to φ in this study (by convention, V is used for nitrogen uptake only when

Figure 6.2 Zooxanthellae: hourly ammonia uptake rates at different incubation ammonia concentrations for zooxanthellae isolated from *Anemonia viridis*. The data was calculated from the eye-fitted curves shown in figure 6.1.

Figure 6.2


0.060
0.050
0.040
0.030
0.020
0.010
0.000
0
10
20
30
40
ρ (µg-at NH3-N.(µg chl a)-1.h-1)
S (µg-at NH3-N.l-1)
Chamber 1
Chamber 2

biomass units are also in terms of N (Dugdale and Goering, 1967)).

V_{max} = maximum uptake rate.

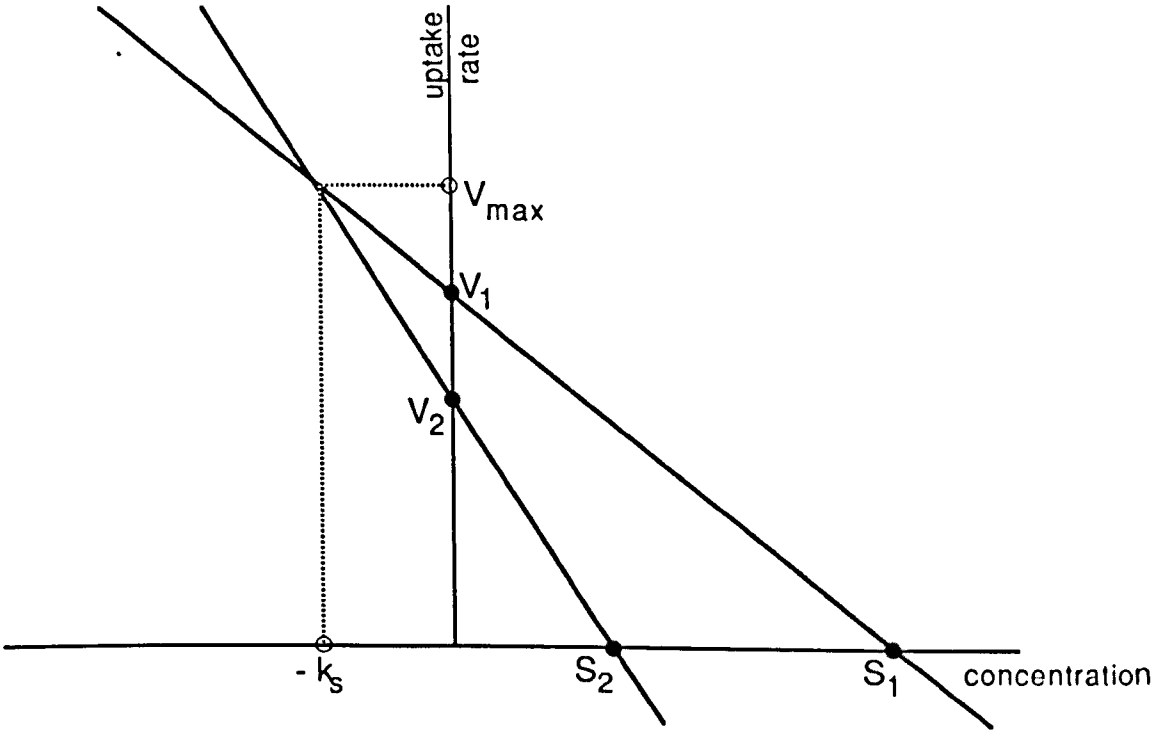
K_S = half-saturation constant, or value of S at which $V=V_{max}/2$.

The terms that define the slope of the V vs. S hyperbola are V_{max} and K_S, and they are normally estimated from linear transformations of the Michaelis-Menten equation (Neame and Richards, 1972). The linear transformations, however, are often subject to undue influence from outlying points, and the parametric statistical methods used to derive the formulae for the transformations may not be appropriate (Cornish-Bowden and Eisenthal, 1974). A method that gives a non-parametric estimate of the two values, and which is not as sensitive to outliers is the direct linear plot (Eisenthal and Cornish-Bowden, 1974). In this, instead of considering the value of (S,V) as a point, it is drawn as a line through the coordinates (S,0), (0,V) on a graph of rate vs. concentration (figure 6.3a). Because of the geometry of the rectangular hyperbola this line passes through the coordinate $(-K_S,V_{max})$. If two values of (S,V) are available, the point where the two lines cross is the best estimate of K_S and V_{max} for the two (S,V) values. With many values of (S,V), each intersection of lines provide an estimate of K_S and V_{max} (figure 6.3b). If all of these are ranked, the median values of K_S and V_{max} may be considered the statistically best estimates of the parameters. This procedure is difficult to carry out manually with more than a small number of (S,V) values, and so a commercially-available computer programme (Enzpack, Elselvier Scientific Publications), based on the direct linear plot method described, but incorporating modifications for replicate samples and negative estimates of K_S and V_{max} (Porter and Trager, 1977; Cornish-Bowden and Eisenthal, 1978) was used to obtain estimates of maximum

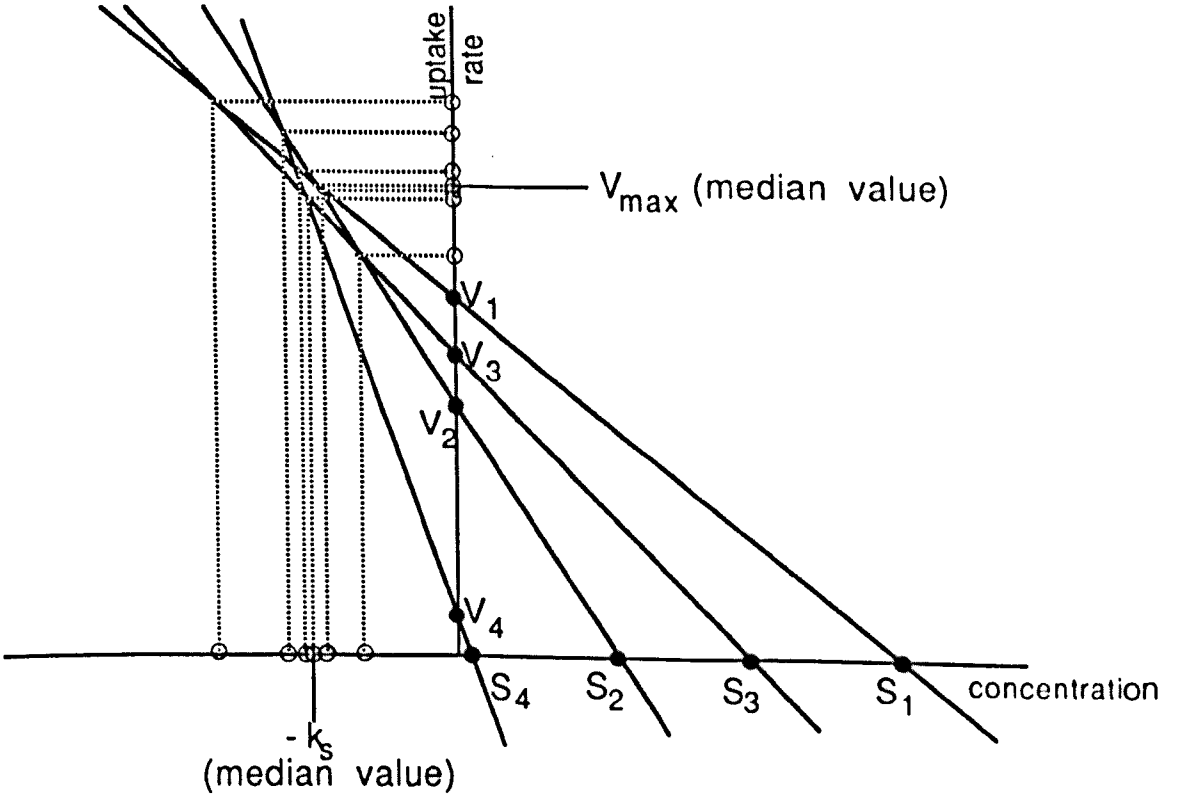
Figure 6.3 Michaelis-Menten kinetics: diagrammatic illustration of the use of the direct linear plot (Eisenthal and Cornish-Bowden, 1974) for the estimation of K_S and V_{max}. Associated values of S and V, instead of being represented as a point (as in figure 6.2), are represented as a line passing through the points (S,0) and (0,V) on a graph of rate vs. concentration. a) For two values of S and V, the point at which the lines cross estimates K_S and V_{max}. b) For several experimental values of S and V, each intersection of lines is an estimate of K_S and V_{max} (if the experiment conformed perfectly to the Michaelis-Menten equation, all lines would intersect at one point, giving the true value of K_S and V_{max}). The best statistical estimate of the parameters is found by ranking the values given by line intersections, and taking the median.

Figure 6.3

a) For experimental values (S_1, V_1), (S_2, V_2)



b) For experimental values (S_1, V_1), (S_2, V_2), (S_3, V_3), (S_4, V_4)



uptake rates and half-saturation constants from experimental data. Estimates of K_s and ρ_{max} for ammonia uptake in isolated zooxanthellae in the light are given in table 6.1.

The depletion experiments run in darkness produced time courses for ammonia depletion that were similar to those of zooxanthellae exposed to light (figure 6.4a). There was a rapid disappearance of ammonia in the first twenty minutes, followed by a slower depletion. Unfortunately these incubations had lower concentrations of zooxanthellae than in the light incubations, and so did not reduce ammonia levels as far as in the latter experiments. Nevertheless the uptake rate vs. ammonia concentration relationships calculated from the depletion time course were close to those for zooxanthellae in the light (figure 6.4b). The K_s and ρ_{max} values, obtained by direct linear plots, were different for the two experimental chambers in darkness (table 6.1), chamber 1 giving lower values for both parameters. This was due to the truncated shape of the depletion curve, which in chamber 1 did not start to become asymptotic during the incubation (figure 6.4a), leading to underestimation of K_s and ρ_{max} values. The depletion curve for chamber 2 had started to become asymptotic, and since it therefore included more of the region of depletion curve corresponding to the curvilinear part of the Michaelis-Menten hyperbola, the estimates of K_s and ρ_{max} were more reliable than for chamber 1. The values for chamber 2 were similar to those for incubations in the light. It would therefore appear that ammonia uptake in freshly isolated zooxanthellae is not light-dependant.

Table 6.1 Zooxanthellae: values of K_s and ρ_{max} calculated for the ρ vs. S relationship for freshly isolated zooxanthellae. Values are given for incubations in light and darkness, with mean values for each light intensity.

Experiment Number	Light level	Estimated K_s ($\mu g\text{-at } NH_3\text{-N}.l^{-1}$)	Estimated ρ_{max} ($\mu g\text{-at } NH_3\text{-N}.(ug\ chl_a)^{-1}.h^{-1}$)
1.1	190 $\mu E.m^{-2}.s^{-1}$	6.49	0.0429
1.2		6.86	0.0246
2.1		6.30	0.0317
2.2		7.09	0.0355
3.1		5.30	0.0303
3.2		3.30	0.0309
Mean		5.89	0.0327
4.1	0 $\mu E.m^{-2}.s^{-1}$	2.39	0.0269
4.2		7.59	0.0331
Mean		4.99	0.0300

Figure 6.4 Zooxanthellae: a) time-course for ammonia depletion in darkness by zooxanthellae freshly isolated from *Anemonia viridis*. The two experimental incubations shown each had chlorophyll *a* concentrations of approximately 0.7 µg Chl*a*.ml^{-1}. The control chamber contained membrane filtered seawater. Curves fitted by eye. b) Hourly ammonia uptake rates at different incubation ammonia concentrations for zooxanthellae incubated in darkness. The data were calculated from the eye-fitted curves shown in figure 6.4a.

Figure 6.4a

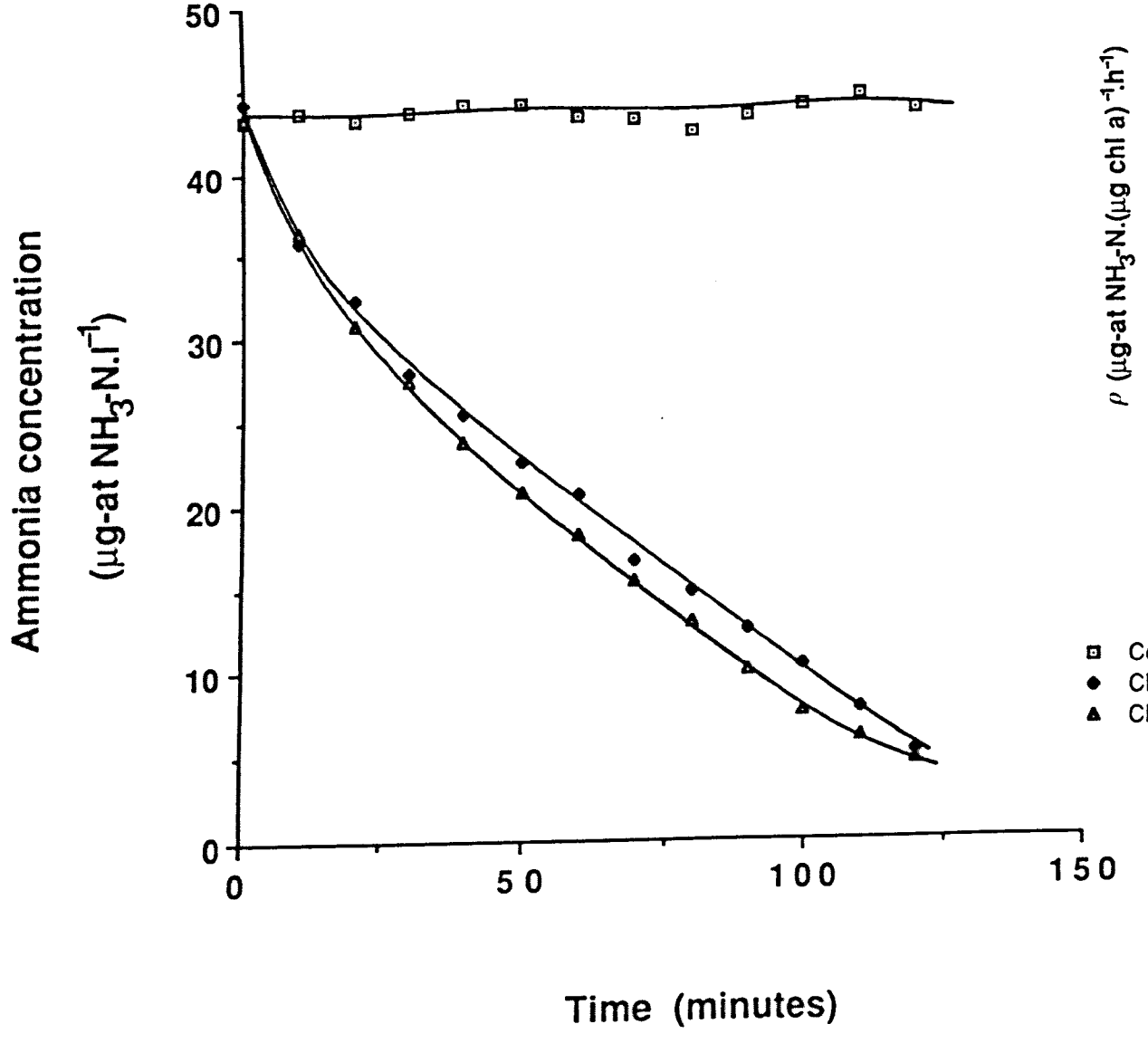
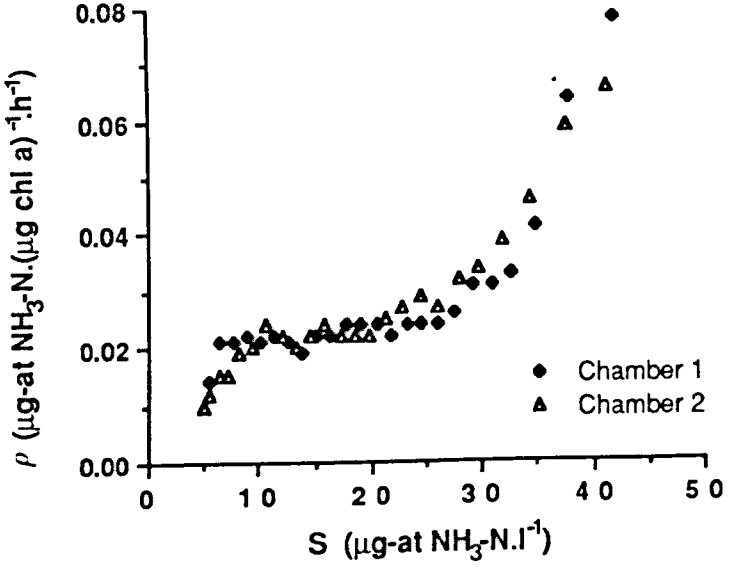


Figure 6.4b



6.3 Incorporation of ammonia by zooxanthellae *in situ*

6.3.1 Introduction

The experiments in Chapters 4 and 5 showed that the intact *Anemonia viridis* association is able to take up ammonia. The depletion experiments in the previous section similarly showed that the zooxanthellae of *Anemonia viridis* have the ability to take up ammonia, at least after they have been isolated from the host. This is not, however, conclusive evidence that the zooxanthellae are taking up ammonia from the surrounding water when in symbiosis. To prove this, it is necessary to show that ammonia molecules from the incubation water are incorporated into the zooxanthellae before they are incorporated into the animal cells. This was investigated by exposing the association to ammonia labelled with the stable isotope ^{15}N.

The use of ^{15}N tracers could not be used to study the kinetics of DIN uptake by zooxanthellae *in situ*, because of the requirement for accurate measurement of the mass of organic nitrogen in the zooxanthellae (see Dugdale and Wilkerson (1986) for discussion of analytical considerations in the use of ^{15}N in studies of uptake kinetics). The method could be used, however, to investigate the incorporation of ^{15}N into the symbiosis by testing for increases in the ratio of ^{15}N:^{14}N in isolated algae and host tissue samples after exposure of symbiotic anemones to water containing elevated concentrations of $^{15}NH_3$. By comparing the relative rates of labelling of animal and algal cells, it was possible to test the hypothesis that assimilation by zooxanthellae is the first stage in the uptake of ammonia by the symbiosis.

6.3.2 Methods

Single *Anemonia viridis* were placed in each chamber of the incubation apparatus described in section 4.3.2, and allowed to settle whilst sea water containing negligible levels of ammonia (<0.1 μg-at NH_3-N.l^{-1}) was constantly pumped through the chambers. The light level used was 190 μE.m^{-2}.s^{-1}, and the chambers were maintained at 10°C. After one hour the water flow was stopped, and sufficient ^{15}N-labelled ammonium sulphate (99 atom-% ^{15}N, Amersham International, Amersham, UK), was added to raise the concentration of ammonia to 20 μg-at NH_3-N.l^{-1}. The anemones were exposed to the elevated concentrations of $^{15}NH_3$ for 0.5, 10 or 30 minutes (time taken from addition of label), after which they were removed from the chambers and rinsed in distilled water. The tentacles of each anemone were excised, homogenized in filtered seawater, and zooxanthellae were isolated by density gradient centrifugation, using the method described in section 2.3.1. By this method, it was possible to obtain samples of zooxanthellae and host tissue that were free of visible contamination by host debris or algal cells respectively. The samples were freeze-dried and ground to a fine powder with a glass rod, and the ^{15}N-enrichment was measured with a mass spectrometer interfaced with an automatic elemental analyser (Preston and Owens, 1983). ^{15}N analyses were carried out by Dr. T. Preston at the Scottish Universities Research and Reactor Centre, East Kilbride, using a VG MM602 mass spectrometer (VG Isogas, Middlewich, Cheshire, UK) linked to an ANA 1400 automatic nitrogen analyser (Carlo Erba, Milan, Italy).

Controls consisted of symbiotic anemones, incubated for 0.5 or 30 minutes with no $^{15}NH_3$ spike, and fractionated as above. Aposymbiotic anemones, incubated for 30 minutes in 20 μg-at $^{15}NH_3$-N.l^{-1}, were used to test for the occurrence of incorporation of ammonia by the anemone

in the absence of algae. The aposymbionts were rinsed in distilled water, freeze-dried and the whole anemone was ground up before analysis for ^{15}N enrichment.

6.3.3 Results

The natural abundance of the ^{15}N isotope is approximately 0.366% of total N. All of the zooxanthella samples from anemones exposed to $^{15}NH_3$ contained higher percentages of ^{15}N than natural abundance (table 6.2). Zooxanthella samples from anemones not exposed to $^{15}NH_3$ contained percentages of ^{15}N very similar to natural levels (table 6.2), showing that the zooxanthellae did not preferentially accumulate ^{15}N from natural background levels.

The percentage of ^{15}N in the zooxanthellae of experimental animals increased almost linearly with incubation duration from 0.5 to 30 minutes (figure 6.5). The experimental zooxanthellae presumably contained similar levels of ^{15}N to the control algae, prior to their exposure to the labelled ammonia, and so a faster enrichment of the zooxanthellae must have occurred over the first 0.5 minute.

The animal tissue samples from symbiotic anemone incubations showed no noticable increase in ^{15}N abundance, regardless of incubation duration (table 6.3). In addition, the lack of ^{15}N in the host fraction indicates that there was little or no ^{15}N contamination from incubation water not rinsed off the anemones.

The aposymbiotic anemones incubated for 30 minutes in the presence of $^{15}NH_3$ showed some ^{15}N enrichment (table 6.3), indicating a low level of $^{15}NH_3$ uptake. The enrichment is likely to have resulted from exchange diffusion processes across the cell membrane.

Table 6.2 Zooxanthellae: ^{15}N enrichment of zooxanthella fractions from symbiotic anemones exposed to 20 µg-at $^{15}NH_3$-N.l^{-1} for varying lengths of time. Enrichments of algal fractions from control anemones are also shown. Individual values for samples are absolute % composition of ^{15}N, mean values have been corrected for natural abundance (0.366 atom % ^{15}N).

Exposure to $^{15}NH_3$	Atom % ^{15}N	Mean atom % excess ^{15}N
20 µM $^{15}NH_3$ for 0.5 mins	0.399 0.398 0.401	0.033
20 µM $^{15}NH_3$ for 10 mins	0.447 0.528	0.122
20 µM $^{15}NH_3$ for 30 mins	0.649 0.606 0.669	0.275
No spike, for 0.5 mins	0.367 0.369 0.362	0.000
No spike, for 30 mins	0.368 0.368 0.378	0.016

Figure 6.5 *Anemonia viridis*: ^{15}N enrichment of anemone and algal fractions of symbiotic anemones exposed to 20 µg-at $^{15}NH_3$-N.l^{-1}, at an irradiance of 190 µE.m^{-2}.s^{-1}, for different lengths of time. Values have been corrected for natural abundance of ^{15}N. Each point is the mean of 3 experiments, error bars show the range of values (where no error bars are drawn, the range is obscured by the symbol). ^{15}N enrichment of aposymbiotic anemones after 30 minutes exposure to the same concentration of $^{15}NH_3$ is also shown.

Figure 6.5

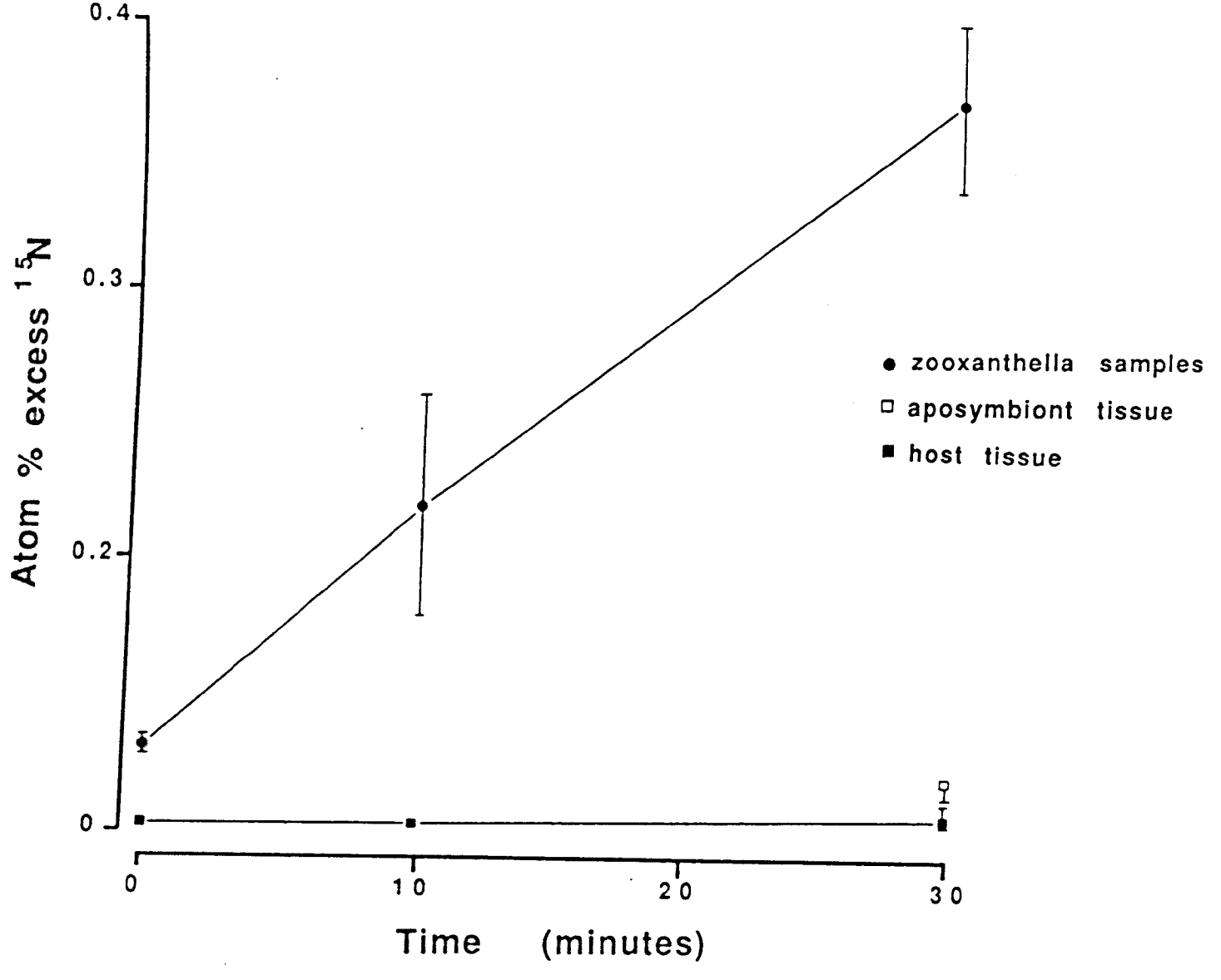

0.4
0.3
0.2
0
Atom % excess ^{15}N
zooxanthella samples
aposymbiont tissue
host tissue
0
10
20
30
Time (minutes)

Table 6.3 *Anemonia viridis*: ^{15}N enrichment of host fractions from symbiotic anemones exposed to 20 µg-at $^{15}NH_3$-N.l^{-1} for varying lengths of time. Enrichments of host fractions from control anemones are also shown. Individual values for samples are absolute % composition of ^{15}N, mean values have been corrected for natural abundance (0.366 atom % ^{15}N). Also shown are the ^{15}N enrichment values for aposymbiotic anemones exposed to similar concentrations of $^{15}NH_3$ for 30 minutes.

Exposure to $^{15}NH_3$	Atom % ^{15}N	Mean atom % excess ^{15}N
20 µM $^{15}NH_3$ for 0.5 mins	0.368 0.369 0.371	0.003
20 µM $^{15}NH_3$ for 10 mins	0.370 0.370 0.371	0.004
20 µM $^{15}NH_3$ for 30 mins	0.372 0.372 0.372	0.006
No spike, for 0.5 mins	0.370 0.369 0.370	0.004
No spike, for 30 mins	0.370 0.371 0.371	0.005
Aposymbionts, in 20 µM $^{15}NH_3$ for 30 mins	0.382 0.387 0.386	0.019

6.4 Discussion

The kinetics of ammonia uptake by freshly isolated zooxanthellae appeared to be well described by the Michaelis-Menten relationship. This was not true of the initial 10-15 minutes of each incubation, however, during which uptake proceeded at higher rates than would be predicted from the application of Michaelis-Menten kinetics to the rest of the data. Michaelis-Menten uptake kinetics for ammonia have been reported in isolated zooxanthellae (D'Elia *et al.*, 1983), cultured zooxanthellae (Domotor and D'Elia, 1984) and are well-known in free-living phytoplankton (e.g. Eppley, Rogers and McCarthy, 1969; MacIsaac and Dugdale, 1969; Caperon and Meyer, 1972). Wilkerson and Muscatine (1984), however, failed to find Michaelis-Menten type uptake kinetics for ammonia in zooxanthellae isolated from *Aiptasia pulchella*, obtaining a linear relationship between ρ and S from depletions with initial concentrations of 11 µg-at NH_3-N.l^{-1}. No previous study has reported an initial rapid uptake of ammonia (apparently independent of ambient ammonia concentration) in zooxanthellae, as was found in this study, although Wilkerson and Trench (1986) reported "surge uptake" of ammonia from high concentrations by the symbiotic coral *Acropora sp.*. Their results were obtained from an intact association, however, and did not show Michaelis-Menten kinetics at lower concentrations, and so any linking of their observed "surge uptake" and the rapid initial uptake shown by *Anemonia viridis* zooxanthellae *in vitro* must remain speculative.

Table 6.4 gives values for K_s and ρ_{max} for zooxanthellae isolated from a range of hosts, and K_s values for some species of phytoplankton. The values for *Anemonia viridis* zooxanthellae compare well with most of those for freshly isolated zooxanthellae from various host species. It is to be noted that, whilst ρ_{max} values for

Table 6.4 Zooxanthellae: K_s and ρ_{max} values for the ρ vs. S relationships of isolated and cultured zooxanthellae, and free-living phytoplankton. Values taken from published literature. Where the literature gives several values the range is shown.

Algal source of data	K_s ($\mu g\text{-at } NH_3\text{-N}.l^{-1}$)	ρ_{max} ($\mu g\text{-at } NH_3\text{-N}$ $(g\ chl_a)^{-1}.h^{-1}$)
Anemonia viridis zooxanthellae[1]	3.30 - 7.59	0.025 - 0.043
Acropora formosa zooxanthellae[2]	6.6	-
Zoanthus sociatus zooxanthellae[3]	5.03 - 7.32	0.007 - 0.014
Zoanthus spp. zooxanthellae[3]	7.06	0.022
Seriatopora hystrix zooxanthellae[3]	17.00	0.013
Montastrea annulgris zooxanthellae[3]	21.99	0.022
Tridacna crocea zooxanthellae[3]	6.97 - 11.71	0.013 - 0.043
Cultured *Zoanthus sociatus* zooxanthellae[4]	0.41 - 1.65 (depending on growth phase)	0.016 - 0.041
Dunaliella sp. (free-living)[5]	0.02 - 0.35	-
Monochrysis sp.(" ")[5]	0.03 - 0.53	-
Range of free-living phytoplankton species[6]	0.1 - 5.7	-

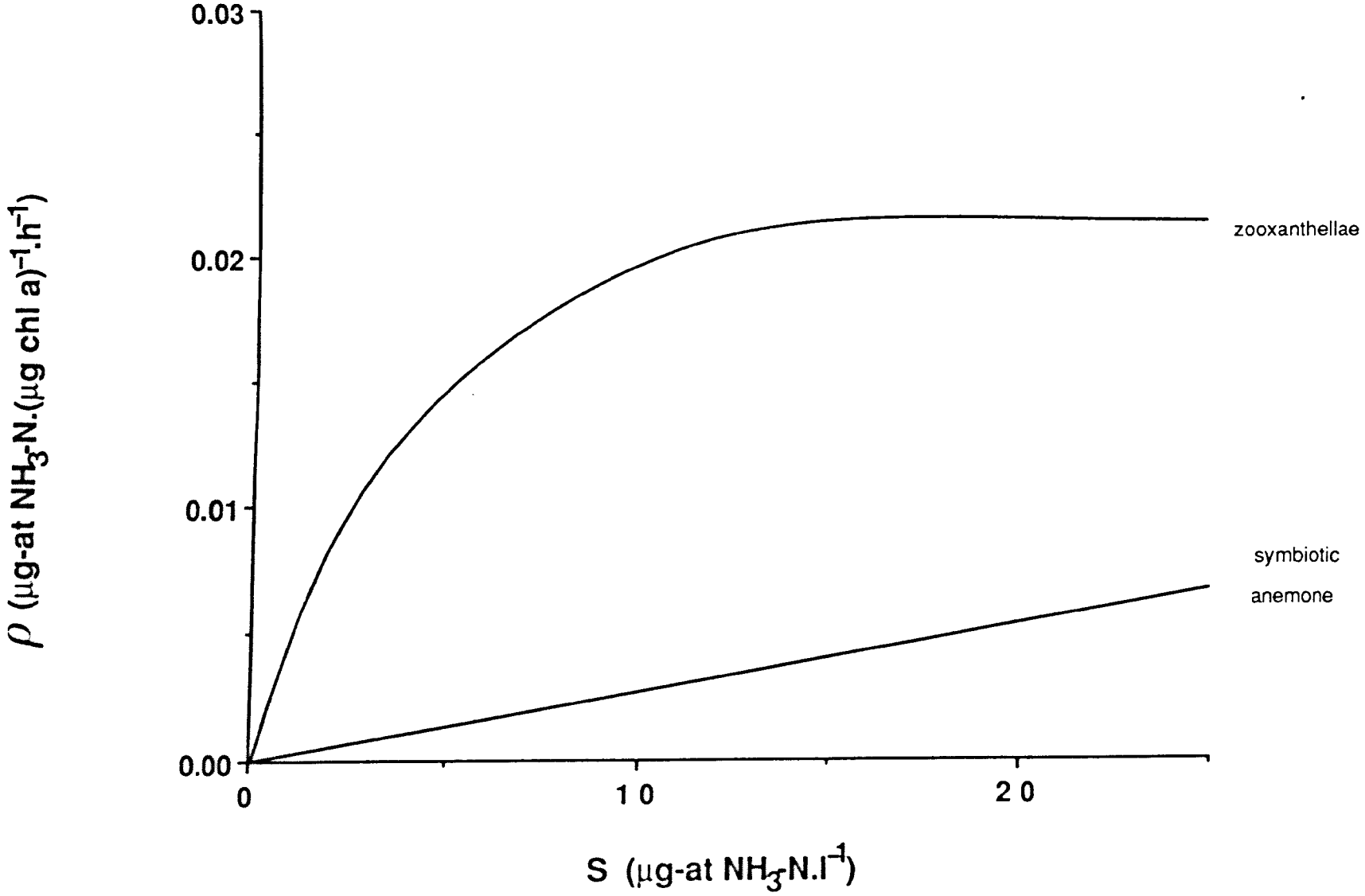
1 This study
2 Gunnersen *et al.*, (1988)
3 D'Elia *et al.* (1983)
4 Domotor and D'Elia (1984)
5 Caperon and Meyer (1972)
6 Eppley, Rogers and McCarthy (1969)

cultured *Zoanthus sociatus* zooxanthellae are also similar, the K_s values for the cultured algae are lower than those for freshly isolated algae, as are those of free-living phytoplankton. A low K_s can be taken as an indication of a high affinity of the algal cells for ammonia, which would be advantageous for algal cells exposed to very low ambient ammonia concentrations. Since the intracellular zooxanthellae are in association with a constant supply of ammonia, via the host nitrogen metabolism, it is to be expected that they might have higher K_s values than oceanic phytoplankton which are exposed to relatively low levels of ammonia.

The kinetics of ammonia uptake by the freshly-isolated zooxanthellae differ markedly from those of the intact *Anemonia* symbiosis. Whilst the zooxanthellae show clear signs of uptake saturation, no such saturation could be found in symbiotic anemones (figure 6.6). In addition, uptake rates of the isolated algae were far higher than uptake rates of the symbiosis at similar incubation concentrations. At 10 μg-at NH_3-N.l^{-1} the zooxanthellae had an estimated uptake rate of approximately 0.02 μg-at NH_3-N.(g Chl*a*)$^{-1}$.h^{-1} (from figure 6.2), whilst a starved symbiotic anemone at the same irradiance (190 μE.m^{-2}.s^{-1}) and incubation concentration would have had an uptake rate of 0.003 μg-at NH_3-N.(g Chl*a*)$^{-1}$.h^{-1}. Wilkerson and Muscatine (1984) also reported that isolated zooxanthellae from *Aiptasia pulchella* took up ammonia seven times faster than the symbiotic association, but other studies have found similar rates of uptake in intact symbioses and their isolated algae (e.g. Muscatine and Marian, 1982; Wilkerson and Trench, 1986), or faster uptake by the symbiosis than by the algae (Muscatine *et al.*, 1979). This variability in reported uptake capabilities by isolated zooxanthellae may result from different strains of algae being used in experiments, or from

Figure 6.6 *Anemonia viridis*: comparison of the relationship between ammonia uptake rate and incubation concentration for the intact symbiosis and freshly isolated zooxanthellae at an irradiance of 190 $\mu E.m^{-2}.s^{-1}$. Intact anemone line recalculated from figure 4.9 using the organic weight-chlorophyll$_a$ content conversion given in appendix I. Isolated zooxanthellae line taken from figure 6.1.

Figure 6.6


0.03
0.02
0.01
0.00
0
10
20
ρ (µg-at NH3-N.(µg chl a)-1.h-1)
S (µg-at NH3-N.l-1)
zooxanthellae
symbiotic
anemone

different isolation and incubation procedures.

Depletion experiments with zooxanthellae in darkness showed that light was not necessary for ammonia uptake by freshly-isolated zooxanthellae. This was unexpected, since symbiotic *A. viridis* that had been incubated in darkness for the same length of time showed clearly reduced uptake rates compared with symbiotic anemones exposed to irradiance of 190 $\mu E.m^{-2}.s^{-1}$ (see Chapters 4 and 5). Similar results were obtained for freshly isolated zooxanthellae by D'Elia *et al.* (1983), whilst Domotor and D'Elia (1984) found that cultured zooxanthellae, isolated from the same host species as those used by D'Elia *et al.* (1983), showed faster uptake rates in light than in darkness. Uptake of ammonia in darkness is typical of nitrogen-depleted algal cells (Syrett, 1981), which are thought to use carbon reserves to drive uptake in the absence of photosynthesis. There is, however, no reason for the algae to become nitrogen-starved immediately after removal from the host, and so the difference in the effects of light on uptake by the symbiosis and the algae alone remains to be explained.

The interpretation of algal metabolic activity in the intact symbiosis based upon the results of experiments with isolated zooxanthellae may be questionable, because of possible damage done to the algal cells during the separation process, and because the physical and chemical conditions in the incubations are unlikely to emulate exactly those of the intracellular environment. These problems may be compounded if the algae are maintained in culture after isolation, since the cells undergo morphological changes (McLaughlin and Zahl, 1957; 1959; Freudenthal, 1962), and are likely to show corresponding physiological differences from freshly isolated zooxanthellae (Domotor and D'Elia, 1986). Despite these problems, it is felt that if freshly isolated zooxanthellae are used, some

indication of the metabolic activity of the algae *in situ* may be obtained.

The ^{15}N-tracer experiments confirm that the zooxanthellae are responsible for the uptake and incorporation of ammonia in the intact association. If the host-uptake hypothesis of Rees (1987) (see section 6.1) had been correct for this symbiosis, ^{15}N would have accumulated in the host fraction to a greater extent than the algal fraction. Even allowing for host contamination of the algal fraction, and the extensive loss from the host fraction of animal cell constituents labelled with ^{15}N, it is unlikely, given the sensitivity of the analytical technique, that there would have been no marked enrichment of the host fraction if the host had assimilated $^{15}NH_3$. That the experiments with zooxanthellae *in situ* give results which agree with predictions based on current understanding of the symbiosis, whilst experiments using isolated zooxanthellae often do not, may be an indication of the unsuitability of isolated algae as a model for the processes occurring in the symbiosis.

The ^{15}N incorporation experiments do not show that the host benefits from the uptake of ammonia by the algae. To demonstrate this, it would be necessary to show ^{15}N enrichment of the host tissues, which could possibly have been done if longer incubation times had been used. The potential for further study of the timescale and pathways of DIN incorporation into the animal cell by ^{15}N tracer methods is great. Similar methods, linked to GC-MS (gas chromatography-mass spectrometry) analysis, have been used to show that ammonia assimilation via the glutamine synthetase-glutamate synthase (GS/GOGAT) enzymic pathway occurs in isolated zooxanthellae (Summons and Osmond, 1981; Summons *et al.*, 1986). Similar methods could be used to follow ammonia incorporation into amino acids in

zooxanthellae *in situ*, and to follow translocation of labelled amino acids to the host. Pulse-chase studies could be used to investigate the time taken for label to accumulate in the host amino acid pool. These methods would be central to a better understanding of the pathways of nitrogen exchange within the symbiosis, because they allow closer observation of processes that can currently only be inferred from indirect observations of exchange with the environment in which the symbiosis is placed.

CHAPTER 7: A PREDICTIVE MODEL FOR AMMONIA UPTAKE IN *ANEMONIA VIRIDIS*

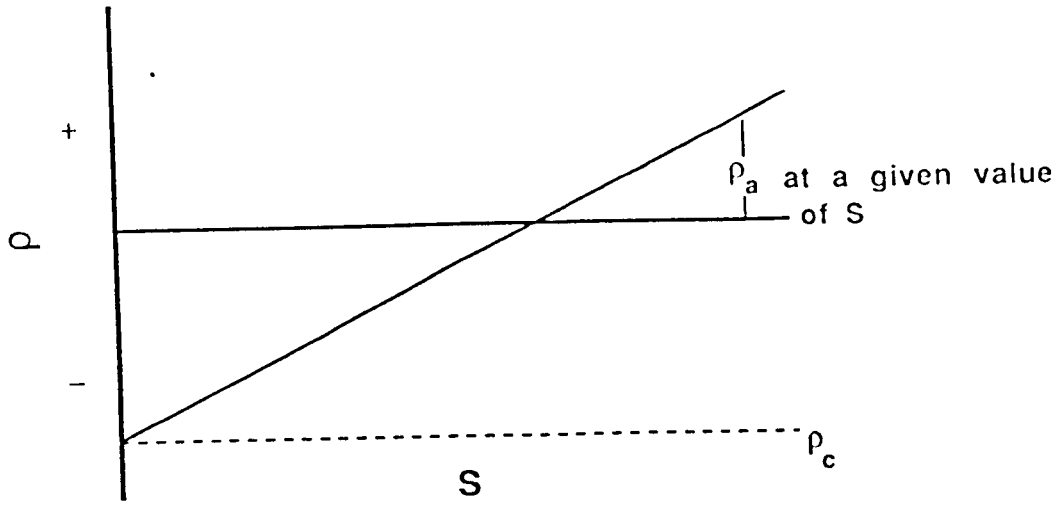
7.1 The mechanism of ammonia uptake

Chapters 4, 5 and 6 investigated some of the factors affecting the flux of DIN in the intact *Anemonia viridis* symbiosis, and also in the anemone and algal tissue alone. In particular, the kinetics of ammonia flux were studied under varying conditions of illumination and ambient ammonia concentration. The processes of solute flux in organic systems can be broadly split into physical (i.e. diffusion) and biological (carrier transport) phenomena (Neame and Richards, 1972), and these exhibit different kinetic and energetic characteristics. Hence it should be possible, by referring to the kinetics observed, to make some inferences about the mechanism by which DIN uptake occurs.

The previous chapters have described experiments which suggest that the observed ammonia flux kinetics of the intact association are the result of two antagonistic flux processes: the catabolic production of ammonia by the animal cell metabolism as an excretory endproduct, and the uptake and assimilation of ammonia by the zooxanthellae. The aim of this section is to describe a model which allows the prediction of the magnitude of these two flux processes under varying conditions of light and ambient ammonia concentration from the kinetics obtained in Chapters 4 and 5.

The likelihood of diffusion being the major process in operation during ammonia efflux from aposymbiotic anemones was discussed in section 4.5.3. The consequences of ammonia loss by diffusion from cells in which ammonia was being produced as a metabolic end-product were also discussed in that section. The graphical representation of the flux rate vs. ambient ammonia concentration relationship is shown

below, and the flux processes are summarized, with symbols, on the same diagram.



Ammonia flux in aposymbiotic *Anemonia viridis*

k_D = diffusion constant

S = ambient ammonia concentration, in μg-at NH_3-N.l^{-1}

φ = any ammonia flux rate, in μg-at NH_3-N.$g^{-1}.h^{-1}$

φ_a = ammonia flux rate of aposymbiotic anemones

φ_c = rate of catabolic production of ammonia by the animal cell

φ_c is estimated from the value of φ_a when S=0 (see section 4.5.3), and remains constant. Thus the variation of φ_a with S is entirely a function of S, as k_D and φ_c do not change.

It is therefore possible, from the results in Chapter 4 (see figure 4.9), to make estimates of k_D, and φ_a for starved aposymbiotic anemones, and these are represented in figure 7.1, for a 1 g organic weight anemone.

The anemone tissues in the intact association will also produce ammonia as an end-product of nitrogen metabolism. It is reasonable to assume that the rates of ammonia production by the animal cells of similarly-sized symbionts and aposymbionts will be the same, if the animals are in equivalent physiological states. As in the aposymbionts, the rate of animal ammonia production, φ_c, is assumed

Figure 7.1 *Anemonia viridis*: schematic diagram of ammonia flux in a 1 g organic weight aposymbiotic anemone. All negative flux values represent rates of ammonia production or efflux, and all positive values represent rates of ammonia uptake or influx.

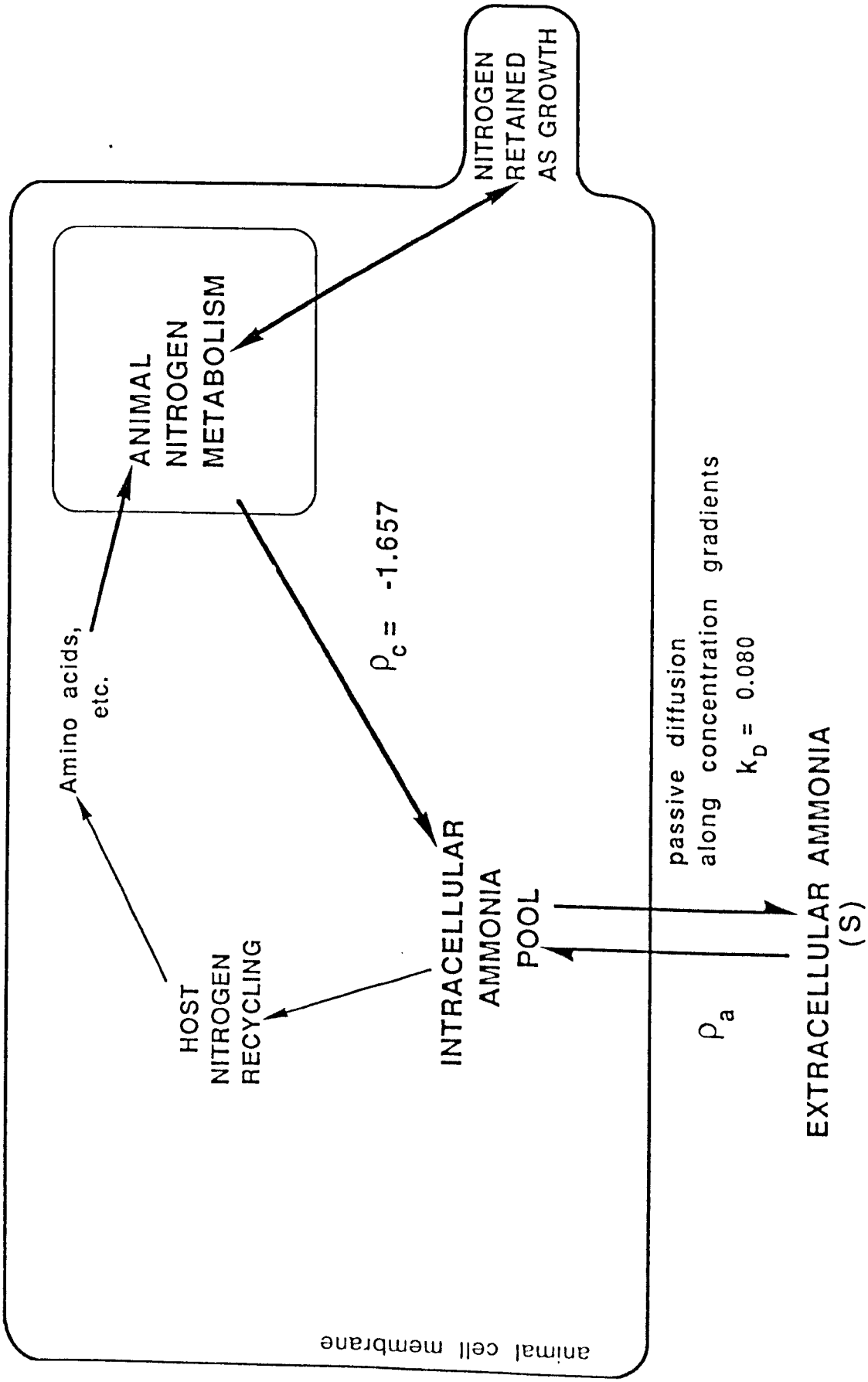
S = ambient ammonia concentration ($\mu g\text{-at } NH_3\text{-N}.l^{-1}$)

φ_a = rate of ammonia flux from an aposymbiont ($\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$)

φ_c = rate of catabolic ammonia production ($\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$)

k_D = diffusion constant

Figure 7.1 Schematic diagram of ammonia flux in an aposymbiont

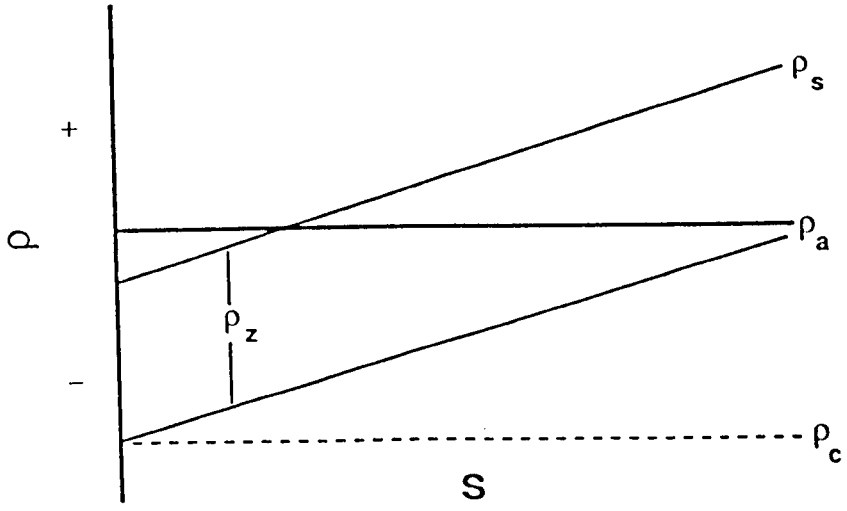


not to change.

The difference between the kinetics of ammonia flux obtained from symbiotic and aposymbiotic anemones is due entirely to the zooxanthellar uptake of ammonia (see Chapter 6). The difference between the flux rates of symbionts and aposymbionts at a given ambient ammonia concentration must therefore represent the *in situ* uptake rate of the zooxanthellae (φ_z). φ_z is therefore calculated from:

$$\varphi_z = \varphi_s - \varphi_a$$

Thus, at low light intensities (<100 $\mu E.m^{-2}.s^{-1}$), φ_z does not change with increasing ambient ammonia concentration, S. This is illustrated below:



Ammonia flux in symbiotic *Anemonia viridis* in low light

Figure 7.2 shows the magnitude of flux processes occurring in a 1 g starved symbiotic anemone under low light conditions, with values derived from the data shown in table 4.6 and figure 4.9. It is clear that although φ_z does not increase with S, it does increase with light intensity, which is to be expected if the ammonia uptake capacity of the zooxanthellae is related to photosynthetic processes (see below).

The symbiotic anemones exposed to higher light intensities than 50 $\mu E.m^{-2}.s^{-1}$ had significantly greater regression coefficients than

Figure 7.2 *Anemonia viridis*: schematic diagram of ammonia flux in a 1 g organic weight symbiotic anemone at different, low light intensities. All negative flux values represent rates of ammonia production or efflux, and all positive values represent rates of ammonia uptake or influx.

S = ambient ammonia concentration ($\mu g\text{-at } NH_3\text{-N}.l^{-1}$)

φ_a = rate of ammonia flux from an aposymbiont ($\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$)

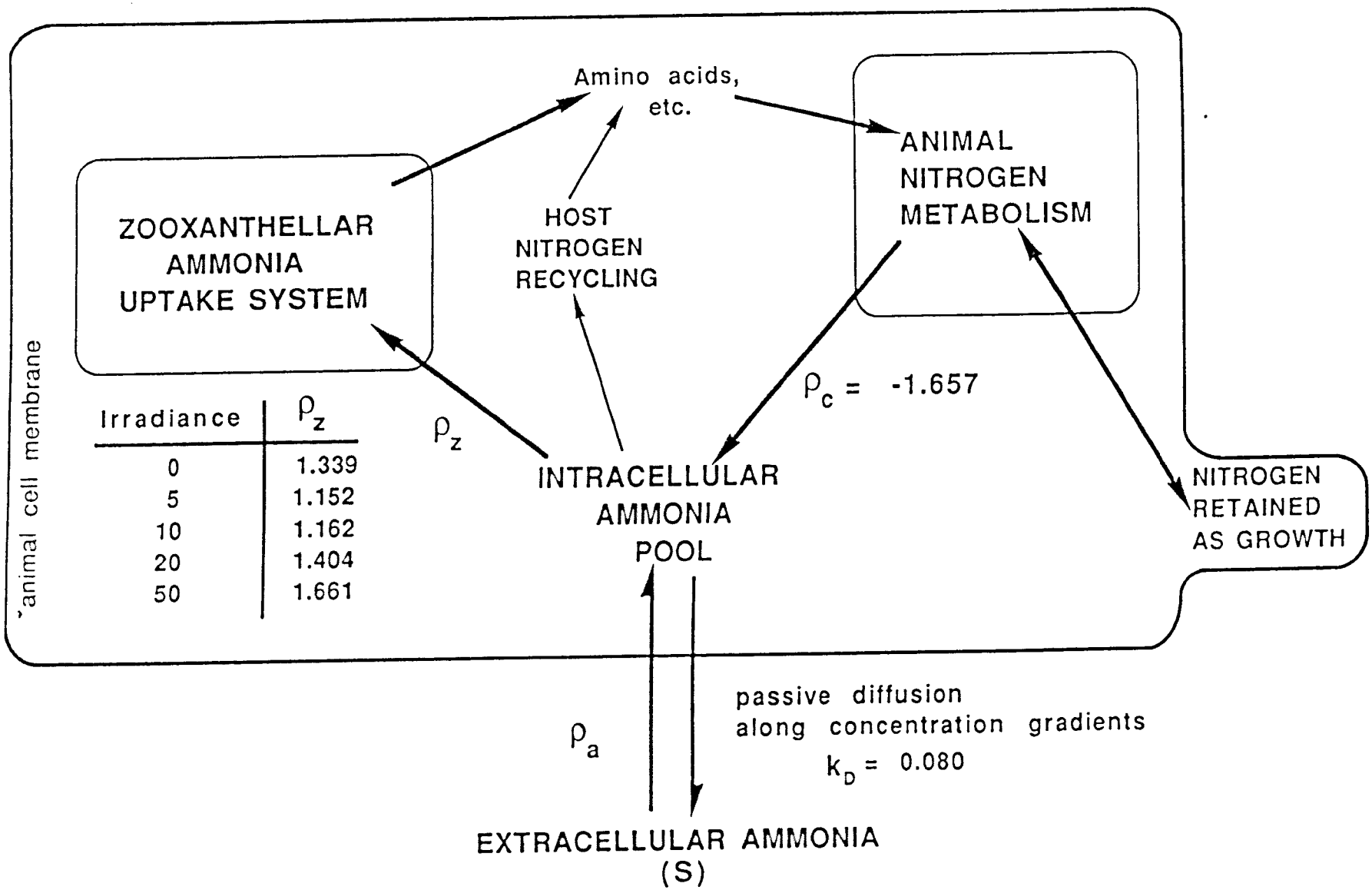
φ_c = rate of catabolic ammonia production ($\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$)

φ_z = rate of ammonia uptake by zooxanthellae ($\mu g\text{-at } NH_3\text{-N}.g^{-1}.h^{-1}$)

k_D = diffusion constant

irradiance = $\mu E.m^{-2}.s^{-1}$

Figure 7.2 Schematic diagram of ammonia flux in a "low light" symbiont

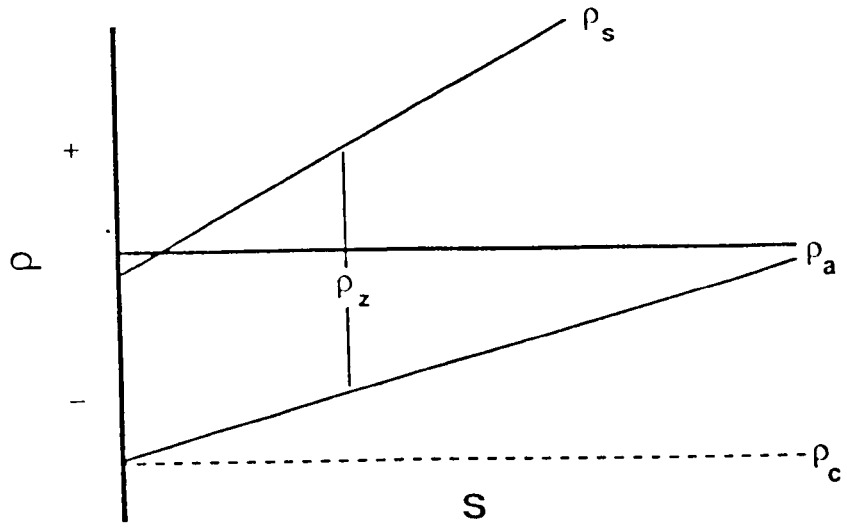


those exposed to low light intensities (see section 4.4.3, and table 4.5). In the general situation, the diffusion coefficient, k_D is dependant upon the solute type, and the physical and chemical characteristics of the membrane across which the solute is passing (see section 4.5.3). Since the solute, ammonia, is the same for all anemones in this study, and the animal cell membrane characteristics of the anemones should have been similar, k_D would not change with light intensity. Some other process must therefore have been operating to produce the changed regression coefficients of the anemones exposed to high light intensities.

In the above description of algal uptake in symbiotic anemones exposed to low light levels, it was apparent that φ_z, the algal ammonia uptake rate, did not change with exogenous ammonia concentration. This meant that the observed kinetics of net flux in the anemone were still controlled by diffusion across the animal cell membrane. The lack of dependence of φ_z on the availability of ammonia is characteristic of a saturated uptake system; although more ammonia was available to the algae as extracellular concentrations increased, the algae did not increase their uptake rate. If φ_z is calculated for "high light" symbionts, using the formula described above:

$$\varphi_z = \varphi_s - \varphi_a$$

it can be seen that φ_z increases with increasing external ammonia concentration. This is illustrated below:



Ammonia flux in symbiotic *Anemonia viridis* in high light

The rise in uptake rate with greater availability of substrate is characteristic of an uptake system that has not reached saturation, whilst the lack of uptake rate dependancy on S, shown by the zooxanthellae of anemones in low light, is typical of a saturated uptake system. It is not entirely clear why the ammonia uptake mechanism of the zooxanthellae should be saturated at low, but not high light intensities (at least over the range of S values used). It is known that ammonia uptake is light-dependent in microalgae, to a greater or lesser extent, depending on the alga's nutritional status (Grant and Turner, 1969; Thacker and Syrett, 1972), but experiments have apparently only been done to compare light and dark assimilation rates, and not to assess the effect of increasing light intensity. The exact cause of the light-dependency of ammonia uptake is uncertain, but possible mechanisms include (from Syrett, 1981):

a) light generation of reduced ferredoxin, which drives the glutamate synthase (GOGAT) reaction of ammonia assimilation.

b) the generation of ATP via photophosphorylation, which may drive the glutamine synthetase (GS) assimilatory reaction.

c) photosynthetic carbon-fixation, providing carbon acceptors for ammonia assimilation.

Since it is known that photosynthetic rates increase with irradiance, until saturation is reached (for instance, at irradiances higher than 160 $\mu E.m^{-2}.s^{-1}$ for the zooxanthellae of *A. viridis in situ* (Tytler, 1982)), it is reasonable to assume that availability of ATP and carbon skeletons, and possibly reduced ferredoxin, will also increase with irradiance. If the rate of ammonia uptake by the algae, at a given light intensity, follows a Michaelis-Menten hyperbolic relationship with ammonia concentration similar to that of freshly isolated zooxanthellae (see Chapter 6), the saturation uptake rate, $\rho_{z.max}$, will therefore depend upon the light intensity. This allows for the increasing elevation of the ρ vs. S relationships of symbiotic anemones at low, but increasing, light intensities. If the half-saturation constant, K_s, is also dependant upon light intensity, then it would be possible for the zooxanthellae in high light to exhibit non-saturated ammonia uptake, whilst zooxanthellae at lower light levels would exhibit saturated uptake across the same range of ammonia concentrations. This is shown in figure 7.3. Figure 7.3c is similar to the graph that would be obtained of ρ_z against S from the results of experiments in Chapter 4.

The values for the ammonia fluxes of a 1 g starved symbiont at higher light intensities are shown in figure 7.4.

All of the above is equally applicable to starved or fed anemones. Chapter 5 showed that the feeding state did not affect the slope of the ρvs. S relationship for the anemones, but that there was a tendency for the elevations of the relationships for fed anemones to be lower than those for starved anemones under similar illumination. This tendency was not always statistically significant, but in those

Figure 7.3 Zooxanthellae: Demonstration of the possible effects of light on the kinetics of ammonia uptake by the zooxanthellae *in situ*. a) The uptake rate/concentration relationship for a single light intensity. $\rho_{z.max}$ is the rate of uptake at saturating ammonia concentrations, k_S is the concentration at which uptake proceeds at half the maximum rate. b) The relationship at two "low" and one "high" light intensity. $\rho_{z.max}$ increases with light intensity, and so does k_S. c) The observed relationship between ρ_z and S for the same three light intensities, if only a small range of S values are tested. This approximates to the relationships predicted for zooxanthellae *in situ* from the model described in section 7.1.

Figure 7.3

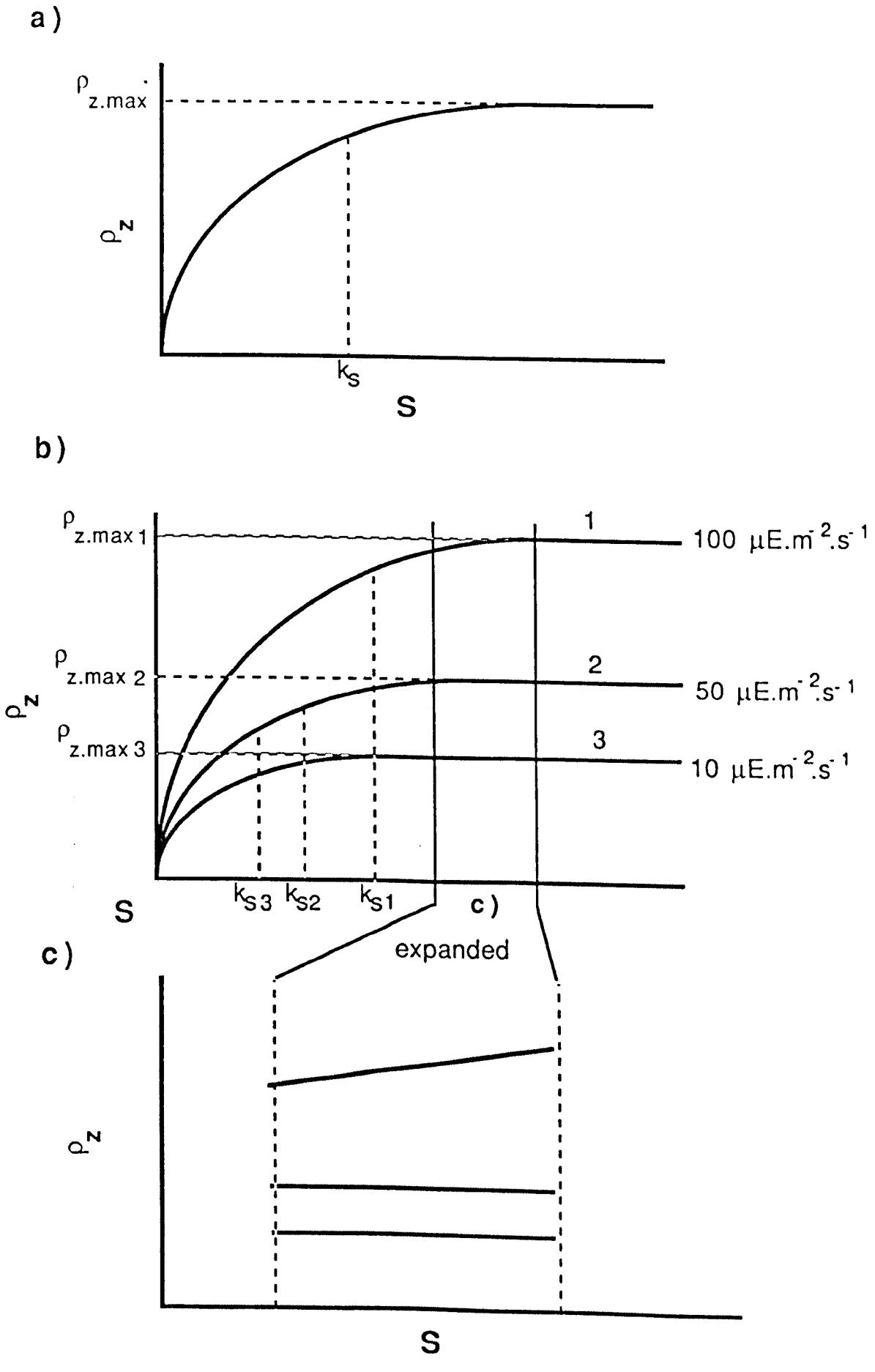

a)
ρz.max
ρz
ks
S
b)
ρz.max 1
ρz.max 2
ρz.max 3
ρz
1
2
3
100 μE.m-2.s-1
50 μE.m-2.s-1
10 μE.m-2.s-1
ks3
ks2
ks1
S
c)
expanded
c)
ρz
S

Figure 7.4 *Anemonia viridis*: schematic diagram of ammonia flux in a 1 g organic weight symbiotic anemone at a light intensity of 100 $\mu E.m^{-2}.s^{-1}$. All negative flux values represent rates of ammonia production or efflux, and all positive values represent rates of ammonia uptake or influx.

S = ambient ammonia concentration (μg-at NH_3-$N.l^{-1}$)

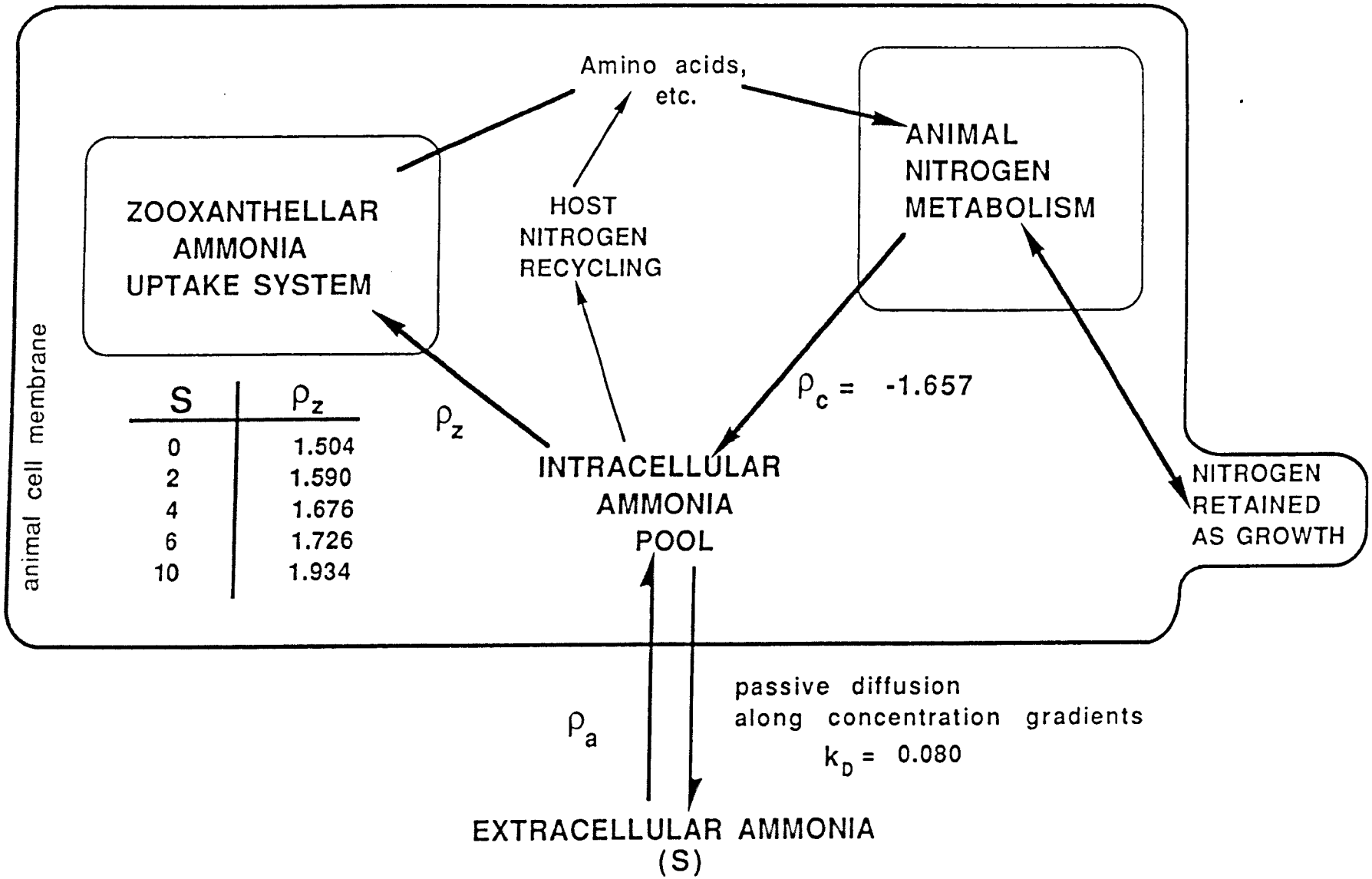
φ_a = rate of ammonia flux from an aposymbiont (μg-at NH_3-$N.g^{-1}.h^{-1}$)

φ_c = rate of catabolic ammonia production (μg-at NH_3-$N.g^{-1}.h^{-1}$)

φ_z = rate of ammonia uptake by zooxanthellae (μg-at NH_3-$N.g^{-1}.h^{-1}$)

k_D = diffusion constant

Figure 7.4 Schematic diagram of ammonia flux in a "high light" symbiont



cases where it was, it is explained by increased animal cell production of ammonia. The increased ammonia production would account for more of the algal cells' uptake capability, and so more ammonia would be free to diffuse out of the cell.

The concept of DIN uptake in algal-coelenterate symbioses as a two-compartment process, with carrier transport and diffusion occurring at separate sites, is not a new one. Muscatine and D'Elia (1978) found that the Michaelis-Menten kinetics typical of a carrier-mediated uptake system could be shown for ammonia uptake by several hermatypic coral species, but only after a correction had been applied for diffusion processes. This has been supported by more recent research (Burris, 1983), and it is now widely accepted that the zooxanthellae are responsible for creating a concentration gradient in the animal cell down which ammonia passively diffuses. Propp (1981) suggested that this type of mechanism was inadequate to explain diffusion of nutrients into the animal cell from low ambient concentrations, and that an active transport system might also be expected in the animal epithelium, although no experimental evidence was presented to support this. The only major departure from general acceptance of zooxanthellae as the site of carrier-mediated uptake of ammonia has been the suggestion by Rees (1987) that the animal tissue is capable of all ammonia uptake observed, and that the algae are supplied with the animal-assimilated ammonia as a control mechanism for algal population size. Although Rees implies that this may occur in coelenterate-*Symbiodinium* symbioses, the hypothesis is based upon work with the green hydra symbiosis, which has significant physiological differences from dinoflagellate-containing associations. The present study (Chapter 6) has demonstrated that the algae, not the host, are the initial sites of ammonia assimilation in the *Anemonia*

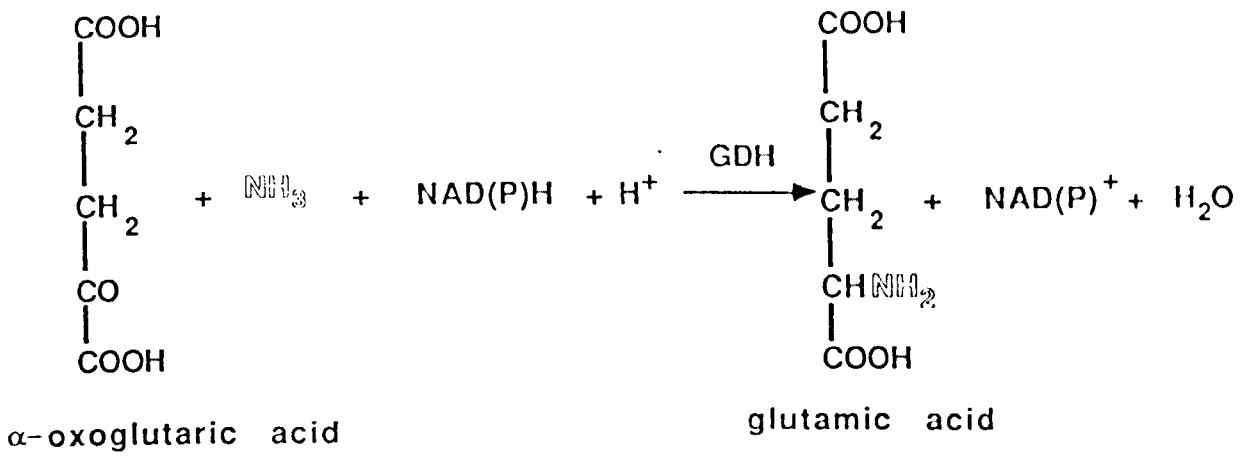
viridis symbiosis.

Muscatine and D'Elia (1978) assumed that the carrier transport system in the zooxanthellae was an active one, since it depended upon energy derived from photosynthesis. It is not clear whether there is a transport system in the zooxathella cell membrane, which concentrates ammonia inside the algal cell where it is subsequently assimilated, or if the assimilation mechanism is sufficient to account for the uptake kinetics observed. Evidence for an active, membrane-associated carrier-transport mechanism in phytoplankton has been produced by Balch (1986), using the ammonium analogue, methylamine, but the mechanism did not seem to be present in all cases. The ammonia assimilatory pathway of plants has been studied extensively, and two different enzyme systems are believed to be involved: glutamic dehydrogenase (GDH), and glutamine synthetase-glutamate synthase (GS-GOGAT). The two systems are shown in figure 7.5. Since the work of Lea and Miflin (1974) showed that the GS-GOGAT system was the more likely pathway in higher plants, a body of literature has built up supporting this in phytoplankton, and also zooxanthellae. Falkowski and Rivkin (1976) proposed that GS was more important in the diatom, *Skeletonema costatum*, because the half-saturation constant for GS (29 µg-at NH_3-N.l^{-1}) was considerably closer to environmental ammonia concentrations than that for GDH (28 mg-at NH_3-N.l^{-1}). Stable isotope tracer studies showed that, in isolated zooxanthellae, $^{15}NH_3$ is incorporated into glutamine, glutamic acid and aspartic acid in proportions that strongly implicate the GS-GOGAT assimilation pathway (Summons and Osmond, 1981; Summons *et al.*, 1986). Low half-saturation constants for ammonia assimilation by GS isolated from zooxanthellae, together with the inhibition of ammonia uptake in intact associations by methionine sulfoximine (an inhibitor of GS) also lead Anderson and Burris (1987) to conclude that this was the major pathway of

Figure 7.5 Zooxanthellae: the proposed enzymic pathways of ammonia assimilation in algae. a) Glutamic dehydrogenase (GDH). b) Glutamine synthetase-glutamate synthase (GS-GOGAT).

Figure 7.5 Pathways of ammonia assimilation in algae

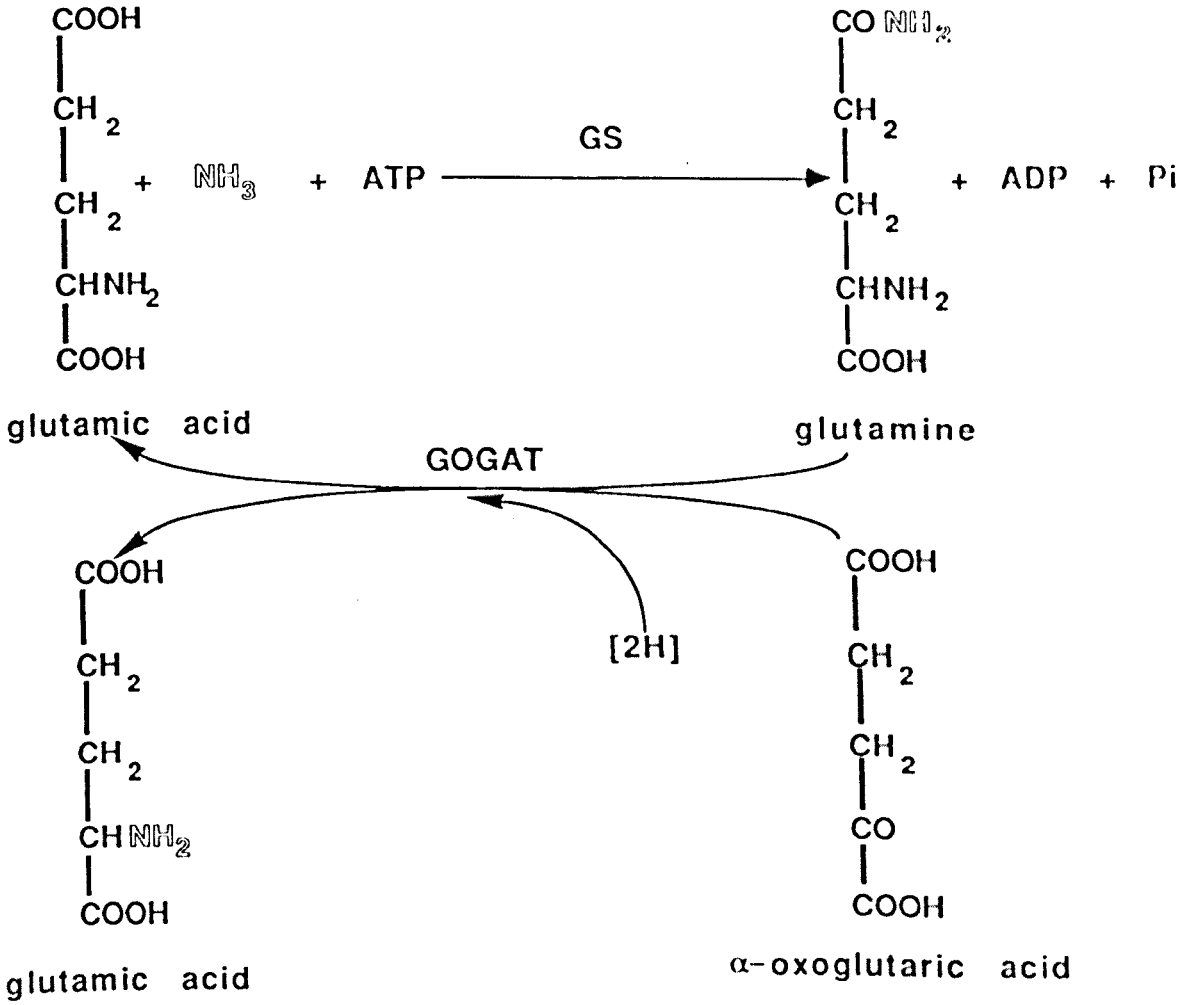
a) Glutamic dehydrogenase (GDH)



α-oxoglutaric acid

glutamic acid

b) Glutamine synthetase (GS)/Glutamate synthase (GOGAT)



glutamic acid

α-oxoglutaric acid

assimilation in anthozoan-dinoflagellate symbioses. GDH may, however, also be involved in assimilation, either in darkness or at very high ammonia concentrations (Summons and Osmond, 1981; Summons *et al*, 1986). At least one study has gone further, and suggests that GDH is the major assimilatory pathway in zooxanthellae isolated from *Acropora formosa* (Dudler and Miller, 1988), and that GS activity is very low in the same algae (unpublished work, cited in Dudler and Miller, 1988).

The exact biochemical pathway of ammonia uptake in algal-coelenterate symbioses therefore remains in question. The model proposed on the basis of the results in this study would be equally valid for a system with a GS or GDH ammonia assimilation pathway, and with or without an additional membrane-bound carrier transport system. Further use of stable isotopes may enable the gross movement of ammonia to be more closely linked to particular biochemical pathways, resulting in an elaboration of the simple model outlined above.

7.2 Effects of symbiosis on the nitrogen balance of *Anemonia viridis*

One of the original aims of this study was to produce laboratory results that would allow the prediction of nitrogen flux in individual *Anemonia viridis* under field conditions of light and DIN availability. This section will make such predictions, and, in comparing the predicted DIN fluxes of symbiotic and aposymbiotic anemones, estimate the gain, in terms of nitrogen, made by the intact association compared to aposymbiotic individuals.

Before making predictions, it is first necessary to define nitrogen "gains" and "losses". The model described in the previous section included several estimates of different flux processes occurring within the symbiosis, and at the host membrane. The

catabolic production of ammonia by an animal cell normally represents a "loss" of useful nitrogen. In symbiotic anemones this rate of loss will be taken as being equal to ρ_c. When the zooxanthellae take up ammonia, of host or exogenous origin, the nutrient is assumed to be assimilated into metabolically-useful compounds. The assimilation of nitrogen into these compounds is therefore a "gain" of useful nitrogen, and, from the model, the rate of gain is represented by ρ_z, the rate of ammonia uptake by the zooxanthellae. ρ_z, unlike ρ_c, is affected by ambient ammonia concentration and light intensity. The net gain or loss of useful nitrogen by the symbiosis will depend upon the relative values of ρ_z and ρ_c, and since ρ_c is constant, the net gain or loss will be affected by environmental light and ammonia availability.

For the symbiotic *Anemonia viridis* on the study site in Linne Mhuirich the ambient ammonia concentrations were negligible throughout the year, and so S will be taken as 0 μg-at NH_3-N.l^{-1} (see section 3.3). The anemones were not observed to feed heterotrophically, and became noticably smaller over the period of observation, and so they were assumed to correspond to the starved anemones used in laboratory experiments. Light levels were calculated on an hourly basis for "ideal" and "average" days in winter and summer (section 3.4, and figures 3.2 and 3.3). Although laboratory experiments were conducted at irradiances of 0, 5, 10, 20, 50, 100, 190 and 300 $\mu E.m^{-2}.s^{-1}$, the effects of light between 0 and 20 $\mu E.m^{-2}.s^{-1}$ could not be separated. The hourly field light values were therefore split into classes as follows:

Field irradiance	Equivalent laboratory irradiance
0- 25	0
26- 75	50
76-150	100
151-250	190
>251	300

(all values in $\mu E.m^{-2}.s^{-1}$)

Table 7.1 gives the number of hours included in each light intensity class for the four field "days". For any given light intensity and value of S, the hourly flux rate of ammonia (φ_s) to or from the intact symbiosis could be calculated from the regression equations given in table 4.6, or the regression lines shown in figure 4.9. The hourly ammonia flux rate was similarly calculated for a hypothetical aposymbiont under the same environmental conditions. From the model in section 7.1, the hourly zooxanthellar uptake rate, φ_z, is given by :

$$\varphi_z = \varphi_s - \varphi_a$$

φ_z could therefore be calculated for each light intensity class. By multiplying φ_z for each light intensity by the number of hours at that light intensity, and summing these values, the daily zooxanthellar ammonia uptake was calculated. This represents the "gain" of useful nitrogen by the symbiosis. Since the animal ammonia production rate is constant, and known, the *net* loss or gain of useful nitrogen could be found. Table 7.2 shows a worked example for a 1 g organic weight symbiotic anemone on the field study site, on an "ideal" summer day, to give a figure for the daily net loss of nitrogen in µg. The corresponding net losses of nitrogen on "average" summer, and "ideal" and "average" winter days, from a 1 g anemone on the study site are

Table 7.1 Field light levels: number of hours of irradiance of each light intensity class (see text) on "ideal" and "average" days in winter and summer.

Light class (and range covered) / $\mu E.m^{-2}.s^{-1}$	NUMBER OF HOURS "Ideal" winter day	"Average" winter day	"Ideal" summer day	"Average" summer day
0 (0 to 25)	15	16	8	9
50 (26 to 75)	2	4	1	2
100 (76 to 150)	1	4	1	2
190 (151 to 250)	2	-	2	2
300 (>250)	4	-	12	9

Table 7.2 *Anemonia viridis*: Calculation of daily loss of metabolically-useful nitrogen from a 1 g organic weight symbiotic anemone at the field study site on an "ideal" summer day. The environmental ammonia concentration is taken to be 0 μg-at NH_3-N.l^{-1} (see section 3.3), and the number of hours at each light level are given in table 7.1.

φ_s = the hourly rate of ammonia flux from a 1 g symbiotic anemone

φ_a = the hourly rate of ammonia flux from a 1 g aposymbiont

(both values calculated at S=0 from the equations given in table 4.6)

φ_z = the rate of ammonia uptake by the zooxanthellae *in situ*

h = number of hours of each light intensity (from table 7.1)

Light intensity	φ_s	−	φ_a	=	φ_z	h	φ_z x h
0	-0.318		-1.657		1.339	8	10.712
50	0.004		-1.657		1.661	1	1.661
100	-0.153		-1.657		1.504	1	1.504
190	0.079		-1.657		1.736	2	3.472
300	-0.116		-1.657		1.541	12	18.492
						Daily φ_z, in μg-at NH_3-N.d^{-1} =	35.841

φ_c, the metabolic loss of ammonia from the host (from data for aposymbionts, table 4.6), is

$$-1.657 \mu g\text{-at } NH_3\text{-N}.h^{-1}$$

so daily φ_c, in μg-at NH_3-N.d^{-1} = 24 x -1.657 = -39.768

Net daily loss of N = $\varphi_z + \varphi_c$

= 35.841 + (-39.768)

= -3.924 μg-at NH_3-N.d^{-1}

= -55.004 μg N.d^{-1}

(corrected for the atomic weight of nitrogen)

All negative values represent losses, positive values represent gains.

given in table 7.3.

It can be seen from table 7.3 that, whilst daily losses of nitrogen are higher in the winter than in the summer, there is relatively little difference in the rate of loss between an ideal or average day at the same time of year. This is because the effects of light on the ammonia flux rates of symbiotic anemones is relatively small at low ambient ammonia concentrations, and the controlling factor becomes the relative number of hours at 0 $\mu E.m^{-2}.s^{-1}$. The table also shows that the rate of nitrogen loss from symbionts, whilst still a drain on the associations nitrogen reserves, is reduced to between 9.9 and 14.3% of the rate of loss predicted for aposymbionts under the same conditions. This means that, on the study site, the zooxanthellae could potentially supply between 86 and 90% of the associations basal nitrogen requirement. These figures exceed the estimation of algal provision of 54% of the basal nitrogen requirements of the temperate coral, *Astrangia danae* (Szmant-Froelich and Pilson, 1984). The value for *Astrangia danae* was the mean of figures for starved and fed corals, at an unspecified ambient ammonia concentration, and so further comparison is difficult. Szmant-Froelich and Pilson (1984) concluded that the zooxanthellae were not of strong selective advantage to the host, but since *Astrangia danae* occurs naturally with a wide range of algal population densities, including complete absence of algae (Szmant-Froelich and Pilson, 1980), unlike *Anemonia viridis*, similar levels of potential host dependance on algal nitrogen provision may not be expected.

It was possible to calculate the daily loss or gain of nitrogen for any ambient ammonia concentration, and figure 7.6 shows graphs of net metabolic nitrogen loss against environmental ammonia concentration for the four types of day. As ambient ammonia concentrations rise, the effects of the shape of daily light curves

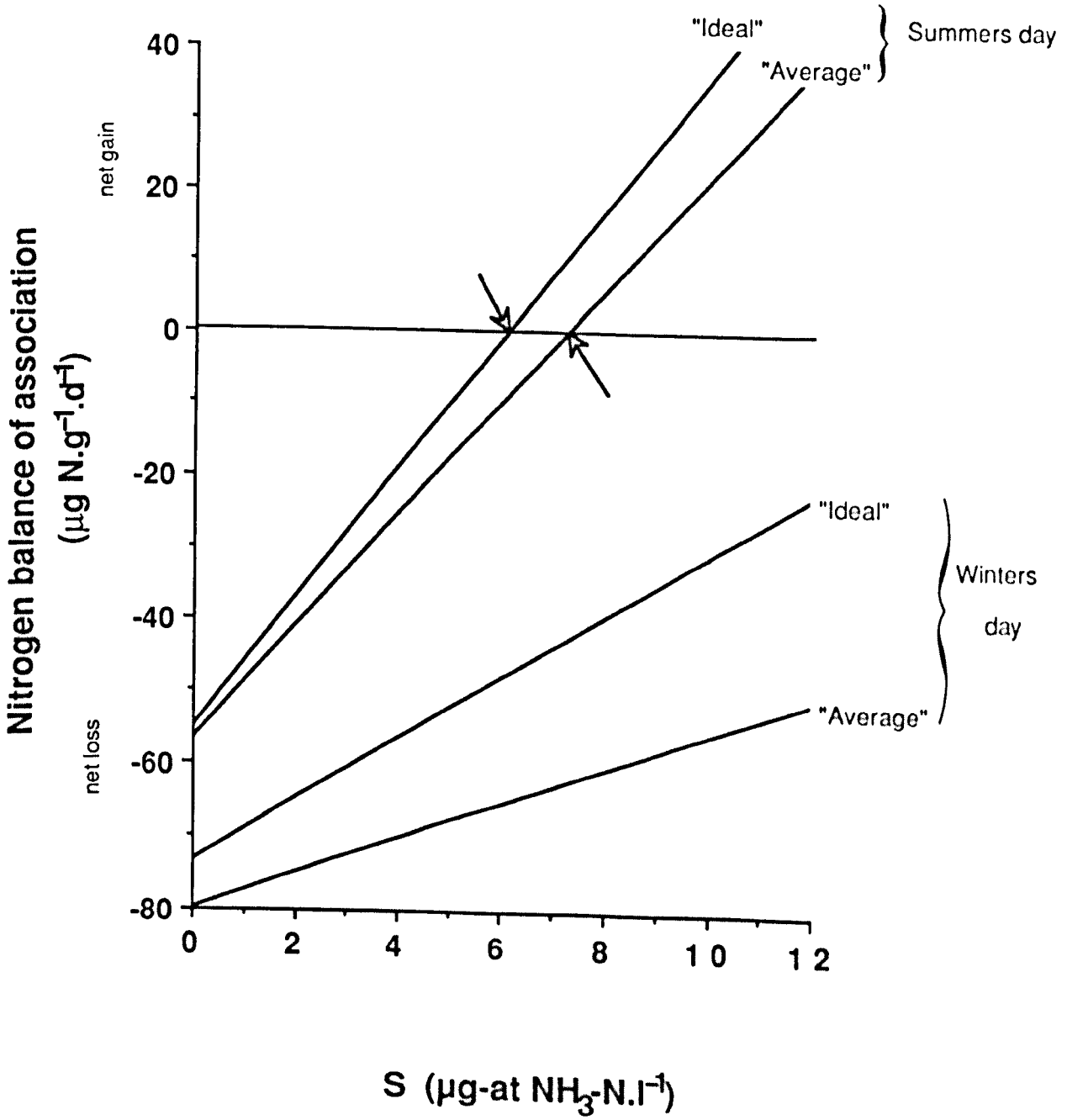
Table 7.3 *Anemonia viridis*: Daily rates of net nitrogen loss from symbiotic anemones on the study site on "ideal" and "average" days in winter and summer. Figures calculated by the method used in table 7.2. Negative values indicate loss. Also shown are the predicted rates of nitrogen loss from a hypothetical 1 g aposymbiont on the study site (derived from the daily loss of nitrogen from host tissue calculated in table 7.2, but corrected for atomic weight of nitrogen, thus converting the figure from -39.768 μg-at NH_3-N.d^{-1} to units of μg N.d^{-1}).

Type of day	Nitrogen loss/ μg N.d^{-1}	
	Symbionts	Aposymbionts
"Ideal" summer	-55.004	-557.018
"Average" summer	-56.671	-557.018
"Ideal" winter	-73.129	-557.018
"Average" winter	-79.614	-557.018

Figure 7.6 *Anemonia viridis*: the net losses or gains of metabolically-useful nitrogen at different environmental ammonia concentrations, by 1 g organic weight symbiotic anemones under field light conditions. Lines are shown for light conditions on "ideal" and "average" days in winter and summer (see sections 3.4 and 8.2). Positive values indicate a net gain of nitrogen, negative values indicate a net loss. The environmental concentrations at which nitrogen losses and gains are balanced, for each type of day, are given below. Arrows on graph indicate these concentrations for "ideal" and "average" summer days.

Type of day	Ambient ammonia concentration at which losses and gains balance ($\mu g\text{-at } NH_3\text{-}N.l^{-1}$)
"Ideal" summer	6.082
"Average" summer	7.238
"Ideal" winter	17.345
"Average" winter	33.046

Figure 7.6


"Ideal"
"Average"
Summers day
40
20
0
-20
-40
-60
-80
net gain
net loss
Nitrogen balance of association
(µg N.g⁻¹.d⁻¹)
"Ideal"
"Average"
Winters
day
0
2
4
6
8
10
12
S (µg-at NH3-N.l⁻¹)

become more pronounced, as the number of hours at higher light intensities increase in relative importance. As with the φ vs. S graphs for intact symbiotic anemones, the relationships between rate of daily nitrogen loss and ammonia concentration are linear. At the point where the line crosses the x-axis, daily metabolic nitrogen losses and gains are balanced. The anemone, at that environmental ammonia concentration and above, is potentially autotrophic with regards to nitrogen. The environmental ammonia concentrations that would result in potential nitrogen autotrophy on the four day types in the field are shown in the figure. It is clear that under no natural light regimes could the anemones in Linne Mhuirich be expected to be self-sufficient with regards to nitrogen. The concentrations at which nitrogen autotrophy is possible in the summer are within the reported range of ammonia concentrations for coastal waters, but they would be very rarely attained in the habitats occupied by *A. viridis*, at least in Scotland. It is possible that such concentrations may be experienced by this species further south in its range, particularly in rockpools containing large numbers of other, non-symbiotic animals, but full nitrogen autotrophy, even if physiologically possible (see Chapter 8), remains an unlikely occurrance.

CHAPTER 8: THE EFFECT OF DISSOLVED INORGANIC NITROGEN UPTAKE ON THE GROWTH OF *Anemonia viridis*

8.1 Introduction

Previous work has shown *Anemonia viridis* to be potentially autotrophic, at least with respect to energy requirements, when kept in a light cycle of 12 hours at 140 $\mu E.m^{-2}.s^{-1}$: 12 hours darkness (Tytler, 1982; Tytler and Davies, 1986). When this was tested in growth experiments, however, the anemones were found to lose weight (Tytler and Davies, 1986). The latter experiments were carried out with negligible concentrations of ammonia in the sea water suurounding the anemones. Under these conditions, the results of the experiments described in Chapter 4 of this study predict that there would be a net loss of ammonia from the anemones over 24 hours. If it is assumed that the anemones in Tytler and Davies' experiment had no other source of exogenous nitrogen, their growth would be limited by the lack of this nutrient. At higher concentrations of ammonia (in excess of 11 μM), however, the model in Chapter 7 predicts that starved symbiotic anemones under the above lighting conditions should experience a net gain of nitrogen over 24 hours. The experiment described in this chapter aimed to test this prediction by comparing the changes in weight of two groups of starved symbiotic *Anemonia viridis*, both exposed to a lighting regime similar to that of Tytler and Davies (1986), but one of which was exposed to higher levels of ammonia in the incubation sea water.

8.2 Methods

Two clear plastic 12 litre aquarium tanks, each containing 10

litres of sea water, were set into the circulating sea water system in the Zoology Department, Glasgow University, so that the circulating water maintained the temperature of the sea water in the tanks at $10^{o}C$, but did not come into contact with it. Each aquarium tank had an airstone, connected to an airpump, to aerate and circulate the water. A set of fluorescent strip lights above the tanks provided light at an intensity of 140 $\mu E.m^{-2}.s^{-1}$ at the water surface for 12 hours of a 12 hours light: 12 hours dark cycle.

Two groups of 10 starved symbiotic *A. viridis* were buoyant weighed as described in section 2.4.1, and one group was placed in each of the aquarium tanks. 130 ml of 1.5 mM $(NH_4)_2SO_4$ solution (made up in artificial sea water) was added to one of the tanks to give an ammonia concentration of 19.3 $\mu g\text{-at } NH_3\text{-}N.l^{-1}$. The anemones in this tank were termed the DIN-fed stock. The second tank had an ammonia concentration of $<0.5\ \mu g\text{-at } NH_3\text{-}N.l^{-1}$, and the anemones in this tank corresponded to Tytlers' (1982) "high light starved" stock of anemones, and Tytler and Davies' (1986) "light-dark" group. The tank containing the DIN-fed stock was respiked after 24 hours with 130 ml of ammonium sulphate solution, and this was repeated every day throughout the experiment. Every second day a 5 ml water sample was taken from the DIN-fed stock tank prior to respiking, and the sample was assayed for ammonia concentration by the method described in section 2.5.1.2, to determine the drop in concentration over the 24 hours since the previous ammonia spike.

The anemones from both experimental stocks were buoyant weighed weekly, for 12 weeks. The anemones were removed from the experimental tanks and placed in artificial sea water (Tropic Marin) on the evening before weighing, to ensure that the water in the anemones' coelenterons was of a similar density to that of the water in which

they were to be weighed (see section 2.4.1). After weighing, the anemones were returned to the experimental tanks. All buoyant weights were converted to ash-free dry (organic) weights, using the relationship given in section 2.4.1.

The water in the tanks was changed each time the anemones were weighed, and the inner surfaces of the tanks were tho roughly cleaned of mucus and algal growth. Water changes were also carried out between weighings if fouling of the tank surfaces became apparent. At all times the water in the tanks remained clear, and the anemones appeared healthy.

After 84 days (for the DIN-fed stock) and 87 days (for the DIN-starved stock), the anemones were finally weighed and the experiment was terminated.

8.3 Results

All of the experimental anemones lost weight over the 12 week period. Tytler (1982) calculated that weight loss during starvation would be an exponential function of time:

$$W = W_0.e^{-kt}$$

Where t = time, in days

W = organic (ash-free dry) weight at time t

W_0 = initial weight

k = rate of weight loss

The linear transformation of this is:

$$\log_e W = \log_e W_0 - kt$$

or

$$\log_{10} W = \log_{10} W_0 - \frac{kt}{2.3026}$$

Linear regression lines of $\log_{10} W$ against time were fitted to the data for each anemone, and the equations of the lines are given in table 8.1. One anemone in the DIN-fed stock, and two in the DIN-starved stock divided during the expreiment, and these were excluded from the results presented here. Figures 8.1 and 8.2 show the data and the regression lines for the DIN-fed and DIN-starved stocks respectively. The slopes of the regression lines within each group were compared by covariance analysis, and no significant differences were found ($F_{slopes,DIN\text{-}fed}$ = 0.717, 8 and 81 d.f., P>0.50, N.S.; $F_{slopes,DIN\text{-}starved}$ = 0.305, 7 and 72 d.f., P>0.75, N.S.), showing that the anemones within each group were losing weight at a uniform rate. The sums of squares and sums of products were pooled for each group to give common regression coefficients of -1.2×10^{-4} and -6.5×10^{-4}, for the DIN-fed and DIN-starved stocks respectively. These common regression coefficients were compared by a further covariance analysis, and were found to be significantly different (F = 54.313, 1 and 168 d.f., P<0.001). The smaller regression coefficient of the DIN-fed stock indicates that those anemones were decreasing in weight more slowly than the DIN-starved anemones.

The ratio of anemone tissue weight to incubation volume, and initial ammonia concentration in the DIN-fed tank, had been selected so that the anemones would decrease the ammonia concentrations to low levels over the 24 hours. This was intended to ensure the subsequent

Table 8.1 *Anemonia viridis*: Equations of linear regression lines fitted to the data for change in organic weight with time in DIN-fed and DIN-starved anemones. The data and the regression lines are shown in figures 8.1 and 8.2, for DIN-fed and DIN-starved anemones respectively.

t = time, in days

W = organic weight in grams, at time t

Stock	Animal No.	n	Equation of regression line
DIN-fed	1	11	$\log_{10} W = 1.12 \times 10^{-4}t + 0.221$
	2	11	$\log_{10} W = -1.93 \times 10^{-4}t + 0.165$
	4	11	$\log_{10} W = -0.80 \times 10^{-4}t + 0.043$
	5	11	$\log_{10} W = -2.59 \times 10^{-4}t - 0.011$
	6	11	$\log_{10} W = -1.88 \times 10^{-4}t - 0.047$
	7	11	$\log_{10} W = -2.34 \times 10^{-4}t - 0.052$
	8	11	$\log_{10} W = -1.12 \times 10^{-4}t - 0.069$
	9	11	$\log_{10} W = 0.49 \times 10^{-4}t - 0.204$
	10	11	$\log_{10} W = -1.84 \times 10^{-4}t - 0.308$
DIN-starved	2	11	$\log_{10} W = -5.78 \times 10^{-4}t + 0.123$
	4	11	$\log_{10} W = -7.47 \times 10^{-4}t + 0.068$
	5	11	$\log_{10} W = -6.75 \times 10^{-4}t + 0.030$
	6	11	$\log_{10} W = -5.31 \times 10^{-4}t - 0.086$
	7	11	$\log_{10} W = -7.69 \times 10^{-4}t - 0.107$
	8	11	$\log_{10} W = -5.95 \times 10^{-4}t - 0.146$
	9	11	$\log_{10} W = -6.84 \times 10^{-4}t - 0.169$
	10	11	$\log_{10} W = -6.21 \times 10^{-4}t - 0.195$

Figure 8.1 *Anemonia viridis*: Change in $\log_{10}$ organic weight with time in nine symbiotic anemones starved of heterotrophic nutrition whilst exposed to elevated concentrations of ammonia (DIN-fed). The ammonia concentration in the incubation tank was spiked to approximately 20 µg-at NH_3-N.l^{-1} every 24 hours. Light levels were set to the anemones' normal aquarium cycle, 12 hours at 140 µE.m^{-2}.s^{-1}: 12 hours darkness. The linear regression lines fitted to the data for each anemone are also shown. Equations for the lines are given in table 8.1.

Figure 8.1

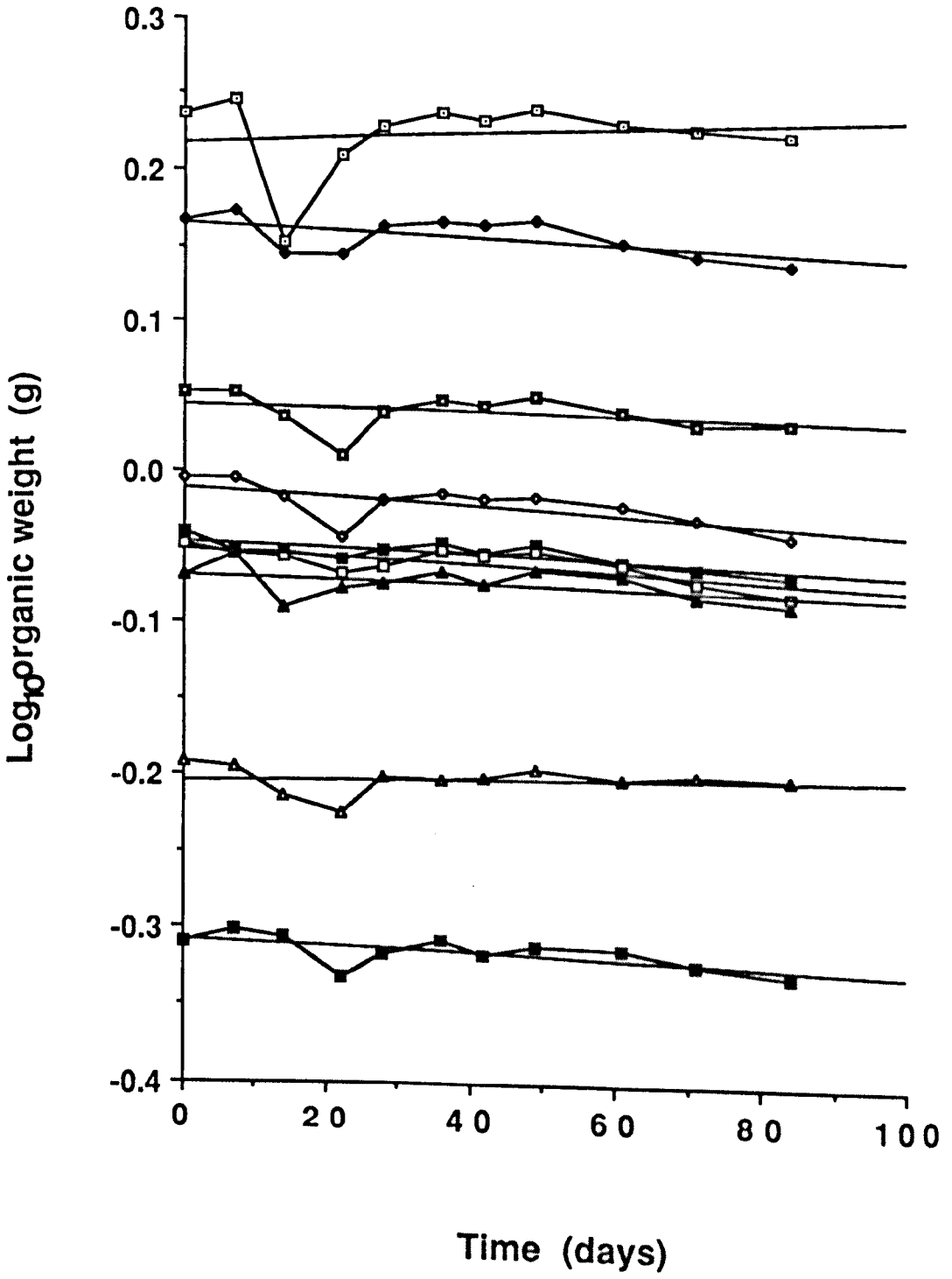
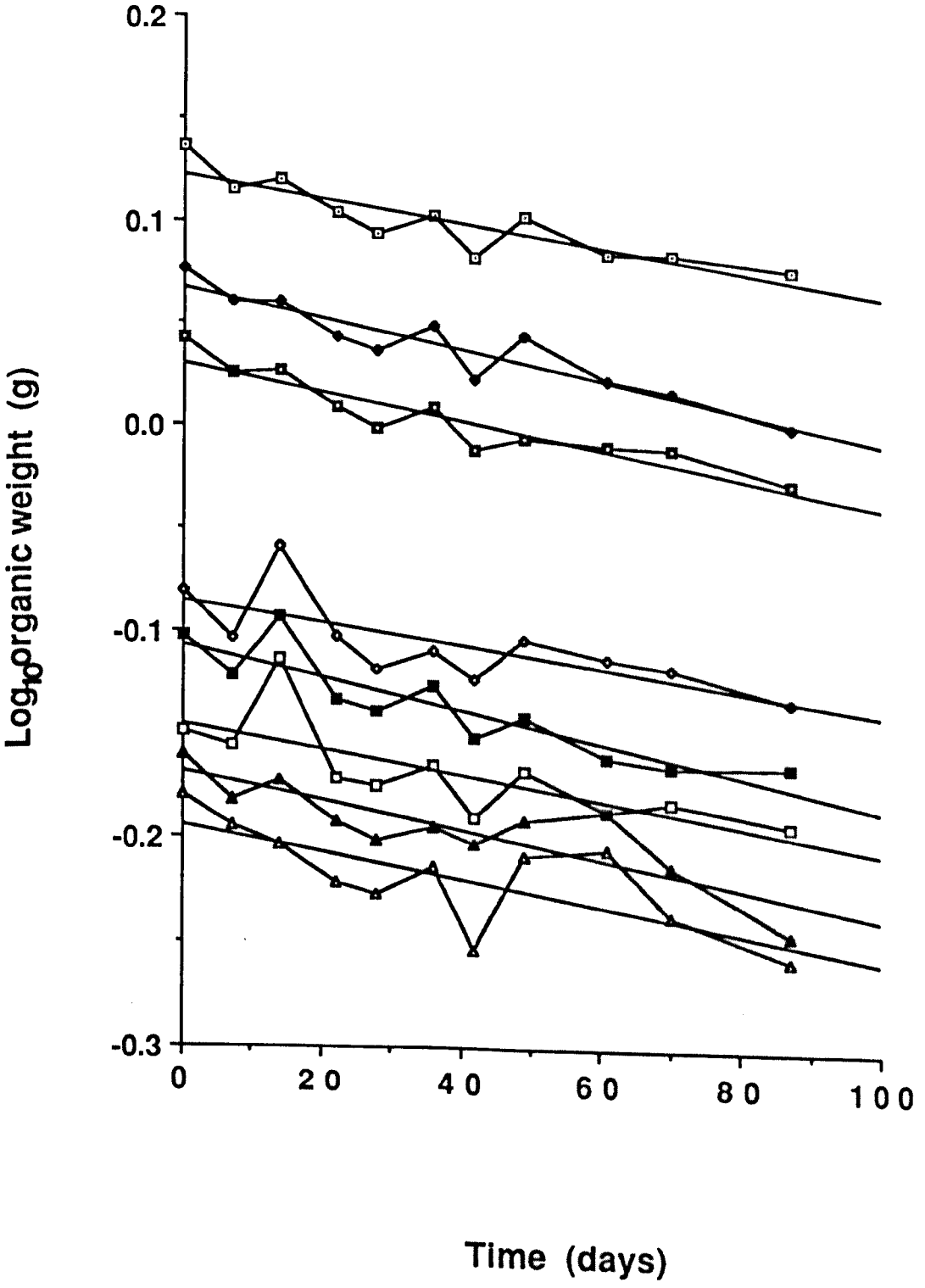

0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0
20
40
60
80
100
Log10organic weight (g)
Time (days)

Figure 8.2 *Anemonia viridis*: Change in $\log_{10}$ organic weight with time in eight symbiotic anemones starved of heterotrophic nutrition whilst exposed to negligable concentrations of ammonia (DIN-starved). Light levels were set to the anemones' normal aquarium cycle, 12 hours at 140 $\mu E.m^{-2}.s^{-1}$: 12 hours darkness. The linear regression lines fitted to the data for each anemone are also shown. Equations for the lines are given in table 8.1.

Figure 8.2


0.2
0.1
0.0
-0.1
-0.2
-0.3
0
20
40
60
80
100
Log10organic weight (g)
Time (days)

re-spiking brought ammonia concentrations back to approximately 20 µg-at NH_3-N.l^{-1}, whilst not leaving the anemones in water with unmeasurable ammonia levels for any length of time. The water sampling, prior to re-spiking, showed that the anemones had not always taken up ammonia at the predicted rate. Sampled ammonia concentrations ranged from 0.870 to 18.260 µg-at NH_3-N.l^{-1}, whereas the concentration predicted 24 hours after spiking, under the light regime used, was approximately 3 µg-at NH_3-N.l^{-1} (using the uptake rate/concentration relationships for starved anemones in darkness and at 100 $\mu E.m^{-2}.s^{-1}$, given in table 4.6). The mean concentration 24 hours after spiking was 5.64 ± 4.33 µg-at NH_3-N.l^{-1} (± one standard deviation, n=18), suggesting that mean uptake rates were slightly lower than predicted from short incubation experiments.

8.4 Discussion

Several studies have investigated changes in weight with time in *Anemonia viridis* starved of heterotrophic nutrition whilst illuminated (Taylor, 1969b; Janssen and Möller, 1981; Tytler and Davies, 1986), and all have found that the anemones lose weight. The predictions made in section 7.2 of net nitrogen loss or gain by symbiotic anemones under various conditions suggested that the ambient ammonia concentration would have to be relatively high before a net nitrogen gain was possible. It therefore seems likley that the weight losses found in the studies mentioned above were due in part to net loss of nitrogen during incubation in sea water with low ammonia levels.

Using the same methods for the calculation of net loss of nitrogen as those outlined in table 7.2, the nitrogen loss rate of a 1 g symbiotic anemone under the experimental protocol of Tytler and

Davies (1986) could be calculated as -79.166 μg $N.d^{-1}$ (with S=0, and 12 hours of 140 $\mu E.m^{-2}.s^{-1}$, 12 hours of darkness). This would give a total loss of nitrogen over the 12 week period, of 6.650 mg N.

Although the protocol used in the present study was based on that of Tytler and Davies (1986), it differed in that a flow-through system for the incubations could not be used, because of the need to enrich the ammonia levels of the water in the DIN-fed incubation. The use of static incubations, where the anemones continue to change the ambient ammonia concentration until the water is changed or re-spiked with ammonia, presents difficulties when trying to apply the calculations used to predict net nitrogen balance for anemones in the field or in a flow-through system (in which the anemones are assumed not to affect the ambient ammonia concentration to any great extent). In order to obtain values for daily φ_S (the ammonia flux rate of symbiotic anemones), it was necessary to carry out iterative calculations, to find the hourly value of φ_S for the ambient concentration (S) at the start of the day, and then to use the calculated φ_S value to predict the value of S at the start of the next hour. The calculation was then repeated to give the value of φ_S for that hour, and to predict the starting value of S for the following hour. In this way, φ_S and S could be determined for each hour of the day, and the daily value of φ_S was calculated from the sum of the hourly values. In this way, it was calculated that a 1 g symbiotic anemone, with a starting concentration of 20 μg-at NH_3-$N.l^{-1}$, would have a daily φ_S value of 16.974 μg-at NH_3-$N.d^{-1}$ (see table 8.2, column 3). Because φ_Z, the zooxanthellar ammonia uptake rate, at any value of S is equal to the difference between φ_S and φ_a, the predicted ammonia flux rate of an aposymbiont at the same value of S (see section 7.1), the hourly values of φ_a had to be calculated and summed, before daily φ_Z could be estimated. The values of φ_a are shown in table 8.2, column 4. In this

Table 8.2 *Anemonia viridis*: Values of S (ambient ammonia concentration), ρ_s (ammonia flux rate of a symbiotic anemone) and ρ_a (ammonia flux rate of an aposymbiotic anemone) at each hour of the day in the DIN-fed incubation. All values obtained by iterative calculation, from a starting ammonia concentration of 20 µg-at NH_3-N.l^{-1} in a 10 litre tank containing ten 1 g anemones, from the ρ vs. S relationships given in table 4.6 for symbionts at 100 µE.m^{-2}.^{s} (approximation to the actual daytime light level of 140 µE.m^{-2}.s^{-1}) and in darkness. Also shown are the calculations of daily values of ρ_s, ρ_a, ρ_z (ammonia uptake rate of the zooxanthellae), ρ_c (ammonia production rate of the animal tissue) and net loss of nitrogen by a 1 g anemone over the 12 weeks of the experiment.

Table 8.2

Time of day	S (μg-at NH_3-N.l^{-1})	Hourly φ_s (μg-at NH_3-N.$g^{-1}.h^{-1}$)	Hourly φ_a
2000-2100*	20.000	1.282	-0.057
2100-2200*	18.718	1.180	-0.160
2200-2300*	17.539	1.085	-0.254
2300-2400*	16.454	0.998	-0.341
2400-0100*	15.465	0.918	-0.421
0100-0200*	14.538	0.845	-0.494
0200-0300*	13.693	0.777	-0.562
0300-0400*	12.916	0.715	-0.624
0400-0500*	12.201	0.658	-0.681
0500-0600*	11.543	0.605	-0.734
0600-0700*	11.938	0.557	-0.728
0700-0800*	10.381	0.512	-0.827
0800-0900	9.869	1.061	-0.867
0900-1000	8.808	0.930	-0.952
1000-1100	7.878	0.816	-1.027
1100-1200	7.062	0.716	-1.092
1200-1300	6.346	0.628	-1.149
1300-1400	5.718	0.550	-1.200
1400-1500	5.168	0.483	-1.244
1500-1600	4.685	0.423	-1.282
1600-1700	4.262	0.371	-1.316
1700-1800	3.891	0.326	-1.346
1800-1900	3.565	0.285	-1.372
1900-2000	3.279	0.250	-1.395
Daily values: (μg-at NH_3-N.d^{-1})		16.974	-20.179

* = hours of darkness

$\varphi_z = \varphi_s - \varphi_a = 16.974 - (-20.179) = 37.153$ μg-at NH_3-N.d^{-1}

From table 7.2, $\varphi_c = -39.768$ μg-at NH_3-N.d^{-1}

Net loss per day = $\varphi_z + \varphi_c = 37.153 + (-39.768)$
$= -2.615$ μg-at NH_3-N.d^{-1}

Over 84 day experimental period, net loss = -219.660 μg-at NH_3-N

= -3076.712 μg N

If 1 μg N = 6.25 μg protein,
weight of protein lost over 84 days = 19.23 mg

way it was estimated that a 1 g anemone at a days starting concentration of 20 μg-at NH_3-N.l^{-1} would have a daily φ_z of 37.153 μg-at NH_3-N.d^{-1}. φ_c, the rate of animal ammonia production (see section 7.2), is unaffected by light or ambient ammonia concentration, and equals -39.768 μg-at NH_3-N.d^{-1} in a 1 g starved anemone (see table 7.2). Thus the predicted daily rate of net nitrogen loss from a 1 g "DIN-fed" anemone is -2.615 μg-at NH_3-N.d^{-1} (table 8.2). This gives a loss of 219.66 μg-at NH_3-N over the 84 days. This can be converted into an estimate of protein loss if it is assumed that 1 μg of nitrogen represents 6.25 μg of protein (Szmant-Froelich and Pilson, 1980), after conversion of μg-at NH_3-N to μg N. A 1 g anemone from the DIN-fed stock would therefore be expected to lose 19.23 mg of protein over the duration of the experiment.

Using the common regression coefficient for the relationship between log weight and time, of -1.2×10^{-4} (see previous section), a 1 g anemone from the DIN-fed stock would have weighed 0.9771 g after 84 days, and so would have lost 22.9 mg in weight. This weight loss is very close to the predicted weight of lost protein. Since the mean ammonia uptake rates for the DIN-fed stock were slightly lower than the rates predicted from results in Chapter 4, the actual net rate of nitrogen loss from the anemones would have been higher than calculated above. Hence the weight of lost protein would have been even closer to the calculated total weight loss. It therefore appears that the model described in the previous chapter predicts well the net loss of nitrogen from symbiotic anemones, if it is assumed that the total weight loss was due to protein catabolism.

A similar calculation could be made for the DIN-starved stock, although the iterative calculation of hourly values of S, φ_s and φ_a had to be done for 5 consecutive days. This was because, unlike the

DIN-fed incubation, which was re-spiked to 20 μg-at NH_3-N.l^{-1} every 24 hours, the water in the DIN-starved incubation was left until it was changed. Thus the anemones were affecting the ambient ammonia concentration constantly for up to a week (the longest period between water changes). The iterative calculation showed that daily φ_s and φ_a reached constant values after 4 days (see table 8.3), because the system reached a set of ambient ammonia concentrations at which the zooxanthellae were able to recapture during the day all of the ammonia lost to the symbiosis during the night. If water changes were made every 7 days, the net loss of nitrogen would be -385.200 μg-at NH_3-N.g^{-1} over the entire experiment (see table 8.3). Using the same conversion factor for protein as above, this represents a potential loss by a 1 g anemone of -33.72 mg protein over the 12 weeks (table 8.3). The actual total weight loss of a 1 g anemone in the DIN-starved stock, using the common regression coefficient for the log weight vs. time relationship of -6.5×10^{-4}, was 118 mg over 87 days, and so it would appear that protein may not be the only component being depleted in these anemones.

The difference between calculated weight of protein loss and total weight loss in DIN-starved anemones is difficult to explain, but must represent loss of carbohydrate or lipid from the anemones. A similar loss of carbohydrate or lipid presumably did not occur in the DIN-fed anemones, since the weight loss observed can be accounted for entirely by the loss of protein. It is possible that the DIN-starved anemones were not exhibiting ammonia flux rates that corresponded with those predicted from Chapter 4, but the difference would need to be quite large to account for the 350% difference between weight of protein lost and total weight loss. A second possibility is that, as structural proteins are catabolized during starvation, other structural components such as lipids tend to degenerate, and hence are

Table 8.3 *Anemonia viridis*: Daily values of φ_s and φ_a for anemones in the DIN-starved stock, over 7 days. Incubation started, on day 1, at a concentration of 0 µg-at NH_3-N.l^{-1}, and water was not changed over the seven days. Light levels were 12 hours at 140 µE.m^{-2}.s^{-1} followed by 12 hours of darkness each day. Also shown are the calculated concentrations of ammonia at the start of each day, calculated in the same way as the figures in table 8.2. The calculation of the weight of protein lost by a 1 g anemone from this stock over the 12 week experiment is shown below the table.

Day no.	S (µg-at NH_3-N.l^{-1})	Daily φ_s (µg-at NH_3-N.d^{-1})	Daily φ_a
1	0	-2.170	-36.869
2	2.171	-0.167	-35.399
3	2.336	-0.013	-35.251
4	2.349	-0.001	-35.277
5	2.350	0.000	-35.277
6	2.350	0.000	-35.277
7	2.350	0.000	-35.277

φ_s for seven days = -2.351 µg-at NH_3-N
φ_a " " " = -248.627 µg-at NH_3-N

φ_z for seven days = $\varphi_s - \varphi_a$ = 246.276 µg-at NH_3-N

φ_c for seven days = 7 x -39.768 = -278.376 µg-at NH_3-N
(see table 8.2)

Net nitrogen loss over seven days = $\varphi_z - \varphi_c$
= -32.100 µg-at NH_3-N

Net nitrogen loss over 84 days (12 weeks) = 12 x (-32.100)
= -385.200 µg-at NH_3-N

= -5395.381 µg N

Using protein conversion factor from table 8.2,

Weight of protein lost over 12 weeks = 33.72 mg

lost. The rate of such protein catabolism in the DIN-fed anemones could have been sufficiently low for the loss of structural lipids to be minimal.

This experiment showed that, in the presence of elevated ammonia concentrations, the rate of weight loss by starved *Anemonia viridis* is reduced, and that this rate reduction can be accounted for by decreased net rates of nitrogen loss. It was not possible to demonstrate autotrophic growth because ambient ammonia levels were insufficient to allow a net nitrogen gain by the symbiosis. This could have been done by spiking the incubation sea water to higher ammonia concentrations, but since the initial concentration used was already at the limit of recorded natural concentrations in coastal waters, such an approach would be undesirable. A more valid method of exposing the experimental anemones to higher concentrations of ammonia would be to incubate them in a flow-through system with a constant addition of a concentrated ammonia solution, resulting in a flow of sea water at a permanently elevated ammonia concentration. This would allow exposure to a steady ammonia concentration of an environmentally-reasonable level (e.g. 12 µg-at NH_3-N.l^{-1}), which would still represent a total ammonia availability in excess of that provided in this study.

Autotrophic growth by this symbiosis therefore remains to be demonstrated. It is possible that another nutrient, such as phosphorus, may become limiting before weight gain is possible, even in conditions of abundant ammonia availability. It is also possible that the host requires certain compounds, such as essential amino acids, that can not be provided by translocation from the algae, or by host synthesis from algal-derived compounds. Both of these limiting factors could prevent autotrophic growth even under conditions allowing a net gain of nitrogen by the association.

Chapter 9: SUMMARY DISCUSSION

It is clear from Chapter 1 that most scientific interest in the physiology of algal-coelenterate symbioses has sought to evaluate the "benefit" of the association, either to the host, the symbiont or both. "Benefit" was defined in Chapter 1, after Laws and Lewis (1983), in terms of increased biological fitness. The fitness of an organism is generally considered to be measured by the number or competitive ability of the offspring it produces, and whilst these factors have been little studied in symbiotic coelenterate species, other partial indices of fitness, such as growth rate, have been the subject of considerable investigation (Smith and Douglas, 1987).

The aims of this study were to investigate some factors affecting one possible source of benefit to the organism, the uptake and retention of dissolved inorganic nitrogen, and then to assess the potential effect this may have on the fitness of the symbiosis. As noted above, the assessment of fitness was based upon the potential effects DIN uptake had on growth rates, as an index of fitness, rather than attempting to determine reproductive fitness. The evaluation of DIN uptake effects on growth rates took two forms: the prediction of net gains and losses of nitrogen by anemones under field conditions, based upon a theoretical model for DIN uptake derived from laboratory experiments (sections 7.1 and 7.2), and an experimental test of growth rates under laboratory conditions favouring different rates of DIN uptake (Chapter 8).

The goal of many studies on symbioses of this type has been to evaluate the relative importance of autotrophy and heterotrophy in the nutrition of the association, and in particular, to assess the potential for the symbiosis to be fully autotrophic. The concept of a totally autotrophic way of life for an algal-coelenterate symbiosis

may be misleading (see below), but the conditions under which nitrogen autotrophy might occur in *A. viridis* were discussed in section 8.2. It would appear that, although on days during the summer the association could be self-sufficient at environmental concentrations of ammonia that are reported to occur around the British coast, nitrogen autotrophy is extremely unlikely at any time of the year for anemones in Scottish sea lochs, because of the low ambient DIN concentrations. Despite this, the results of the growth experiment in Chapter 8 suggest that, even when full autotrophy is not possible, the rate of weight loss in starving anemones may show an inverse correlation with availability of ammonia in the seawater surrounding the anemones. This is an indication of an improvement of biological fitness, as described above.

In previous studies on the potential benefits to host and algae in association in terms of fixed carbon or energy, the most useful approach has proved to be the construction of a budget of losses and gains by each partner (e.g. Davies, 1984; Muscatine *et al.*, 1984; Edmunds and Davies, 1986). The prediction of algal gains and host losses of ammonia-nitrogen on summer and winter days, in section 7.2, form the basis for such an approach for nitrogen. For a realistic nitrogen budget for *A. viridis* in the field, however, several other factors merit investigation. *A. viridis* is known to be a voracious carnivore in part of its geographical range, and heterotrophic feeding is likely to change both the net gain or loss of metabolic nitrogen by the association (see Chapter 5), and the relative importance of DIN uptake by the zooxanthellae. Rates of heterotrophic feeding in the field are therefore required, as is further investigation of the rates of animal ammonia flux immediately after feeding. In addition, there is a certain amount of evidence to suggest that the uptake of dissolved organic matter by this association may make a significant

contribution to the nutritional requirements of the host (Schlichter, 1975). In particular, *A. viridis* appears to have several distinct uptake systems for charged and neutral amino acids (Schlichter, 1973; 1974; 1978), and these processes would have to be accounted for in a comprehensive nitrogen budget.

No study has yet succeeded in showing that growth may occur in this association in the absence of heterotrophic feeding, although the results in Chapter 8 and the predictions in section 7.2 suggest that this might be possible under certain conditions of light and ammonia availability. The possibility remains that growth may be prevented by the requirement for essential amino acids, even in the presence of adequate levels of ammonia for net nitrogen gain (see Chapter 8). Further work is needed on the requirement of the host for specific nitrogenous compounds that may not be provided by the algae, or by host conversion of algal-derived compounds. A particularly interesting line of research associated with this would be to investigate the fate of algal-assimilated nitrogen in the host. Extension of the ^{15}N tracer methods used in Chapter 6, with the coupling of gas chromatography and mass spectrometry, holds great potential for identifying the compounds translocated from the zooxanthellae to the host, and their subsequent metabolic fate. Until this is done, the true nature of the benefits to the nitrogen balance of the symbiosis will remain speculative.

Hallock, in 1981, produced a mathematical model of algal-invertebrate symbiosis which incorporated the utilization efficiencies of the host and symbiont, and which she used to illustrate the benefits of polytrophic nutrition to the system. The model was used to compare organic production rates in similar environments containing plant and animal cells in symbiosis or in isolation from one another. If conditions resulted in a greater net production by algae and invertebrates in association than by free-living plants and animals,

symbiosis was predicted. From Hallock's model it is clear that, if particulate food for animals was unavailable, but dissolved nutrients and light were adequate for plant growth, there would be plant production, but no animals (presumably until herbivores appeared in the environment to graze the plant production). These appear to be the conditions postulated by those studies looking for total autotrophy in symbioses. If there are adequate nutrients for plant growth, there seems little reason for mutualistic symbiosis, and the host would effectively become parasitic on the algae. Hallock's model suggests that the environment in which symbiosis would be most beneficial to both partners would be one where light was abundant, growth-limiting nutrients were very scarce and almost entirely bound up in particulate matter, and considerable energy had to be expended to capture that particulate matter. Under these conditions the intimate metabolic relationship between the symbiotic partners, based on efficient recycling of all nutrients captured, would give symbioses selective advantage over free-living plants and animals. The conditions are descriptive of the tropical reef environment, and possibly explain the predominance of symbioses in coral reefs. The description also tallies well with the conditions of negligible nutrient concentrations and low prey availability experienced by *A. viridis* on the study site in Linne Mhuirich, despite the classical distinction of tropical and temperate waters on the basis of nutrient availability.

No single beneficial factor of symbiosis is likely to be fully responsible for the improvement of biological fitness experienced by symbiotic associations, but it seems certain that the capability to take up dissolved inorganic nitrogen is an important factor in the success of *Anemonia viridis* around British coasts in general, and in Loch Sween in particular.

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APPENDICES

Appendix I, Table 1: Calibration of biomass estimates. Ten starved symbiotic "calibration" anemones and the 10 "DIN-starved" anemones from the growth experiment (Chapter 8) were buoyant weighed, and then sacrificed to obtain values for total zooxanthella numbers and chlorophyll *a* content (see section 2.4.2). Buoyant weights were converted to organic weights as described in section 2.4.1. The growth experiment anemones number twelve because two individuals divided during the experiment. Although excluded from the growth experiment data analysis, they are included here.

The relationships between organic weight and zooxanthella numbers, and zooxanthella numbers and chlorophyll *a* content were calculated by least squares regression, to give the following equations:

$$z = 3.987w_d + 0.401$$

$$chl_a = 226.192z - 229.732$$

z = total number of zooxanthellae in anemone, x 10^8.

w_d = organic weight, in g.

chl_a = total chlorophyll *a* content of anemone, in µg.

Thus a 1 g organic weight symbiotic anemone contains 4.388 x 10^8 zooxanthellae

4.388 x 10^8 zooxanthellae contain 488.498 µg chlorophyll *a*

∴a 1 g organic weight anemone contains 488.498 µg chlorophyll *a*

Table I.1

Anemone no.	Buoyant weight (g)	Organic weight(g)	Zooxanthella numbers ($x10^8$)	Chlorophyll *a* (μg)
1	0.1596	0.8174	3.951	566.699
2	0.1253	0.6430	2.598	363.905
3	0.1145	0.5880	3.026	694.452
4	0.2490	1.2722	5.710	1040.893
5	0.2596	1.3261	6.515	1494.401
6	0.0761	0.3927	1.736	492.255
7	0.1191	0.6114	4.024	839.844
8	0.1385	0.7101	4.205	1040.618
9	0.1277	0.6552	3.596	648.104
10*	0.1819	0.9309	4.040	808.662
11*	0.2345	1.1984	4.503	398.052
12*	0.1962	1.0034	3.758	405.432
13*	0.1842	0.9426	3.555	289.333
14*	0.1063	0.5463	2.610	256.025
15*	0.1438	0.7371	3.169	348.120
16*	0.1337	0.6857	3.353	302.796
17*	0.1569	0.8037	3.081	398.245
18*	0.1051	0.5402	2.929	417.005
19*	0.1252	0.6424	2.877	344.069
20*	0.1219	0.6257	2.332	344.577
21*	0.1077	0.5534	2.115	280.612
22*	0.1106	0.5682	2.082	255.711

* = "DIN-starved" growth experiment anemone

Appendix II

Data from experiments testing weight-specific dissolved inorganic nitrogen (DIN) uptake, φ, of starved *Anemonia viridis* at different ambient DIN concentrations, S. The related φ and S values, obtained as described in in section 4.3.2, are given for each anemone tested.

Tables 1 to 8: Ammonia uptake/concentration values for starved symbiotic anemones in darkness and at 5, 10, 20, 50, 100, 190 and 300 $\mu E.m^{-2}.s^{-1}$.

Tables 9 to 11: Ammonia uptake/concentration values for starved aposymbiotic anemones in darkness and at 190 $\mu E.m^{-2}.s^{-1}$.

Table 12: Nitrate uptake/concentration values for starved symbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$.

Tables 9 and 11 contain data obtained from incubations of 1/2 hour duration, all other data shown was obtained from incubations of 1 hour duration.

φ = weight specific uptake rate, in μg-at N.g organic $weight^{-1}.h^{-1}$.

S = ambient DIN concentration, in μg-at $N.l^{-1}$.

Table II.1 Starved symbiotic anemones in darkness. Ammonia.

Anemone number	S	φ	S	φ	S	φ
A0/ 5.1	7.961	0.472	7.774	0.106	6.619	0.019
	4.715	0.067	3.029	-0.145	0.781	-0.241
A0/ 5.2	9.023	0.580	8.305	0.149	6.462	-0.093
	4.714	-0.056	3.122	-0.299	2.904	0.131
A0/ 5.3	8.898	1.179	8.430	0.076	6.275	0.115
	4.340	-0.191	2.685	-0.152	0.843	-0.114
A0/25.1	8.797	1.349	7.544	0.885	5.971	0.604
	4.515	0.183	2.621	0.253	0.699	0.141
A0/25.2	10.107	0.227	8.884	0.095	7.573	0.059
	5.272	-0.081	3.466	-0.095	1.718	-0.124
A0/25.3	10.399	0.516	8.942	0.030	8.097	0.485
	5.447	0.031	3.554	-0.121	1.864	-1.091
A0/29.1	10.071	0.021	7.008	0.141	5.711	0.190
	4.663	-0.176	2.483	-0.070	0.828	-0.155
A0/29.2	8.443	0.864	8.056	0.663	5.077	0.922
	3.394	0.130	2.042	-0.058	0.579	0.216
A0/29.3	9.657	0.140	8.029	-0.128	6.318	-0.082
	4.416	-0.093	2.649	-0.047	2.676	-1.060

Table II.2 Starved symbiotic anemones at 5 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
05/44.1	10.769	0.240	10.000	-0.264	8.604	0.000
	7.521	-0.144	5.697	-1.103	3.760	-0.623
05/44.2	10.740	0.250	9.344	-0.167	8.518	-0.250
	7.492	-1.139	5.811	-1.111	3.846	-0.805
05/44.3	7.236	0.593	6.410	0.578	5.498	0.401
	4.501	0.312	3.390	0.255	1.339	0.182
05/46.1	9.258	1.042	8.136	0.764	4.987	0.355
	3.896	0.286	2.587	0.316	0.748	0.170
05/46.2	12.500	0.223	10.162	-0.826	7.138	-0.984
	6.827	-0.604	4.707	-0.730	2.805	-1.144
05/46.3	8.603	1.165	6.671	0.662	5.206	0.331
	5.019	0.675	2.556	0.371	1.184	0.424

Table II.3 Starved symbiotic anemones at 10 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
10/42.2	8.882	0.317	6.357	0.506	6.594	0.155
	6.179	0.619	4.129	0.556	3.713	0.246
10/42.3	10.842	0.124	9.238	-0.371	8.139	-0.643
	6.446	-0.025	4.515	-0.742	3.891	-0.420
10/43.1	8.953	1.408	6.625	0.971	4.328	0.619
	2.269	0.486	2.299	0.595	0.895	0.340
10/43.2	11.341	-1.066	8.715	-0.178	6.417	-0.266
	4.536	-0.592	4.238	-0.770	3.133	-1.392
10/43.3	10.953	0.506	9.401	-0.094	6.805	-0.112
	4.238	-0.375	4.805	-0.281	2.507	-0.450

Table II.4 Starved symbiotic anemones at 20 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
20/39.1	7.899	1.332	7.504	-0.349	4.011	1.395
	4.223	0.222	1.732	-0.602		
20/39.2	12.243	-0.084	11.514	-1.708	9.813	0.644
	6.957	-0.924	4.466	-1.204	4.253	-0.224
20/39.3	8.293	1.311	5.954	1.245	5.408	0.609
	3.798	0.622	2.491	0.291	0.942	0.278
20/41.1	7.132	0.583	6.244	0.559	5.840	0.184
	4.871	0.359	3.230	0.239	1.318	0.184
20/41.2	8.693	0.995	8.047	0.618	6.352	0.699
	5.113	0.645	3.794	0.080	1.479	0.457
20/41.3	9.823	0.122	8.854	-0.269	7.240	-0.171
	5.544	-0.245	4.737	-0.196	2.853	-0.587

Table II.5 Starved symbiotic anemones at 50 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
A1/ 7.1	8.905	0.784	7.380	0.669	5.996	0.512
	4.009	0.240	2.555	0.083	0.142	-0.021
A1/ 7.2	8.799	0.708	8.160	0.375	6.174	0.208
	4.080	0.219	2.448	0.198	2.377	0.385
A1/ 7.3	8.728	0.763	8.231	0.687	5.748	0.432
	4.754	0.025	2.448	0.191	0.320	0.114
A1/12.1	6.872	0.751	6.196	0.512	5.436	0.267
	3.239	0.223	2.225	0.299	0.338	0.054
A1/12.2	7.999	0.966	6.900	0.760	5.323	0.367
	3.774	0.161	1.859	0.125	0.507	0.107
A1/12.3	9.154	0.779	7.605	0.547	6.084	0.437
	3.605	0.191	2.619	0.451	0.676	0.301

Table II.6 Starved symbiotic anemones at 100 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
A2/11.1	8.472	0.764	6.071	0.712	4.871	0.501
	3.018	0.282	1.784	0.193	0.343	0.039
A2/11.2	9.981	1.072	6.963	0.813	5.797	0.695
	3.636	0.400	2.641	0.153	0.549	0.024
A2/11.3	8.197	1.097	7.271	0.652	5.659	0.576
	3.533	0.358	3.396	0.141	0.275	0.087
A2/26.1	7.954	1.040	7.187	0.640	5.273	0.493
	4.065	0.293	2.445	0.093	0.707	0.027
A2/26.2	7.423	0.701	6.451	0.574	5.391	0.306
	4.389	0.186	2.357	0.089	1.355	0.134
A2/26.3	8.985	0.808	7.747	0.690	6.157	0.414
	4.566	0.296	2.563	0.020	0.825	-0.158

Table II.7 Starved symbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
A3/ 1.1	8.993	1.013	6.828	1.060	5.662	0.696
	3.964	0.491	1.732	0.348	0.300	0.142
A3/ 2.1	9.051	0.602	6.867	1.128	5.601	0.827
	4.019	0.326	1.931	0.225	0.127	0.101
A3/ 2.2	7.753	1.836	6.519	1.166	5.506	0.408
	4.178	0.349	2.152	0.408	0.349	0.204
A3/ 3.2	9.351	0.897	7.501	1.046	6.021	0.448
	4.238	0.399	3.599	0.548	0.203	-0.066
A3/ 4.1	7.067	1.399	6.300	1.231	5.180	0.944
	4.061	0.758	3.134	0.405	0.863	0.118
A3/ 4.2	6.140	1.081	5.788	0.768	4.893	0.655
	3.230	0.408	2.399	0.180	0.959	0.284

Table II.8 Starved symbiotic anemones at 300 $\mu E.m^{-2}.s^{-1}$. Ammonia.

Anemone number	S	φ	S	φ	S	φ
A4/ 9.1	6.923	1.017	5.758	0.748	5.066	0.389
	3.934	0.289	1.574	0.140	0.158	-0.010
A4/ 9.2	7.017	0.811	5.947	0.691	4.657	0.501
	3.021	0.301	1.605	0.250	0.787	-0.010
A4/ 9.3	7.395	0.673	6.734	0.531	5.004	0.559
	4.059	0.313	2.171	0.218	0.094	-0.028
A4/10.2	7.675	0.809	6.832	0.692	5.084	0.477
	3.854	0.322	3.077	-0.146	0.389	-0.098
A4/10.3	6.315	0.778	5.279	0.579	4.242	0.392
	3.368	0.281	3.400	0.029	0.195	-0.012

Table II.9 Starved aposymbiotic anemones in darkness. Ammonia.

Anemone number	S	φ	S	φ	S	φ
C0/35.1	15.437	-0.286	12.412	-0.745	10.107	-0.730
	8.144	-0.767	7.084	-0.857	4.060	-0.880
C0/35.2	14.150	-0.116	12.249	-0.628	11.470	-0.967
	8.322	-0.729	9.786	-0.794	6.015	-0.830
C0/35.3	15.085	-0.225	11.875	-0.326	9.569	-0.468
	9.257	-0.539	6.795	-0.711	4.488	-0.605
C0/40.1	15.197	-0.066	14.177	-0.460	12.953	-0.636
	11.525	-0.650	9.826	-0.913	9.214	-0.884
C0/40.2	18.257	-0.613	16.455	-0.712	14.177	-0.877
	13.395	-1.055	12.647	-1.094	10.199	-1.160
C0/40.3	14.279	0.098	15.061	-0.057	12.681	-0.856
	11.967	-0.636	10.981	-0.742	7.207	-1.027

Table II.10 Starved aposymbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$ (1 hour incubations). Ammonia.

Anemone number	S	φ	S	φ	S	φ
C3/33.1	12.962	-0.709	14.461	-0.878	11.075	-1.012
	9.382	-0.993	8.827	-1.175	7.189	-1.266
C3/33.2	12.935	-0.579	13.518	-0.722	10.853	-0.816
	9.216	-0.579	7.356	-0.816	5.968	-0.827
C3/33.3	11.103	-0.326	12.047	-0.458	10.020	-0.790
	7.689	-0.657	7.190	-0.778	5.829	-1.001
C3/34.1	13.349	-0.811	11.665	-0.928	10.257	-1.077
	7.991	-1.035	6.521	-1.269	4.990	-1.461
C3/34.2	13.074	-0.815	12.492	-1.017	9.706	-0.837
	7.716	-0.953	6.552	-1.292	4.592	-1.313
C3/34.3	12.001	-0.318	11.848	-0.727	10.991	-0.878
	9.337	-0.994	7.164	-1.036	6.460	-1.229

Table II.11 Starved aposymbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$ (1/2 hour incubations). Ammonia.

Anemone number	S	φ	S	φ	S	φ
C3/50.1	12.236	-0.748	11.123	-0.690	9.062	-0.848
	6.837	-0.762	5.856	-1.308	3.075	-0.949
C3/50.2	10.829	-0.132	9.618	-0.490	8.473	-0.810
	6.739	-0.867	4.547	-1.074	2.944	-1.243
C3/50.3	12.334	-0.744	12.039	-1.001	9.127	-0.852
	7.950	-1.149	6.837	-1.528	4.613	-1.420
C3/51.1	12.797	-0.636	14.005	-0.886	9.977	-1.388
	8.211	-1.407	7.188	-1.464	4.524	-1.233
C3/51.2	13.261	-0.641	13.757	-0.993	9.234	-0.833
	7.963	-1.009	5.515	-0.833	5.515	-1.698
C3/51.3	12.735	-0.276	13.137	-0.849	10.473	-1.826
	8.304	-1.274	6.817	-1.274	4.586	-1.231

Table II.12 Starved symbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$. Nitrate.

Anemone number	S	φ	S	φ	S	φ
N1/14.1	3.537	-0.069	3.537	-0.137	3.942	0.137
	6.989	0.103	5.770	-0.034	6.177	-0.240
N1/14.2	5.364	-0.098	3.943	-0.065	2.318	0.000
	6.786	0.000	5.771	-0.098	4.755	-0.196
N1/14.3	4.349	0.067	3.943	0.000	2.318	0.067
	7.192	0.000	6.177	0.167	3.942	0.134

Appendix III

Data from experiments testing weight-specific dissolved inorganic nitrogen (DIN) uptake, φ, of fed *Anemonia viridis* at different ambient DIN concentrations, S. The related φ and S values, obtained as described in section 5.2, are given for each anemone tested.

Tables 1 to 5: Ammonia uptake/concentration values for fed symbiotic anemones in darkness and at 50, 100, 190 and 300 $\mu E.m^{-2}.s^{-1}$.

Table 6: Ammonia uptake/concentration values for fed aposymbiotic anemones in darkness.

Table 6 contains data obtained from incubations of 1/2 hour duration, all other data shown was obtained from incubations of 1 hour duration.

φ = weight specific uptake rate, in μg-at N.g organic weight$^{-1}.h^{-1}$.

S = ambient DIN concentration, in μg-at $N.l^{-1}$.

Table III.1 Fed symbiotic anemones in darkness

Anemone number	S	φ	S	φ	S	φ
B0/27.1	12.889	-0.583	11.191	-0.547	8.707	-0.291
	7.513	-0.680	5.595	-0.459	6.036	-0.971
B0/27.2	13.046	-0.808	10.405	-0.340	7.953	-0.316
	6.947	-0.762	6.853	-1.453	4.307	-0.715
B0/28.1	9.323	0.280	6.319	0.129	5.214	0.121
	3.457	0.061	3.542	-0.265	0.623	-0.106
B0/28.2	9.748	0.213	7.736	0.053	7.396	0.160
	5.214	0.128	3.230	-0.277	1.870	-0.363
B0/28.3	11.816	-0.280	9.040	-0.280	8.841	-0.379
	5.838	-0.454	5.667	-0.666	3.174	-0.606

Table III.2 Fed symbiotic anemones at 50 $\mu E.m^{-2}.s^{-1}$

Anemone number	S	φ	S	φ	S	φ
B1/23.1	8.449	0.545	17.320	0.427	6.070	0.288
	4.362	0.117	2.928	0.128	1.495	0.096
B1/23.2	10.523	-0.119	9.334	-0.079	7.748	-0.475
	5.765	-0.277	4.697	-0.343	3.050	-0.950
B1/23.3	10.706	0.027	9.700	-0.283	8.663	-0.371
	6.924	-0.610	4.911	-0.539	3.020	-0.627
B1/24.1	7.604	0.604	8.096	0.699	5.736	0.508
	3.934	0.466	2.917	0.480	0.655	-0.110
	8.391	1070				
B1/24.2	7.309	0.791	7.211	0.491	7.539	0.491
	5.474	0.376	4.294	0.130	2.556	0.215
	0.262	0.061				
B1/24.3	10.522	0.341	9.146	0.150	9.178	0.300
	7.375	-0.014	5.736	-0.259	3.537	-0.178
	1.475	-0.205				

Table III.3 Fed symbiotic anemones at 100 $\mu E.m^{-2}.s^{-1}$

Anemone number	S	φ	S	φ	S	φ
B2/21.1	8.615	0.588	6.586	0.424	4.969	0.337
	3.969	0.144	2.646	0.077	2.087	-0.048
B2/21.2	9.673	0.553	6.763	0.458	6.174	-0.108
	4.880	-0.243	3.763	-0.351	2.381	-0.202
B2/21.3	10.026	0.275	8.232	0.232	6.879	0.058
	4.880	-0.087	3.704	-0.376	1.999	-0.232
B2/22.1	10.895	1.016	7.815	0.663	6.583	0.240
	4.702	0.183	3.275	0.155	2.204	0.113
B2/22.2	7.944	0.808	7.231	0.630	5.739	0.471
	4.377	0.258	3.081	0.133	1.362	-0.035
B2/22.3	8.755	0.675	6.550	0.648	5.350	0.385
	4.410	0.230	3.080	0.074	1.816	-0.162

Table III.4 Fed symbiotic anemones at 190 $\mu E.m^{-2}.s^{-1}$

Anemone number	S	φ	S	φ	S	φ
B3/18.1	7.406	0.353	5.967	0.368	5.127	0.113
	4.138	-0.105	2.908	-0.173	1.439	-0.210
B3/18.2	6.897	0.807	5.877	0.531	4.708	0.588
	3.418	0.208	1.739	0.092	1.379	-0.092
B3/18.3	6.927	0.705	6.387	0.666	4.708	0.203
	3.508	0.087	2.849	0.068	0.899	-0.270
B3/30.1	1.585	-0.263	7.927	0.247	7.242	0.200
	7.212	-0.016	5.750	-0.295	4.134	-0.088
B3/30.2	9.760	0.421	9.014	0.306	7.709	-0.498
	5.937	0.287	4.912	-0.383	1.306	-0.727
B3/30.3	8.392	1.000	7.647	0.273	5.688	0.197
	4.849	0.788	2.549	0.030	0.840	-0.318
B3/32.1	8.604	0.746	7.340	0.664	5.565	0.355
	5.053	0.018	2.219	0.064	0.614	-0.127
B3/32.2	7.715	0.752	5.701	0.744	5.428	0.564
	6.282	-0.392	1.775	0.065	0.614	-0.065

Table III.5 Fed symbiotic anemones at 300 $\mu E.m^{-2}.s^{-1}$

Anemone number	S	φ	S	φ	S	φ
B4/19.1	9.600	0.597	8.059	0.597	6.061	0.448
	4.064	0.298	2.313	0.027	0.736	-0.258
B4/19.2	9.145	0.571	7.849	0.583	6.131	0.790
	5.781	0.352	1.892	0.194	1.016	0.061
B4/31.1	9.274	0.845	6.991	0.674	5.691	0.704
	3.864	0.402	2.564	0.251	1.334	0.141
B4/31.2	9.837	0.547	8.220	0.377	6.991	0.273
	5.374	0.047	3.935	-0.189	2.565	-0.104
B4/31.3	8.010	0.730	6.393	0.600	6.218	0.202
	4.285	0.222	2.318	0.091	1.475	0.000

Table III.6 Fed aposymbiotic anemones in darkness

Anemone number	S	φ	S	φ	S	φ
D0/36.1	14.432	-0.580	15.166	-1.064	11.782	-0.941
	10.696	-1.064	9.100	-1.363	7.120	-1.487
D0/36.2	17.082	-0.818	18.519	-1.807	14.624	-0.811
	12.420	-1.245	10.633	-1.131	8.812	-1.480
D0/36.3	17.401	-0.517	14.368	-0.747	11.080	-0.914
	9.578	-0.716	8.269	-1.057	7.216	-1.288