INORGANIC NITROGEN AND PHOSPHORUS NUTRITION IN TRIDACNID CLAMS AND THEIR ALGAL SYMBIONTS

Thesis submitted by

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ABSTRACT

This study examined inorganic nutrition in tridacnids to address the question of nutrient limitation of their zooxanthellae, and the influence of elevated nutrient concentrations on calcification. Experiments consisted of three-month-long exposures of cultured *Tridacna gigas* in outdoor tanks and indigenous *T. maxima in situ* to increased levels of ammonia (N) and phosphate (P), alone or in combination (N+P). Following the incubation experiments, biomass and nutrient-depletion of the symbiotic partners were measured. In addition, a more detailed study was carried out on the effect of elevated P on the zooxanthellae, both *in situ* and in culture.

Major findings from the outdoor experiments were confirmed in the field study, while laboratory experiments elucidated host involvement in nutrient limitation of the zooxanthellae. In particular, zooxanthellae are N-limited *in vivo*, as demonstrated by the increased zooxanthellae density in N-supplemented clams. The decrease in ammonium-depletion by zooxanthellae isolated from these clams demonstrates that zooxanthellae have access to increased N in sea water. N-limitation of zooxanthellae, therefore, is a function of sea water concentrations of inorganic N, particularly ammonia, as this nutrient can diffuse across cell membranes depending on its concentration gradient.

Also, zooxanthellae are P-limited *in vivo*. Regardless of ambient phosphate concentrations in sea water, zooxanthellae inside the animal host were not affected, and they exhibited N:P ratios (>30:1) and P-depletion rates similar to

those of P-starved cultured zooxanthellae. Host involvement in P availability to the zooxanthellae is strongly implicated by: (1) the unaffected acid phosphatase activity (P-liberating enzymes) and undetectable polyphosphates (P reserves) in zooxanthellae, regardless of the clams' P environment; and (2) rapid growth, decreased N:P ratio, and decreased P-depletion by zooxanthellae grown under P-sufficient culture conditions. Host influence on the algae's P environment *in vivo* is further substantiated by the consistently low levels of inorganic phosphate (< 0.1 μM)) in the haemolymph surrounding the zooxanthellal tubes, despite the clams' uptake and assimilation of P from sea water (i.e., decreased N:P ratio). P-limitation of zooxanthellae, therefore, is independent of ambient sea water concentrations of phosphate, which cannot passively cross cell membranes. The host's role in P availability to its zooxanthellae is either: (1) a natural consequence of the morphological and spatial relationships between the symbiotic partners, with the host retaining phosphate for its own use before the nutrient can reach the zooxanthellae; or (2) through active P restriction by the host to control its algal population.

Investigation of calcification demonstrated that increased ambient levels of N and P modified shell formation in *T. gigas* as follows: (1) enhanced shell-extension rates, but reduced shell weights at equivalent size; (2) changes in crystal lattice parameters based on X-ray diffractometry on the shells; and (3) structural alterations in the outer shell layer, with misshapen aragonite crystals, irregular crossed-lamellar orientation, and increased porosity. Such skeletal weakening has adverse implications for calcifying organisms in eutrophic reef waters.

STATEMENT ON SOURCES

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

C. A. BELDA

May 1994

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 GENERAL INTRODUCTION

Giant clams of the family Tridacnidae are a unique group of bivalves. Apart from their enormous size, they differ from ordinary bivalves in having an exposed fleshy mantle densely populated by unicellular dinoflagellates, commonly known as zooxanthellae. These zooxanthellae photosynthesize carbon-rich compounds (Griffiths and Streamer, 1988), much of which is translocated to the animal host for use in its respiration, growth, and reproduction (Gladfelter, 1985). In return, the clam host shelters its algal symbionts and provides them with dissolved nutrients, such as inorganic nitrogen (see Wilkerson and Trench, 1986), phosphorus (see Yonge, 1936), and inorganic carbon (see Yellowlees et al., 1993) from sea water and host metabolic processes. Thus, this symbiotic relationship confers to giant clams a nutritional advantage over other bivalves in that they are not only heterotrophic bivalves that filter-feed on particulates (Klumpp et al., 1992) and take up dissolved organic molecules from sea water (Fankboner, 1971; Goreau et al., 1973; Southgate, 1988); but, they are also autotrophic bivalves capable of taking up dissolved inorganic molecules from sea water (Yonge, 1936; Wilkerson and Trench, 1986; Yellowlees et al., 1993). Such dual mode of nutrition in giant clams is the key factor in their survival in the nutrient-poor, tropical reef waters of the Indo-Pacific region (Yonge, 1975).

Recently, heavy exploitation and habitat degradation have threatened the status of giant clams throughout most of their geographic range, leading to international efforts on their conservation, management, and mariculture (Copland

and Lucas, 1988). This concern, coupled with the continuing scientific interest in symbiosis as a biological phenomenon, has intensified investigations of the clam-zooxanthellae symbiosis.

1.1 PARTNERS IN SYMBIOSIS

1.1.1 The Invertebrate Host

There are eight extant species of giant clams, six of which belong to the genus *Tridacna* (*Tridacna gigas*, *T. derasa*, *T. tevoroa*, *T. squamosa*, *T. maxima*, and *T. crocea*), and two to the genus *Hippopus* (*Hippopus hippopus* and *H. porcellanus*) (Rosewater, 1965; Rosewater, 1982; Lucas et al., 1991). A ninth species, *T. rosewateri*, is only known from shell type specimens (Sirenko and Scarlato, 1991). *T. gigas* is the largest (Yonge, 1975) and fastest-growing of all bivalves, reaching a size of up to 137 cm (Rosewater, 1965) with a growth rate (exponential growth stage) of up to 1 cm mo^{-1} (Belda, 1989; Gomez and Mingoa, 1993). *T. crocea*, on the other hand, is the smallest, reaching a maximum size of 14 cm (Lucas, in press). Attainment of such large sizes of these bivalves has been attributed to their dual mode of nutrition (Yonge, 1975).

Scientific interest in the nutrition of giant clams originated more than a century ago as a result of their unusual bivalve morphology (Brock, 1888).

Association with zooxanthellae has profoundly modified tridacnid structure in that the siphonal mantle regions, where the zooxanthellae predominate, have been extended over the upper surface for maximum exposure to light (Yonge, 1936). Yonge postulated that such change occurred through a 180° rotation of the viscero-pedal complex in relation to the mantle-shell complex, with a corresponding displacement of the hinge and ligament to a mid-ventral position. Stasek (1962, 1963) disputed Yonge's view, however, as it would suggest that the shell and mantle were independent of the rest of the clam's body components. Instead, he proposed that a morphologically-posterior direction of growth, instead of a ventral direction typical of bivalves, gave rise to the unique tridacnid morphology. Stasek's hypothesis is supported by the juvenile tridacnids' pattern of growth, which proceeds through a series of functional stages (LaBarbera, 1975).

The only other bivalves which have developed symbiosis with zooxanthellae are some cockles, which also belong to the superfamily Cardiacea. There are at least three known species, namely, *Corculum cardissa* and *Fragum fragum*, which have translucent shell regions for exposure of their symbionts to light (see Goreau et al., 1973), and *F. unedo,* where the zooxanthellae are packed within the posterior mantle spreading over the substrate beyond the shell margins (Kawaguti, 1983). Among other molluscs, symbiosis is found in Gastropoda (in nudibranchs) (reviewed in Kempf, 1984).

Apart from Mollusca, symbiotic associations with dinoflagellates are confined to four other phyla, namely, Cnidaria, Porifera, Platyhelminthes, and Protozoa (reviewed in Kokke and Spero, 1987; Hinde, 1988). Most tropical cnidarians are symbiotic with zooxanthellae, symbiosis being universal among hermatypic corals (Goreau et al., 1973; reviewed in Szmant-Froelich and Pilson, 1980). In temperate regions, the association is confined to some sea anemones (reviewed in Fitt et al., 1982), hydroids, and a few species of scleractinian corals (e.g., see Szmant-Froelich and Pilson, 1980). Porifera and Platyhelminthes have only a few known symbiotic associations with zooxanthellae (see Nicol, 1960; Hinde, 1988). In Protozoa, the symbionts are found in foraminiferans (McEnery and Lee, 1981; Jorgensen et al., 1985), radiolarians (reviewed in Rogerson et al., 1989), ciliates, and flagellates (Nicol, 1960).

1.1.2 The Zooxanthella

Among the various algal groups symbiotic with marine invertebrates, the gymnodinioid dinoflagellates of the class Dinophyceae predominate in the marine environment (Taylor, 1973; Hofmann and Kremer, 1981; Trench and Blank, 1987). Association of these dinoflagellates with reef communities has made them predominantly circumtropical in distribution (Muscatine, 1980a).

These unicellular algae are coccoid in shape and yellow-brown in colour (Taylor, 1973). Inside the animal host, they are in a vegetative state, with no

flagella nor a distinct girdle; outside the host, they are typical motile (flagellated) gymnodinioid swarmers (Zahl and McLaughlin, 1957; McLaughlin and Zahl, 1959). Their photosynthetic pigments include chlorophylls a and c, β-carotene, peridinin, neo-peridinin, dinoxanthin, neo-dinoxanthin, diadinoxanthin, and xanthophyll (Jeffrey and Haxo, 1968).

Until recently, *Symbiodinium (=Gymnodinium) microadriaticum* was regarded as a single, pandemic species of symbiotic dinoflagellates. The use of independent biochemical, physiological, morphological, and behavioural studies, however, resulted in a re-evaluation of the taxonomy of these zooxanthellae. Trench and Blank (1987) described four new species, although the authors acknowledged that evidence for sexual recombination remains to be presented before the existence of distinct biological species can be experimentally assessed. Recently, Rowan and Powers (1991a, b) characterised zooxanthellae isolated from taxonomically diverse hosts (corals, anemones, gorgonians, zoanthids, and jellyfishes), based on genetic sequences of the small ribosomal subunit of ribonucleic acid (ssRNA). They found the same alga in individuals of the same host species from single localities, but similar zooxanthellae in different host species, and estimated several distinct taxa distributed among the different hosts, regardless of host taxonomic status.

Overall, there is a consensus of a clear variability within the genus *Symbiodinium*, and recent advances in taxonomic studies show promise in

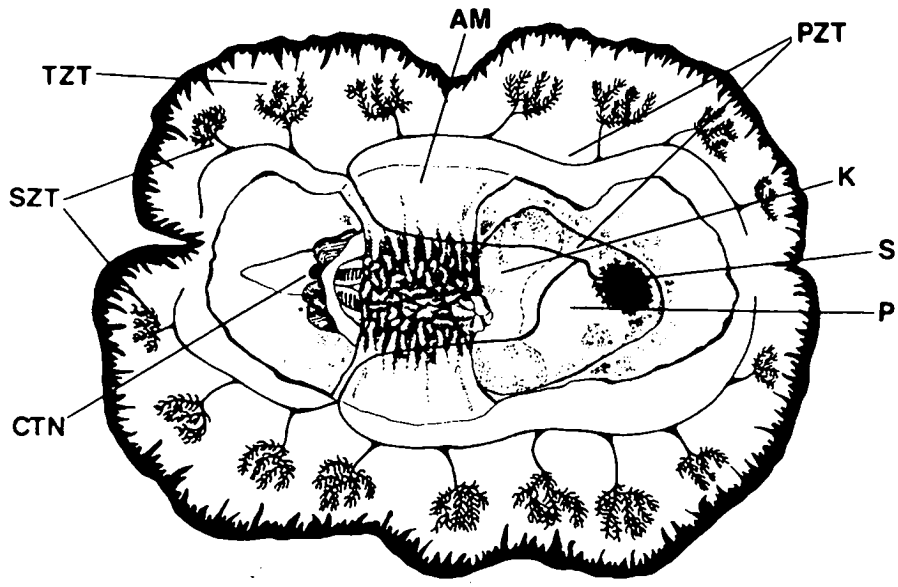
demonstrating the existence of distinct biological species of symbiotic zooxanthellae. Until this task is completed, it is appropriate to refer to symbiotic dinoflagellates as *Symbiodinium* sp, with the exception of those species described by Trench and Blank (1987).

Until recently, the exact location of zooxanthellae in giant clams was controversial. Yonge (1936) initially observed that the zooxanthellae were contained within the haemolymph cells in the haemal spaces of the mantle. Later on, his own electron-microscopy studies showed that the zooxanthellae were not within haemolymph cells (Yonge, 1980), but he nevertheless continued to argue for the presence of zooxanthellae in the haemolymph. This was despite Mansour's (1946) observation, based on serial sections, that the zooxanthellae were contained within tubular structures, which he hypothesised to be connecting the mantle regions with the stomach. Mansour's observation of a complex duct system originating from the stomach was dismissed as extremely atypical among molluscs (Yonge, 1953) and even absurd (Morton, 1978). More recently, however, Norton et al. (1992) and Norton and Jones (1992) confirmed Mansour's findings when they demonstrated from their extensive histological studies on giant clams that the zooxanthellae are mainly in the mantle contained within the blind terminal branches of a diverticulum of the gut. This duct system arises as a single primary tube, extending from a diverticular duct of the stomach. It splits into right and left tubes above the digestive organs, both tubes passing ventrally through the kidney, crossing posteriorly through the connective tissue sheath of

the adductor muscle, and proceeding dorsally into the root of the siphonal mantle. Then each tube splits anteriorly and posteriorly on each side of the mantle into two secondary branches, which further divide into fine tertiary branches with blind ends within the exposed mantle surface (Fig. 1.1).

This confirmation brings the clam-zooxanthellae association in common with many other symbioses where the zooxanthellae are associated with the host's digestive system (Norton et al, 1992). In invertebrate hosts other than the clams and cockles, zooxanthellae are intracellular, residing within vacuoles surrounded by host-produced membranes within endodermal or gastrodermal cells (Trench et al., 1981; Trench and Blank, 1987).

Fig. 1.1. Dorsal diagrammatic view of a giant clam showing the zooxanthellal tube system. AM - adductor muscle; CTN - ctenidia (gill); K - kidney; P - pericardium (heart); PZT - primary zooxanthellal tube; S - stomach; SZT - secondary zooxanthellal tube; TZT - tertiary zooxanthellal tubes. (reproduced with permission from Norton and Jones, 1992)


AM
PZT
TZT
SZT
K
S
P
CTN

1.1.3 Establishment of Symbiosis

Acquisition of dinoflagellate symbionts by most animal hosts is by reinfection of offspring at each successive generation (Trench, 1987). This occurs in two ways. One is through access of the free-swimming algae to the host, possibly through some chemotrophic behaviour (Fitt, 1984). This has been shown in tridacnids (Fitt and Trench, 1981; Fitt et al., 1984), gorgonians, jellyfish (see Trench, 1987), nudibranchs (Hoegh-Guldberg and Hinde, 1986; Kempf, 1984), and cockles (see Fitt and Trench, 1981). Another is through ingestion of intermediate hosts, such as protozoans, plankton, and planulae harbouring algal symbionts, as has been demonstrated for *Aiptasia* sp. (Taylor, 1973). A less common mode of symbiont acquisition may be through maternal or cytoplasmic inheritance, where the algae are either partitioned between daughter cells or polyps during fission or budding (Trench, 1987), or transmitted directly via the egg or indirectly prior to the release of the offspring (Taylor, 1973). This has been demonstrated for many corals (reviewed in Taylor, 1973), alcyonaceans (Benayahu et al., 1988), and hydroids (reviewed in Trench, 1987).

In larval tridacnids, ingested zooxanthellae remain in the stomach for over a week (Fitt et al., 1986), and after metamorphosis, eventually migrate to microscopic channels within the developing mantle, via a duct (Fitt and Trench, 1981), which is the beginning of the zooxanthellal tube system described by Norton et al. (1992) and Norton and Jones (1992). Similarly, in coelenterates,

Symbiodinium sp. is phagocytosed by endodermal cells (Fitt and Trench, 1983), which eventually migrate into the mesoglea to become the "amoebocytes" where algal proliferation occurs (Colley and Trench, 1985). In both cases, the algae resist digestion by their hosts (Fitt and Trench, 1981; Fitt and Trench, 1983).

1.2 NUTRITION OF THE INVERTEBRATE HOST

1.2.1 Autotrophic Sources of Nutrition

Evidence for translocation of photosynthate from the zooxanthellae to the host comes from a wide range of studies employing radioisotopes. Use of this technique is based on the assumption that the zooxanthellae exclusively fix inorganic carbon into organic carbon and translocate some of it to the animal host. In *in vitro* studies, isolated zooxanthellae are incubated with radioactively-labelled bicarbonate and the incubation medium analysed for labelled products released by the algae. In *in vivo* studies, the whole organism is incubated with labelled bicarbonate and subsequently separated into its plant and animal tissue constituents. Examination of the extent of translocation is based on ^{14}C levels detected in the animal-tissue fraction (Muscatine, 1980b; Trench, 1987).

Short-term experiments demonstrated that 20-95% of the total fixed ^{14}C is released *in vitro* and *in vivo* by zooxanthellae from hosts ranging from protozoa to

molluscs (Muscatine, 1967; Muscatine and Cernichiari, 1969; Schmitz and Kremer, 1977; Trench, 1971a; Steen and Muscatine, 1984; Hoegh-Guldberg and Hinde, 1986; Drits et al., 1987; Griffiths and Streamer, 1988). Evidence has been presented that the most commonly translocated photosynthetic products are glycerol, lipids, glucose, and alanine, with some leucine, glutamine, glutamate, and organic acids. Presence of host tissues or some "host factor" is believed to augment the release of photosynthate *in vitro*, but the nature of such augmentation is unknown (Muscatine and Cernichiari, 1969; Trench, 1971a, b, c; Patton and Burris, 1983; Gladfelter, 1985).

More recent studies have provided evidence for qualitative and quantitative translocation differences among various hosts. For the giant clam *Tridacna gigas*, Griffiths and Streamer (1988) showed that glucose is the major photosynthate translocated to the host *in vivo*, and not glycerol, as previously shown by Muscatine (1967) *in vitro*. Griffiths and Streamer found that glycerol only occurs in significant quantities *in vitro* in the presence of host tissue extracts. That glucose is the major photosynthate released by the algae to their clam host is supported by the much higher glucose concentrations (up to 620 μM) in the haemolymph of *T. gigas*, varying diurnally with day/night light regimes the way photosynthesis does, whereas glycerol concentrations were consistently low (< 4 μM) (Rees et al, 1993a).

Photosynthesis: respiration ratio (P:R), while useful in investigating community metabolism, has limited applications in symbiotic associations because it yields limited information about the nutritional contribution of the zooxanthellae to the host (Gladfelter, 1985). For this reason, Muscatine et al. (1981) developed the concept of CZAR (contribution of zooxanthellae to animal respiration), which quantitatively assesses the portion of the host's daily metabolic energy demand that could be satisfied by algal production. Reported CZAR values range from over 92-100% for tridacnids (Fisher et al., 1985; Fitt et al., 1986; Mingoa, 1988; Klumpp et al., 1992), 9-158% for corals under various conditions of illumination or depth (Muscatine et al., 1981; McCloskey and Muscatine, 1984 in Gladfelter, 1985; Muscatine et al., 1984), 13-79% for sea anemones depending on the organism's nutritional history or intertidal position in the field (Fitt et al., 1982; Shick and Dykens, 1984; Zamer and Shick, 1987), and 13-48% for zoanthids (Steen and Muscatine, 1984).

Apart from respiration, translocated carbon may be used for other purposes. It may serve as a lipid reserve and reproductive energy source, and it may also be used for synthesis of new tissues, organic skeletal matrix, and mucus (Gladfelter, 1985; Rinkevich, 1989).

1.2.2 Heterotrophic Sources of Nutrition

The exogenous sources of nutrition for symbiotic hosts are dissolved organic compounds and particulate food. In the aquatic environment, dissolved organic compounds include amino acids, carbohydrates, and vitamins; while particulate food consists of phytoplankton, zooplankton, bacteria, and animal detrital material (Taylor, 1973).

Tridacnid mantle, mantle cavity, and gills constitute a relatively large and potentially permeable surface area for uptake and loss of dissolved organic matter (Lucas, in press). For instance, evidence has been presented for pinocytosis of amino acids, possibly through the micropinocytic channels of the microvillous border of the clams' siphonal mantle epidermis (Fankboner, 1971). Similarly, Goreau et al. (1973) showed that [^{3}H]-leucine in ambient sea water was rapidly taken up by *T. maxima* into its mantle and ctenidial (gill) epithelia. Such uptake of dissolved organic matter has been suggested to be of most nutritional benefit to tridacnids during their larval and early juvenile stages, when they have the highest ratio of surface area to body mass (Lucas, in press). In fact, other bivalve larvae are known to take up dissolved organic matter from sea water (Manahan, 1983).

Other invertebrate hosts, such as corals, have been shown under laboratory conditions to accumulate amino acids and [^{14}C] glucose from modest concentrations in seawater (Stephen, 1962 in Fankboner, 1976). Such

heterotrophic transfer of dissolved substances may also occur *in situ*, as indicated by the transfer of kelp-exudated dissolved carbon to a coral (Fankboner, 1976). Similarly, zoanthids can absorb amino acids and sugars from very low concentrations in seawater, and it has been suggested that these substances may be incorporated into the hosts' reproductive tissue (Trench, 1974).

For a majority of symbiotic hosts, ingestion of particulate food such as phytoplankton, zooplankton, bacteria, and animal detrital material may constitute a significant source of essential metabolites (Goreau et al., 1973; Taylor, 1973). It has been suggested that phytoplankton is a major source of particulate food for tridacnids (Goreau et al., 1973), although this is questionable considering the low phytoplankton levels in reef waters. They are also capable of taking zooplankton into their stomach, where "rapid disintegration and probably digestion" were observed by Mansour (1945), in contrast to Ricard and Salvat (1977) who noted that *T. maxima* filter, but do not assimilate, phytoplankton and zooplankton. Fitt et al. (1986), however, demonstrated that *T. gigas* ingested and digested [^{14}C]-labelled *Isochrysis galbana* in sea water. Furthermore, unicellular algae have been used successfully in culture of clam larvae (Braley, 1990).

More recently, *T. gigas* has been shown to efficiently utilise particulate organic matter, mostly in the form of detritus, available in reef waters (Klumpp et al., 1992). The clams filtered three quarters of 2-50 um particles passing through their mantle cavity, and absorbed at least half of the particulates' carbon content.

Such particulate food provides from a quarter to over half of the carbon used for growth and respiration in moderate- (ca. 190 mm shell length) and small-sized clams (ca. 35 mm), respectively, with the nutritional benefit obtained declining with clam size (Klumpp et al., 1992).

In the case of other symbioses, corals and zoanthids are capable of ingesting and assimilating animal detrital material and bacteria, but they feed primarily on zooplankton (reviewed in Muscatine, 1973; Johannes, 1974). For foraminiferans, zooplankton appears to be the main food source (Be et al., 1981 in Jorgensen et al., 1985). Similarly, it has been suggested that symbiosis in poriferans is probably sustained to a large extent by host filter-feeding (see Taylor, 1973); while in anemones, host phagotrophy is evidently indispensable for the total growth of the association (see Muscatine, 1973).

As regards the possible digestion of the zooxanthellae by the host, Yonge (1936) claimed that zooxanthellae are "farmed" by giant clams in their mantle, conveyed by amoebocytes via blood vessels, and digested *en route* to or in the digestive gland's interdiverticular spaces. This has been invalidated by the recent confirmation of Mansour's (1945) zooxanthellal tube system (Norton et al., 1992; Norton and Jones, 1992). Similarly, Fankboner (1971) interpreted the "culling" of senescent and atypical zooxanthellae by amoebocytes of the digestive gland as indicative of lysosome-derived hydrolytic activity. This histochemical demonstration of acid phosphatase activity, near or in the algae, is also rendered

ambiguous by the presence of intrinsic acid phosphatases in the algae themselves (see Trench, 1987; Jackson et al., 1989) and possible algal autolysis (Trench, 1974). Muscatine and Greene (1973) and Muscatine (1973) pointed out that there was no direct evidence for such hydrolysis of algal substrates, nor had there been any demonstration of host assimilation of the digestive products (Muscatine, 1973). More importantly, however, long before Norton and co-workers' significant histological findings, Mansour (1945) argued that the algae are not contained within amoebocytes and are defaecated undigested. Indeed, Fitt et al. (1986) showed that *T. gigas* juveniles released up to 80% of [^{14}C]-labelled freshly-isolated zooxanthellae within 72 h following feeding. The persistent release of dark-brown faeces, packed with viable zooxanthellae, was documented by Ricard and Salvat (1977), Trench et al. (1981), and Lucas (in press). Lucas (in press) suggested that the zooxanthellae travel down the zooxanthellal tube system into the alimentary canal and are expelled undigested. Also, the epithelial cells lining the primary and secondary sections of the tube system are equipped with long cilia (Norton and Jones, 1992). This suggests that the clam host drives the zooxanthellae out of the tubular system, as one way of controlling their population size.

A 'medium' sized *T. maxima* was estimated to annually release 17-26 g of protein as zooxanthellae in its faeces (Ricard and Salvat, 1977). Lucas (in press) pointed out that such non-digestion of zooxanthellae is a surprising 'waste' of potential food. Algal densities are approximately 10^6 cells cm^{-2} colony surface in

corals, 10^9 cells g^{-1} tissue in sea anemones, and 2 x 10^8 cells g^{-1} mantle tissue in giant clams, comprising 3 to 14% of these associations' protein biomass (see Muscatine, 1980a). While digestion of the zooxanthellae constitutes a potential source of food, available evidence suggests that it is a minor source of nutrition to the clam host, if at all.

1.2.3 Autotrophy Versus Heterotrophy

The nutritional benefits derived by the host from autotrophy, which are much easier to quantify even in field situations, have received more attention than the host's gains from exogenous sources. Most investigators have assessed the zooxanthellae's nutritional contribution and considered the rest of the host's nutritional needs as satisfied by heterotrophy.

The contribution of the zooxanthellae to the nutrition of their symbiotic hosts cannot be over-emphasised. However, while there is substantial evidence that energy-rich compounds derived from zooxanthellae contribute significantly to the metabolic energy demands of symbiotic hosts, the adequacy of symbiont production to fully meet the metabolic energy requirements in many associations, particularly under suboptimal conditions, has not been demonstrated (see CZAR values above). More importantly, there is no evidence for the ability of the symbionts to supply all the nutrients necessary to support host tissue synthesis. While carbon production by the zooxanthellae may meet the clam host's

requirements for respiration and structural carbon, heterotrophic sources of nutrition would certainly play a major supplementing role.

Yonge (1975) and Fankboner and Reid (1990) drew attention to the intact and functional filter-feeding and digestive apparatuses of giant clams, being comparable to those of heterotrophic bivalves. Fankboner and Reid (1990) pointed out the potential nutritional benefit that can be derived by clams from filtering large volumes of reef waters which contain dissolved organic carbon, particulates (zooplankton, little phytoplankton, and detritus), and mucus flocs. Indeed Klumpp et al. (1992) demonstrated the considerable nutritional benefit derived by *T. gigas* from its highly-efficient filtering activity, although these workers dealt mainly with carbon.

While inorganic nutrients, such as nitrogen and phosphorus, are typically present at very low concentrations in reef waters (Crossland, 1983; Kinsey, 1991), symbiotic associations may take advantage of occasional large inputs from natural sources such as oceanic upwellings and intrusions. The suggestion that the tridacnids may be capable of storing phosphorus in phosphorite concretions in the kidney (Trench et al., 1981), for instance, deserves exploration. Such potential capacity to store nutrients, in addition to nutrient recycling and conservation, would contribute to the tridacnids' success in a nutrient-poor environment.

1.3 NUTRITION OF THE ZOOXANTHELLAE

1.3.1 Nutrient Requirements

Growth studies on cultured zooxanthellae have shown that photosynthesis can provide their total energy requirements (reviewed in Taylor, 1973). In addition, the zooxanthellae obtain inorganic and organic substances from their hosts and the seawater.

Host-derived substances useful to the zooxanthellae include end-products of metabolism and directed fluxes of specific metabolites from the host. In tridacnids and coelenterates, studies suggest that the zooxanthellae may receive nitrogen and phosphorus from their hosts' metabolic waste products (Yonge, 1936; D'Elia, 1977; Cates and McLaughlin, 1979; Wilkerson and Trench, 1986). Ammonium ions may be derived by zooxanthellae from host metabolic sources such as deamination, pyrimidine breakdown, and adenosine monophosphate (AMP) deaminase activity (McGilvery, 1983). As regards phosphate, Taylor (1973) suggested that glycero-phosphoric, cytidylic, adenylic, and guanylic acids may serve as phosphorus sources, just as urea, uric acid, guanine, adenine, and several amino acids can serve as nitrogen sources. *In vitro* studies on nutrient uptake by cultured zooxanthellae suggest that zooxanthellae are capable of uptake and utilisation of organic compounds potentially present in the host (reviewed in Steen, 1986). There is some evidence for an active transport mechanism for

uptake of cysteine, methionine, and taurine in cultured zooxanthellae from the giant clam *T. maxima*, and of alanine in zooxanthellae isolated from the jellyfish *Cassiopeia xamachana* (reviewed in Steen, 1986). Coelenterate zooxanthellae, which are incapable of synthesising their own glycine from CO_2 or serine (von Holt, 1968), have been reported to show preferential uptake and assimilation of host glycine. Also, the "back transport" technique, where labelled food substrates are introduced to the hosts, followed by assay of the algae, has shown that zooxanthellae in anemones can assimilate ^{35}S-amino acids from their hosts (Carroll and Blanquet, 1984; Steen, 1986).

The preceding studies, however, mostly dealt with determining the capacity of zooxanthellae for uptake of substances potentially present in the host by: (1) monitoring host excretion of inorganic nutrients, (2) measurement of uptake by zooxanthellae in culture, or (3) measurement of uptake by zooxanthellae isolated from the host. Detailed analysis of actual metabolite movement from host to algae, such as the "back transport" study of Carroll and Blanquet (1984), is wanting. Available evidence for host-derived nutrition of the zooxanthellae pales in comparison with that for zooxanthellae-derived nutrition of the host. It is important to clarify and quantify this less-studied aspect of symbiosis nutrition to further understand nutrient recycling and conservation, as well as the relative contribution of exogenous sources of nutrition to the symbiotic association.

Nutrients from seawater, such as dissolved inorganic and organic compounds, may be taken up by the zooxanthellae via the host (see Cates and McLaughlin, 1979; Hinde, 1988) through diffusion or active transport (see Taylor, 1973; Wilkerson and Trench, 1986). Of the mineral nutrients available, nitrogen and phosphorus have received the most attention because of their possible limiting effects on zooxanthellae growth. While these nutrients may be acquired through the host, the ultimate source is the sea water.

Crossland (1983) presented an extensive tabulation of nutrient concentrations in coral-reef waters prior to 1980, showing wide ranges of values around different island types and locations (e.g., oceanic reefs, high islands). Dissolved organic nutrient concentrations ranged from 0.1 to 7.5 μM for nitrogen, and 0.02 to 5 μM for phosphorus. Nitrate concentrations were from 0.01 to 5 μM; nitrite from undetectable values to 5 μM; ammonia from 0.07 to 11 μM; and phosphate from 0.01 to 5.5 μM. Nevertheless, typical values for these dissolved inorganic nutrients were < 1 μM. In areas of known chronic reef pollution (e.g., Kaneohe Bay), typical ranges for inorganic nitrogen were 2 to 4 μM, and 0.3 to 1.2 μM for inorganic phosphorus (see Kinsey, 1985).

1.3.2 Nutrient Acquisition Through The Host

The zooxanthellae are separated from the clam's haemolymph by a thin layer of epithelial cells, which comprise the tertiary tube branches in the mantle (Norton et al., 1992; Norton and Jones, 1992). These epithelial cells are likely to be similar to those lining the digestive tract of most organisms, in that they are permeable to nutrients (Yellowlees, pers. comm.). Exchange of nutrients or metabolites can, therefore, occur between the zooxanthellae and the haemolymph across the zooxanthellal tube (Rees et al., 1993b). As discussed previously, nutrients may be taken up by the intact association from sea water through the gills and other body surfaces (e.g., mantle) via diffusion or active transport. These nutrients are likely made available to the zooxanthellae through the haemolymph.

Active transport is generally held responsible for nutrient uptake by zooxanthellae (reviewed in Muscatine, 1980b). Giant clams, other corals, and some anemones, however, do not show clear-cut saturation kinetics for nitrogen uptake (see Wilkerson and Trench, 1986). One explanation for this is that net uptake has both diffusive and active components, with the diffusive component of uptake through the animal tissue masking any active uptake at the site of the algae (see Wilkerson and Trench, 1986).

D'Elia et al., (1983) proposed the "depletion-diffusion" model as a mechanism of nutrient uptake in symbiosis. That is, zooxanthellae deplete the

animal tissue of inorganic nutrients, creating a concentration gradient through which nutrients from seawater passively diffuse into the animal tissue. D'Elia and Cook (1988) further extended this hypothesis to include regulation of the host's cytoplasmic nutrient concentration by the nutritional status of the symbiotic partners. That is, when algal uptake exceeds host nutrient regeneration, host cytoplasmic concentration must decrease and *vice versa.*

Evidence presented in favour of this hypothesis includes localisation in zooxanthellae of assimilatory enzymes for inorganic nitrogen, and similarity of nutrient uptake kinetics between freshly isolated zooxanthellae and intact symbioses (D'Elia. et al., 1983). Other observations which implied the role of the zooxanthellae in the uptake of inorganic nitrogen are: ammonium-uptake by symbiotic hosts and ammonia-excretion by aposymbiotic and non-symbiotic hosts (e.g., Muscatine and D'Elia, 1978; Muscatine and Marian, 1982); reduction of ammonia release by the host in the presence of zooxanthellae (reviewed in Muscatine, 1980b); and ammonium-uptake by freshly-isolated zooxanthellae (e.g., Wilkerson and Muscatine, 1984).

This depletion-diffusion hypothesis, however, was developed to account for nutrient uptake by intracellular zooxanthellae. In this model, D'Elia and Cook (1988) emphasised that uptake kinetics of symbioses may be complicated by the spatial and morphological relationships of the symbiotic partners. As discussed previously, tridacnid zooxanthellae are intercellular, being separated from the

haemolymph by a tube system. Thus, any hypothesis explaining the acquisition of nutrients by zooxanthellae in the clam has to account for the morphological relationship between the symbiotic partners. That is, nutrients taken up from sea water will have to go across the gill or mantle membranes, into the haemolymph, across the zooxanthellal tube cells, and through the zooxanthellae membranes via diffusion or active transport, whichever is applicable.

Recently, Miller and Yellowlees (1989) suggested that the depletion-diffusion model is adequate to describe uptake of ammonia (i.e., uncharged species NH_3), which can readily diffuse across membranes, but not phosphate and nitrate, which are always present as charged species. Additionally, intracellular phosphate concentrations are always high relative to those in sea water (McGilvery, 1983). Thus, they proposed that uptake of these charged nutrients from sea water and across the perialgal membrane (in the case of intracellular zooxanthellae) must be carrier-mediated (i.e., active transport or facilitated diffusion via a non-specific carrier)

The predominant species of ammonia (NH_3), ammonium (NH_4^+), nitrate, and phosphate within the physiological pH range have been tabulated by Miller and Yellowlees (1989). At pH 7.4, NH_3 constitutes only about 1% of the total NH_3-NH_4^+ species; at pH 8.2, its concentration increases to 8%. In contrast to ammonia, nitrate and phosphate, are always charged regardless of pH, the predominant species being NO_3^-, HPO_4^{2-}, and $H_2PO_4^-$ (Miller and Yellowlees,

1989). Thus, uptake of these nutrients against a concentration gradient would only be via active transport across host membranes (Miller and Yellowlees, 1989).

In *T. gigas*, haemolymph pH varied on a diel cycle from 7.4 to 8.1, with highest values at noon and lowest at night, reflecting ambient light levels and zooxanthellae photosynthetic activity (Fitt et al., in prep.). Similarly, the pH of the culture medium surrounding zooxanthellae isolated from *T. crocea* increased during photosynthesis (D'Elia et al., 1983). The increase in pH is understood to be brought about by the zooxanthellae's removal of CO_2 from the haemolymph (or culture medium) during photosynthesis (i.e., lower $[H^+]$). Natural ammonium levels in the haemolymph vary inversely with haemolymph pH, with greatest ammonium concentration found at night when haemolymph pH was lowest (Fitt et al., in prep.). Such increase in ammonium concentration at lower pH is understood to be brought about the the availability of more H^+ for protonation of ammonia (into ammonium) present in the haemolymph. It has also been observed that in clams exposed to increased ammonia in sea water, ammonium accumulated faster in the haemolymph at a lower than a higher haemolymph pH, suggesting that ammonia diffuses from sea water (pH 8.1) down a concentration gradient and accumulates in the haemolymph at night (Fitt et. al., in prep.). On the other hand, ammonium concentration in the haemolymph was lower at noon when pH in the haemolymph was similar to that of sea water.

These observations support the depletion-diffusion hypothesis in clams. Ammonia, being an uncharged molecule, diffuses across the animal membrane (gill, mantle, or zooxanthellal tube system) depending on the pH gradient, which in turn determines this nutrient's concentration gradient across the animal membrane. On the other hand, ammonium, being a charged molecule, cannot diffuse back from the haemolymph into the sea water (or from the tube system back into the haemolymph), and instead accumulates inside the host for active uptake by the zooxanthellae and/or the host (see following discussion of host ammonium-assimilation enzymes).

There are two basic requirements that would enable the zooxanthellae to induce a net uptake of ammonium by the intact symbiosis, as proposed by the depletion-diffusion of D'Elia and co-workers. These are: (1) a system for active uptake, and (2) an ability to assimilate ammonium (Miller and Yellowlees, 1989). Indeed, cultured and freshly-isolated zooxanthellae from corals take up ammonium from sea water (e.g., Gunnersen et al., 1988). Also, assimilation enzymes (glutamine synthetase, GS; glutamine dehydrogenase, GDH) are present in the zooxanthellae of corals (Summons and Osmond, 1981; Gunnersen et al., 1988; Dudler and Miller, 1988) and tridacnids (Rees et al., in press).

However, recent studies also showed very high levels of ammonium assimilation enzymes in tridacnid hosts (GS) (Rees et al, in press) and some corals hosts (NADPH-GDH: Catmull et al., 1987; GS: Yellowlees et al., in press).

Miller and Yellowlees (1989) and Rees et al. (in press) pointed out that available enzyme-activity data imply the involvement of symbiotic host tissue in ammonium assimilation, as has previously been suggested for the *Hydra-Chlorella* symbioses (Rees, 1987). It has also been suggested that ammonium uptake and retention by zooxanthellae may only be indirectly dependent on the zooxanthellae. Instead, the host may assimilate ammonium, using translocated carbon from its zooxanthellae as amino-group acceptors (Miller and Yellowlees, 1989). Miller and Yellowlees (1989) pointed out that this host-mediated assimilation is compatible with available evidence for dependence of nutrient uptake by intact associations on light and presence of zooxanthellae. It is evident that both partners in the symbiosis have the capacity to both acquire and assimilate ammonium.

Light stimulation of uptake and retention of ammonium and nitrate has been demonstrated for tridacnids (Wilkerson and Trench, 1986), reef corals (Muscatine and D'Elia, 1978), anemones (Wilkerson and Muscatine, 1984), and cultured zooxanthellae (Domotor and D'Elia, 1984). This light stimulation of nutrient uptake is believed to occur through photoreduction, supply of energy for uptake via photophosphorylation, or photosynthetic supply of carbon skeletons as nitrogen acceptors (see Muscatine, 1980b). Ammonium-assimilation rate by *T. gigas* decreased with time in continuous darkness, with net ammonia release occurring after several days (Wilkerson and Trench, 1986). Such release of ammonia under prolonged incubation in darkness has also been shown for symbiotic corals and anemones, suggesting that ammonium assimilation by the

zooxanthellae is prevented due to depletion of zooxanthellae carbon reserves (Muscatine and D'Elia, 1978; Wilkerson and Muscatine, 1984). Similarly, inorganic phosphorus is taken up in the light only by symbiotic corals, while uptake may diminish in the dark (D'Elia, 1977).

1.3.3 Nutrient Limitation

It has generally been assumed that the host cell, where most zooxanthellae live, is a nutrient-rich environment (see Taylor, 1973). Recently, however, some investigators suggested that zooxanthellae, including those located intercellularly, may be nutrient-limited.

Evidence consistent with such a view includes: a) high zooxanthellae densities inside the host, suggesting a great nutrient demand that may deplete intracellular concentrations for further algal growth; b) intact associations taking up dissolved inorganic nutrients from very low concentrations in seawater, and uptake kinetics implying low nutrient concentrations within host tissues; and c) nitrate uptake, a characteristic of nitrogen-starved algae, (Cook and D'Elia, 1987), since nitrate must first be reduced to ammonium before it can be assimilated, making ammonium the preferred species for assimilation.

Of the major nutrients, nitrogen is considered by many investigators as the most limiting. The possibility that the clam zooxanthellae may be isolated from

host ammonium sources is suggested by *T. gigas* being capable of taking up nitrate (Wilkerson and Trench, 1986; Fitt et al., in prep.), and that ammonium was undetectable in the haemolymph (Deane and O'Brien, 1980). Furthermore, zooxanthellae freshly-isolated from *T. gigas* took up nitrate from haemolymph only when ammonium concentration was low enough not to inhibit nitrate uptake (Fitt et al, in prep.). Addition of ammonium promoted photosynthesis by zooxanthellae in clam tissue slices (Summons et al., 1986), and exposure of clams to increased ammonia or nitrate in sea water increased their shell-extension rates and tissue weights (Braley, 1992; Hastie et al, 1992; Fitt et al., 1993). Such increased growth of the clam host has generally been assumed to be a direct consequence of increased translocation of photosynthate from the zooxanthellae. Furthermore, surge uptake for ammonium and nitrate have been demonstrated for tridacnids, with nitrate uptake being saturated at 8-10 μM (Wilkerson and Trench, 1986).

For cnidarians such as corals and anemones, ammonium concentrations in tissue homogenates have been reported to be about 5-50 μM and 40 μM (Crossland and Barnes, 1977; Wilkerson and Muscatine, 1984). However, Wilkerson and Muscatine, (1984) pointed out that disruption of animal tissue generates ammonium from deamination reactions when cellular structure is destroyed. Furthermore, homogenisation evenly distributes tissue metabolites, thus precluding detection of possible small-scale local depletion of ammonium existing near endosymbionts (see D'Elia and Cook, 1988).

Also, nitrogen limitation occurs at high zooxanthellae densities, as indicated by further increase in population density, chlorophyll *a* content, and maximum photosynthetic rate in coral colonies incubated in ammonium-enriched seawater (Hoegh-Guldberg and Smith, 1989). It has also been suggested that intracellular ammonium concentration is very low and dependent on the feeding history of the host, as corroborated by the ammonium uptake rate by zooxanthellae being greatest in starved anemones, and internal levels of ammonium in well-fed hosts being elevated enough to competitively inhibit uptake of the ammonium analogue, methylammonium (D'Elia and Cook, 1988).

While these studies suggest nitrogen limitation in zooxanthellae, other studies presented contradictory evidence. For instance, the ammonium-uptake activity of zooxanthellae isolated from corals was lower than that of nitrogen-starved cultured zooxanthellae (Gunnersen et al., 1989). The same isolated zooxanthellae had very low levels of ammonium-assimilation enzymes. Furthermore, cnidarian zooxanthellae have a general carbon to nitrogen ratio of 6, which is typical of algae grown in nitrogen-sufficient conditions (D'Elia et al., 1983).

In contrast to nitrogen, phosphorus nutrition in symbiosis has received little attention. While it has been demonstrated that the giant clam *T. crocea* took up more phosphate than the non-symbiotic clam, *Spondylus* (Yonge, 1936); and that cultured zooxanthellae from tridacnids took up phosphate from the medium (see

Jackson et al., 1989), the possibility of phosphorus limitation in clam zooxanthellae has not been investigated.

Recently, Miller and Yellowlees (1989) pointed out that available evidence favours phosphate limitation over nitrogen limitation as a means of host control over algal growth. Unlike ammonium, phosphate is always present as a charged species ($H_2PO_4^-$ and HPO_4^{2-}) in the normal cytoplasmic pH range, making it simpler for the host to regulate phosphate availability to the algae through a carrier system. Furthermore, by regulating the activity of this carrier system, the animal host can sustain its own normally high (millimolar) cytoplasmic concentrations of phosphate (Miller and Yellowlees, 1989).

Relevant data are provided by Jackson et al., (1989), showing very high phosphate uptake activities of freshly isolated zooxanthellae from the coral *Acropora formosa* and suggesting that the bulk of phosphatase activity associated with zooxanthellae reflects algal response to phosphate limitation (cf. Fankboner, 1971; Fitt and Trench, 1983). They observed that phosphatase activity, which makes inorganic phosphate available to the zooxanthellae, was always high in freshly isolated zooxanthellae, but repressed when incubated in a medium containing 2 m*M* phosphate (similar to host cytoplasmic phosphate concentration). This implies that the perialgal phosphate concentration experienced by the zooxanthellae *in vivo* may be below that of the host cytoplasm.

To date, there is a general disagreement as to which nutrient is actually limiting the growth of zooxanthellae. Externally-supplied inorganic nitrogen enhances densities of coral zooxanthellae *in vivo* (Muscatine et al., 1989; Hoegh-Guldberg and Smith, 1989), while inorganic phosphorus does not (Muscatine et al., 1989). On the other hand, carbon:nitrogen ratios of zooxanthellae are comparable to those of algae grown in nutrient-sufficient conditions (D'Elia et al., 1983), while phosphate-uptake and phosphatase activities of zooxanthellae freshly isolated from corals suggest phosphorus limitation (Jackson et al., 1989). As has been pointed out by Miller and Yellowlees (1989) and others for other algae and plankton, however, it is possible that limitation by a single nutrient may be an over-simplification. Instead, they suggested a possible oscillation between nitrogen and phosphorus limitation as a consequence of host nutritional status and light availability, or concurrent limitation by more than one nutrient. Recently, Rees et al. (in press) suggested that both the clam host and its zooxanthellae are nitrogen-deficient, and that the glutamine-synthetase activity by the host has a major role in assimilating available ammonia from sea water. Clearly, further research is needed before this major issue in marine symbiosis can be resolved.

1.3.4 Nutrient Recycling

The question of whether symbiotic associations are nutrient-limited seems to be confounded by the apparent disparity between the high rates of production in coral reef communities and the low concentrations of nutrients in the surrounding

waters. Such seeming incongruity lends much appeal to the widely accepted notion of tight nutrient recycling within symbiotic associations.

That nitrogen is recycled in tridacnids is consistent with available evidence. The zooxanthellae release some amino acids to their animal host, as has been shown *in vivo* for *T. gigas* (e.g., Griffiths and Streamer, 1988). Oxidative and biosynthetic metabolism by the animal host, in turn, produces ammonia, which may be taken up by the zooxanthellae for incorporation into amino acids. Indeed, tridacnids and some cnidarians do not release, or have reduced excretion of, ammonia in the presence of their zooxanthellae (see Muscatine, 1980b; Wilkerson and Trench, 1986), except under prolonged incubation in the dark (Muscatine and D'Elia, 1978; Wilkerson and Muscatine, 1984); while aposymbiotic and non-symbiotic cnidarians release ammonia in significant amounts (Muscatine and D'Elia, 1978; Muscatine and Marian, 1982). These results have been interpreted to suggest that the zooxanthellae retain the latter's metabolic waste product (i.e., ammonia) that might otherwise be excreted.

Phosphorus recycling within the intact association is not clear. Of interest, however, is the presence of numerous phosphorite concretions in the tridacnid kidney. Trench et al. (1981) pointed out that such phosphorus pools seem incongruous with the clams' existence in a phosphate-depleted environment, unless these concretions serve as a reservoir of phosphorus (Trench et al., 1981).

Although nutrient recycling is plausible, more detailed studies are needed to demonstrate the actual fate of these nutrients within the intact association. In particular, considering that the photosynthate translocated by the zooxanthellae to their animal host is largely non-nitrogenous (e.g., Griffiths and Streamer, 1988), it would appear that only a small amount of nitrogen is recycled between the symbiotic partners. Also, the observation of significant amounts of nitrogen-assimilation enzymes (glutamine synthetase) in tridacnids (Rees et al, 1989; Rees et al., in press) and some cnidarians (Catmull et al., 1987; Yellowlees et al., in press) would indicate that retention of ammonia, as host waste product, may not only be attributable to the zooxanthellae, but also to the host (see Miller and Yellowlees, 1989).

While significant nutrient recycling may occur, recycled nutrients are inadequate for the total growth of the association. As mentioned earlier, amino acids translocated by the algae to the host seem to be but a minor fraction of the largely non-nitrogenous photosynthate.

For tridacnids and some corals, algal supply of reduced carbon, which may be more than enough to meet the hosts' metabolic energy demands (see CZAR values in earlier sections), is of possible additional benefit in terms of conserving essential amino acids from respiration. Nevertheless, heterotrophic contribution to the nutrition of symbiotic organisms must play an important role.

Marine symbioses, unlike their non-symbiotic counterparts, are capable of occupying different trophic levels within their ecosystems, e.g. as primary producers, primary consumers, or secondary consumers, enabling them to more conveniently avail themselves of autotrophic and various heterotrophic nutritional sources at all times. It is, thus, likely that such trophic adaptability, coupled with nutrient recycling and conservation, is behind their success in tolerating relatively low nutrient concentrations in their environment.

1.4 EFFECTS OF P ON CALCIFICATION

While zooxanthellae in symbiotic associations benefit from increased availability of dissolved inorganic nutrients from sea water, several studies have suggested that elevated levels of these nutrients may be harmful to the calcification process of the animal host. In particular, increased phosphorus may markedly suppress deposition of calcium carbonate in corals.

Kinsey and Domm (1974) carried out an eight-month long, discontinuous fertilisation (i.e., for 3 h during low tide each day) of a patch reef at One Tree Island, Great Barrier Reef, Australia, with 2.0 μM phosphate and 20 μM urea plus ammonium. They found a 50% increase in the rate of net community photosynthesis, and attributed such effect to increased production by benthic algae, possibly including the zooxanthellae. However, the calcification data from the

same experiment, analysed based on an alkalinity anomaly approach (Kinsey and Davies, 1979), showed > 50% suppression of net calcification of corals during the fertilisation period. Kinsey and Davies (1979) attributed this effect primarily to phosphate, which is in accordance with laboratory studies suggesting that phosphates may act as crystal poisons of calcification (Simkiss, 1964). There was no available experimental evidence for suppression (or otherwise) of calcification by increased nitrogen. Similarly, recent work by Rasmussen (1989) showed rapid skeletal extension of branches of *Acropora formosa* coral colonies exposed to up to 4 μM phosphate (superphosphate) in outdoor tanks, but this extension was accompanied by considerable thinning of skeletal diameter. Also, scanning-electron micrography indicated significant alteration of the internal morphological structure of the corals exposed to increased phosphate concentrations.

To date, no similar investigations on tridacnids have been carried out by other workers. Giant clams, like hermatypic corals, are among the coral reefs' calcifying constituents, which live in symbiosis with zooxanthellae. Potential effects of elevated nutrients on these different symbiotic associations may be comparable, and together, may have significant implications for calcification in reef waters receiving enhanced inputs of nutrients from anthropogenic sources.

1.5 ASSESSMENT OF STATUS OF RESEARCH

Some of the major issues in tridacnid nutritional physiology have now been satisfactorily resolved. These questions, which had prevailed over the decades, include: (1) the location of zooxanthellae in the clams and (2) whether clams derived a major portion of their nutrition from translocated photosynthate or digestion of the zooxanthellae themselves. The confirmation of the existence of the zooxanthellar tube system has much to do with helping to clarify the nutritional relationship between the clam host and its algal symbionts.

Nevertheless, a great scope for investigation remains, with the current trend of research gearing toward symbiotic interactions, in the context of nutrient fluxes and metabolism. In particular, there is a need to quantify many aspects of the nutritional physiology of tridacnids. Whereas the contribution of zooxanthellae photosynthesis to host nutrition has been widely addressed in many types of hosts, little work has been carried out on the contribution of host-derived substances, as well as the contribution of particulates and exogenous sources of dissolved organic and inorganic molecules on zooxanthellae nutrition. This information is necessary in order to determine the relative significance of the various sources of autotrophic and heterotrophic nutrition of the clam-zooxanthellae symbiosis. Other interesting aspects for further investigation include pathways of nutrient assimilation, detailed mechanisms of nutrient uptake, nutrient recycling and conservation, and the possible detrimental effects of nutrients (e.g. pollution). More importantly, the

question of nutrient limitation of the zooxanthellae still remains. Which nutrient is actually limiting? Or is it possible that more than one nutrient is limiting?

These still unresolved issues are not unique to tridacnids. In fact, similar investigations are presently being carried out on other invertebrate-zooxanthellae symbioses, notably, the hermatypic corals. Tridacnids do not only offer a wide open field for investigations along these lines; but, they also present an excellent opportunity for comparing the nutritional features of their intercellular zooxanthellae with those of the coelenterate's intracellular algal symbionts.

1.6 OBJECTIVES OF THIS STUDY

This study was undertaken to further understand the nature of inorganic nutrition in giant clams, with respect to exogenous nitrogen (N) and phosphorus (P), as these are the two major nutrients shown to limit the growth of marine phytoplankton. In particular, the study aimed to:

1. determine whether growth of clams and their zooxanthellae is N- and/or P-limited under normal environmental conditions based on a number of growth parameters;
2. ascertain the extent to which the host regulates the availability of nutrients to its algal symbionts; and
3. demonstrate any effects of elevated N and P concentrations on clam calcification.

This study is significant in having both scientific and practical relevance. Apart from its scientific value in helping elucidate the nutritional physiology of tridacnids and their zooxanthellae, it has applications to clam mariculture in terms of growth enhancement of clam stocks, as well as potential implications for calcifying organisms in reef waters with elevated nutrient concentrations.

Chapter 2 GENERAL MATERIALS AND METHODS

The experimental and analytical procedures routinely used throughout this study are presented in this chapter. Techniques unique to particular experiments are presented elsewhere in appropriate chapters.

2.1 EXPERIMENTAL ORGANISMS

2.1.1 *Tridacna gigas*

Tridacna gigas specimens were collected from cultured stocks maintained on the intertidal reef flat in Pioneer Bay, opposite the James Cook University of North Queensland's (JCUNQ) Orpheus Island Research Station, Great Barrier Reef, Australia. These giant clams were produced by the Giant Clam Project of the Australian Centre for International Agricultural Research under the supervision of Prof. John Lucas of JCUNQ. Giant clams of various size-classes (shell lengths), namely, 4 to 6 cm, 16 to 20 cm, and 22 to 26 cm, were used for the different nutrient experiments.

2.1.2 *Tridacna maxima*

Tridacna maxima specimens used in the multi-agency ENCORE Project (Enrichment of Nutrients on a Coral Reef) of the Great Barrier Reef Marine Park

Authority (GBRMPA) (see Chapter 5 for explanation of this project) were indigenous to the reef areas surrounding the experimental micro-atolls at One Tree Island, Southern Great Barrier Reef, Australia. Sixty clams, ranging from 17 to 23 cm shell length, were collected from the reef crest and randomly allocated to twelve micro-atolls by Dr. Ove Hoegh-Guldberg of University of Sydney (ENCORE Permit No. G92/382).

2.1.3 *Symbiodinium* sp.

Symbiodinium sp. were isolated from *T. gigas* as described in Section 2.2.2 below. These freshly-isolated zooxanthellae were used for nutrient-uptake experiments and biomass analyses.

Cultured *Symbiodinium* sp. (Culture CS-163 from *T. gigas)* was purchased from the Micro-algae Culture Collection Unit of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Marine Laboratories in Hobart, Tasmania, Australia. This stock culture was subcultured and then purified using a 24-h triple-antibiotic treatment. Effective dosage was found to be approximately 125.0 mg l^{-1} penicillin G (K or Na salt), 62.5 mg l^{-1} streptomycin sulphate, and 12.5 mg l^{-1} chloramphenicol. To prepare the triple-antibiotic solution, 2.5 g penicillin and 1.25 g streptomycin were dissolved in 18.0 ml distilled water. To this solution was added 0.25 g chloramphenicol previously dissolved in 2.0 ml 95% ethanol. The mixture was mixed thoroughly, filter-sterilised, and used on the

day of preparation. One ml of the triple-antibiotic solution was added to 1 l of healthy zooxanthellae culture, to which 0.1 ml of sterility test medium (1.0% w/v nutrient broth) had been previously added. The sterility test medium introduced a little organic matter that would induce cell division in the bacteria (penicillin acts on dividing bacteria). After 24 h of incubation, the treated cultures were subcultured into antibiotic-free medium. The subcultures were then incubated for at least two weeks and checked for bacterial contamination. Purified subcultures were scaled up in culture to produce quantities enough for experimentation.

2.2 MEASUREMENT OF BIOMASS PARAMETERS

2.2.1 Clam Host

Before collecting *T. maxima* from the experimental micro-atolls, a wooden wedge was inserted between the gaping valves in order to keep the valves open for later extraction of the soft tissues from the shell. This procedure was not necessary for *T. gigas* as this species does not close its valves completely. Once in the laboratory, the clams were emptied of most of sea water trapped inside their mantle cavities by inverting them onto a beaker. Using a scalpel, the soft-tissue mass was extracted from the shell by cutting through the mantle and adductor muscles attached to the inner surface of both valves, being careful not to rupture

any visceral organs in the process.

2.2.1.1 Shell length and soft-tissue weight

Shell length (longest axis) was measured to the nearest mm using calipers. After removing the clam from its shell, the mantle was excised from the rest of the soft tissues, if necessary. Any remaining sea water and haemolymph were blotted off the tissues with paper towel. The tissues were then weighed separately to the nearest 0.01 g.

2.2.1.2 C:N:P ratio of soft tissues

Whole-clam, viscera, and mantle were freeze-dried and analysed for C and N composition using a CHN elemental analyser (LECO CHN-600, Australian Institute of Marine Science; CARLO ERBA EA-1108-CHN, JCUNQ), and for N and P composition using the hydrogen peroxide-sulfuric acid digestion technique of Allen (1974).

2.2.1.3 Nutrient levels in haemolymph

During extraction of the soft tissues from the shell, the haemolymph was collected in a beaker and the volume recorded. The haemolymph was centrifuged at 2000 *g* for 10 min to remove cells and debris. Thirty ml of the

haemolymph was immediately analysed for ammonia (see Section 2.4.2 below). The rest of the haemolymph was kept at -20°C for later determinations of phosphate and total phosphorus (see Section 2.4.2 below). All assays were done in triplicate for each clam.

2.2.2 Zooxanthellae

The mantle was cut into small pieces and homogenised in 0.45-um-filtered sea water (MFSW) using a blender. The homogenate was centrifuged at 1000 *g* for 2.5 min and the animal tissue fraction decanted. The algal pellet was then resuspended in MFSW and the animal tissues were filtered on a double layer of clinical gauze cloth. Most of the remaining zooxanthellae from the filtered animal tissues were washed through the filter with MFSW. The total zooxanthellae filtrate was then centrifuged at 2000 *g* for 2.5 min, the supernatant was discarded, and the resulting algal pellet was resuspended in MFSW. The zooxanthellae were washed two more times or until the supernatant was clear. Finally, the algal pellet was resuspended in 20 ml of MFSW. Prior to nutrient-uptake experiments, the final zooxanthellae suspension was kept in the dark by wrapping in aluminium foil.

2.2.2.1 Density and size

A known volume (0.25 ml to 1.0 ml) of the final zooxanthellae suspension was preserved with buffered 10% formalin solution in a 1:4 volume ratio (formalin : zooxanthellae suspension). Zooxanthellae density and size were determined under a compound microscope for 6 replicate subsamples from each algal suspension using a haemocytometer.

2.2.2.2 C:N:P ratio

A known volume (5 ml to 10 ml) of the final zooxanthellae suspension was centrifuged at 1000 *g* for 2.5 min and the algal pellet stored at -20°C. The frozen pellets were later lyophilised over 24 h and weighed to the nearest 0.1 mg. The samples were then stored with silica gel at -20°C until analysed for C and N composition using a CHN elemental analyser (CHN-600, AIMS; EA-1108-CHN, JCUNQ), and for N and P composition using the hydrogen peroxide-sulfuric acid digestion technique of Allen (1974) (see Section 3.5.3 below).

2.3 MEASUREMENT OF NUTRIENT UPTAKE BY FRESHLY-ISOLATED ZOOXANTHELLAE (FIZ)

The density of the remaining final zooxanthellae suspension was estimated, and, if necessary, an appropriate dilution was made to obtain 10^8 cells ml^{-1}.

Twenty ml solutions of 20 µ*M* ammonia (N) or 10 µ*M* phosphate (P) in MFSW were prepared in small glass scintillation vials. To obtain a final density of 10^6 cells ml^{-1} in these incubation vials, 0.2 ml of the zooxanthellae suspension was added in each of triplicate vials at 15-s intervals. Triplicate vials without zooxanthellae were kept as controls for bacterial N- or P-uptake in 0.45 µ MFSW. The zooxanthellae were then incubated in ammonia for 30 to 60 min and in phosphate for 3 to 8 h under natural light conditions (50 µE m^{-2} s^{-1} to 200 µE m^{-2} s^{-1}; 26°C to 28°C). After incubation, the FIZ were successively filtered on GF/C glass-fibre filters at 15-s intervals and the filtrates were immediately analysed for ammonia or phosphate, as appropriate (see Section 2.4.1 below).

2.4 DETERMINATION OF DISSOLVED NUTRIENTS

2.4.1 Ammonia and Phosphate in Sea Water

Ammonia in sea water was determined using a modification (Wilkerson and Trench, 1986) of the method of Liddicoat et al. (1975), while phosphate was measured using the method of Strickland and Parsons (1972). All assays were done in triplicate.

2.4.2 Ammonia, Phosphate, and Total Phosphorus in the Haemolymph

Ammonia in the haemolymph were determined as in Section 2.4.1 above. However, after adding the appropriate reagents, the samples were centrifuged at 1000 *g* to spin down the precipitates before reading the clear supernatant on the spectrophotometer. For phosphate and total phosphorus determinations, 10% (w/v) trichloroacetic acid was first added to the haemolymph to precipitate its protein content (see Deane and O'Brien, 1980). The protein precipitate was then centrifuged down at 2000 *g* and the supernatant was analysed for phosphate (Strickland and Parsons, 1972) and total phosphorus (Koroleff, 1983). All assays were done in triplicate.

Chapter 3 N- AND P-SUPPLEMENTED *Tridacna gigas* IN TANKS: NUTRIENT-LIMITED GROWTH OF SYMBIOTIC PARTNERS

3.1 INTRODUCTION

As discussed previously, symbiotic zooxanthellae are associated with the digestive system of the host in many invertebrate symbioses. In tridacnids in particular, the zooxanthellae occupy the terminal branches of a diverticulum of the stomach (Norton et al., 1992). This anatomical situation requires that all nutrients acquired by the zooxanthellae have to be obtained from the host's tissues and fluids, and not directly from the water column. Thus, dissolved nutrients must be acquired from the sea water, across the host's cells (i.e., gill, mantle), through the haemolymph, and across the thin layer of epithelial cells comprising the zooxanthellal tube system.

This supply of nutrients to the zooxanthellae is a major factor in the reciprocated supply of photosynthate to the host. Indeed, the control of nutrient supply is the basis of current thought on how zooxanthellae numbers are controlled within the host (e.g., Muscatine and Pool 1979, Trench 1987).

The association between host and zooxanthellae is therefore a complex one. The host, while trying to maximise its supply of photosynthate from the zooxanthellae, needs to control the numbers of zooxanthellae in its tissues. This dichotomy has generated much debate in the literature, particularly as to whether

Chapter 3 N- AND P-SUPPLEMENTED *Tridacna gigas* IN TANKS: NUTRIENT-LIMITED GROWTH OF SYMBIOTIC PARTNERS

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zooxanthellae are nutrient-limited, and if so, which nutrient is limiting (Cook and D'Elia, 1987; Miller and Yellowlees, 1989; Rees, 1991).

One of the tools used to investigate this question has been to observe the response of the intact organism to the presence of elevated nutrients, particularly nitrogen and phosphorus. The majority of the experiments conducted thus far have involved cnidarian symbioses. The general consensus is that increasing the supply of nitrogen causes an increase in the density of zooxanthellae in the host (Hoegh-Guldberg and Smith, 1989; Dubinsky et al., 1990; Fitt et al., 1992), whereas, phosphate has little effect on algal density (Stambler et al., 1991). However, while nitrogen levels in sea water are important in determining zooxanthellae numbers, the increase in available nitrogen need not be reflected in the physiological status of these algae, nor in the metabolic relationship between the symbiotic partners. While an increase in nutrient levels in sea water has a direct effect on free-living phytoplankton, in these symbiotic associations the host may exert some control over the algae both through the supply of these nutrients and the efficacy of "host factor" (Trench, 1971; Muscatine et al., 1972).

This study investigated the effects of elevated nutrient levels on various biological parameters, including host-tissue biomass, zooxanthellae population, and individual C:N:P ratios of the symbiotic partners. C:N:P ratios, in particular, are widely used to determine the nutritional status of algae in general (see Atkinson and Smith, 1983; Domotor and D'Elia, 1984; Stambler et al., 1991).

Based on the algae's relative N and P composition, it is possible to interpret which nutrient is relatively limiting to their growth. Examination of C:N:P composition is especially relevant to symbiotic zooxanthellae, since it has been hypothesised that host control over algal numbers is through nutrient supply (Cook and D'Elia, 1987).

The major objectives of this study were: (1) to determine whether growth of the clams and their zooxanthellae is N- and/or P-limited under normal environmental conditions; and (2) to examine if increasing the ambient nutrient concentrations in the sea water could shed light on the role of the animal host in the supply of inorganic nutrients to its symbiotic algae. The experiment consisted of exposing one-year-old *Tridacna gigas* to elevated levels of ammonium and phosphate in outdoor tanks over an extended period, and then analysing the clams' soft tissues and zooxanthellae. Data on the effects of these elevated nutrient levels on calcification are reported in Chapter 4 (Belda et al., 1993a).

3.2 MATERIALS AND METHODS

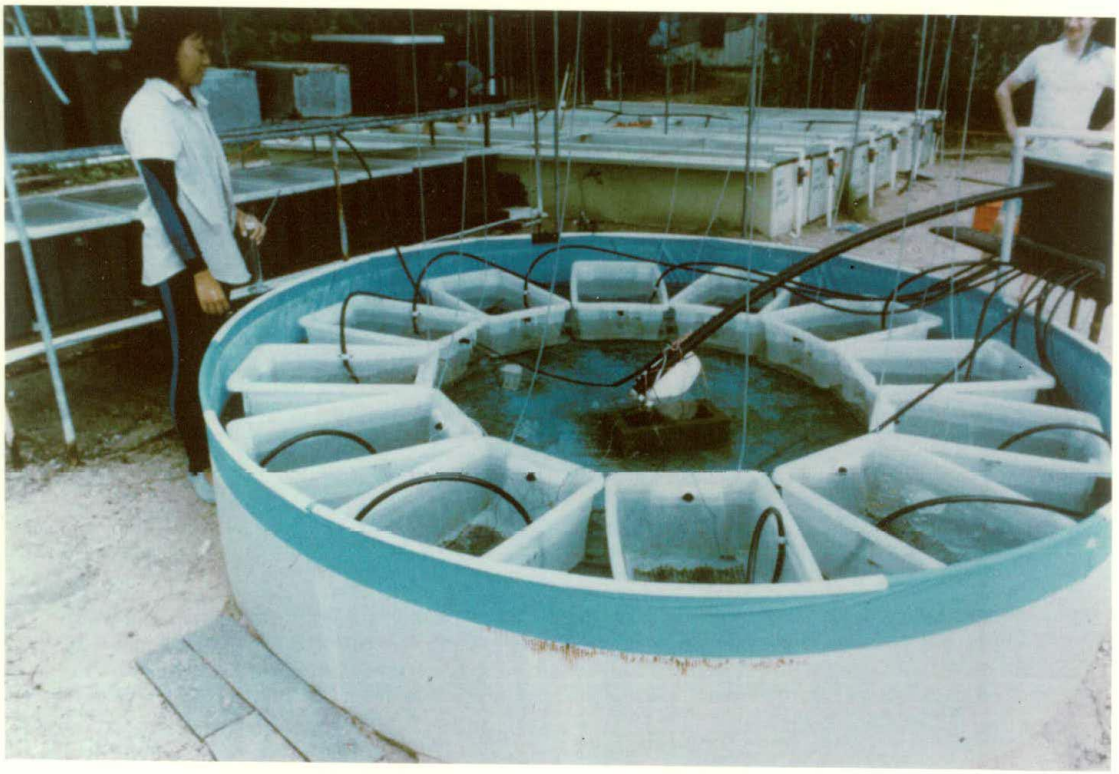
3.2.1 Experimental Design and Maintenance

This study was conducted over three months during the summer of 1990-1991 at Orpheus Island Research Station, North Queensland, Australia, following a

preliminary experiment performed the previous summer. Year-old *Tridacna gigas* juveniles (40 - 60 mm shell length) were taken from a single cohort of clams being grown in a nearby ocean nursery, and randomly allocated into groups of 20 clams each. Each clam was cleaned of epibionts and tagged with a numbered plastic tape using marine epoxy. Each group of clams were then placed in an open, aerated 60-l tank containing a plastic mesh and pieces of gravel for substrate. Filtered sea water (80-100 µm, FSW) flowed into the clam tanks at a rate of 1 l min^{-1}. Using peristaltic pumps, concentrated solutions of analytical-grade NH_4Cl (N) and KH_2PO_4 (P) were added to each inflowing sea-water stream at 1 ml min^{-1}, giving final nutrient concentrations of 5 and 10 µ*M* N (5N, 10N); 2, 5, and 10 µ*M* P (2P, 5P, 10P); and 5N+2P, 5N+5P, 5N+10P, 10N+2P, 10N+5P, and 10N+10P. An unenriched group of clams served as the control. Background N and P levels were less than 0.1 µ*M*.

The experimental tanks were partially immersed in running sea water within a large circular tank (Fig. 3.1) for temperature control, and the circle of tanks moved one position clockwise each week for light control. Tanks and clams were cleaned weekly, followed by replenishment of nutrient stocks in light-proof reservoirs. Flow rates were checked regularly, water temperature monitored daily, salinity noted every other day, and ambient irradiance and nutrient levels (see Section 2.4.2, Chapter 2) in tanks measured periodically. Final nutrient levels and sea water pH (8.1-8.2) remained essentially unchanged throughout the experiment.

Fig. 3.1. Experimental set-up at Orpheus Island Research Station. *Tridacna gigas* juveniles were placed in white plastic tanks, with nutrient supply coming from gray reservoir tanks shown in the background.



3.2.2 Weight and C:N:P Ratio of Soft Tissues

At the termination of the experiment, soft tissues were carefully removed from the shells, blotted dry, and individually weighed to the nearest 0.01 g. The tissues were then freeze-dried and analysed for C, N, and P composition as previously described (Section 2.2.1.2, Chapter 2).

3.2.3 Chlorophyll *a* Content, Density, and C:N:P Ratio of Zooxanthellae

Zooxanthellae samples were prepared as described in Section 2.2.2, (Chapter 2). For pigment analysis, a known volume was pipetted from the algal suspension and filtered through a Whatman GF/C glass-fibre filter using a low vacuum pressure. The filter was thoroughly drained, folded in half, and kept covered with aluminium foil at -20°C. The frozen filters were later ground in 8 ml of chilled, double-distilled acetone, and chl *a* was extracted over 24 h in a refrigerator. The samples were then centrifuged at 1000 *g* for 7 min and the absorbance of the supernatant was immediately read at 630, 647, 664, 665, and 750 nm. The amounts of pigments were calculated using the equations of Jeffrey and Humphrey (1975). For density and C:N:P ratio of the zooxanthellae, see Section 2.2.2, Chapter 2.

3.2.4 Mitotic Index, Specific Growth Rate, and Doubling Time of Zooxanthellae

In an independent preliminary study using smaller clams (ca. 5 mm shell length), the zooxanthellae's mitotic index was examined over a 24-h period to determine if there was diel periodicity in the rate of division. Juvenile clams were obtained from one of the Research Station's culture tanks where post-harvest juvenile clams were being reared. A group of juveniles were pre-incubated in either 10 μ*M* N or 10 μM P in shallow, rectangular plastic containers (40 cm x 25 cm x 10 cm) by letting concentrated solutions of N or P (contained in elevated, light-proof containers) constantly drip into the FSW flowing into the incubation containers. The nutrient and sea-water flow rates were adjusted to achieve the desired nutrient concentrations in each incubation medium. A group of unenriched clams served as control. After three days of incubation, ten clams were collected from each group every three h over a 24-h period. Considering the small size of the juvenile clams, the zooxanthellae were examined by squashing the tiny juveniles with a scalpel and thoroughly grinding the tissues on a glass slide prior to an immediate examination under a compound microscope. The number of dividing cells per 100 zooxanthellae was determined for each clam. A cell was considered to be dividing if it appeared as a doublet with a clear cell plate. This procedure of examining zooxanthellal mitotic index from tiny clams was based on the assumption that the doublet and non-doublet zooxanthellae were randomly distributed within the clam tissues after thorough grinding. Also, this

procedure was considered economical and acceptable for the purpose of determining the occurrence of diel periodicity in zooxanthellal division rates, assuming that diel periodicity does not vary with clam size (also see Fitt et al., 1993).

Zooxanthellae samples from the bigger clams were then subsequently collected before dawn (0500-0600 h), when peak division of zooxanthellae was observed to occur (see Results Section below). Mantle tissues were removed from the shell, thoroughly homogenised, and resuspended in a known volume of FSW. One ml of this algal suspension was preserved with 0.2 ml of buffered 10% formalin solution. The percentage of dividing cells was determined using a haemocytometer. The dividing cells in 4 subsamples of 500 zooxanthellae from each algal suspension were averaged and the resultant percentage taken as the mitotic index (MI). The specific growth rate (SGR) and doubling time (T) were then calculated (Wilkerson et al., 1983).

3.2.5 Polyphosphate Content in Zooxanthellae

To determine polyphosphate content, zooxanthellae samples were prepared as described in Section 2.2.2, (Chapter 2). The resulting algal pellet was immediately frozen until analysis.

Prior to polyphosphate extraction, 1 ml of 20 m*M* NaF was added to each frozen algal pellet to inhibit phosphatase activity. Boiling 50 m*M* EDTA was then added to the sample in a volume to volume ratio of 1:3 (cells:EDTA), and the mixture was heated at 100°C for 7 min. The cells in the sample were lysed (80 to 90% breakage) by passing them twice through a French Press (140 000 kPa). The homogenates were then centrifuged at 12 000 *g* for 10 min, lyophilised, and subsequently kept at -20°C. Immediately prior to analysis by ^{31}P-nuclear-magnetic-resonance spectroscopy (^{31}P NMR), the samples were dissolved in 1.75 ml of 10 m*M* EDTA and 10% D_2O in double-distilled water, and adjusted to pH 8-9 with 1 *M* NaOH. ^{31}P-NMR analysis was then carried out on 2 ml samples in 10 mm-diameter tubes, using a Bruker CXP 300 spectrometer, operating at 121.5 MHz. Spectra were collected with a 1 s repetition time, at 60° pulse, 4096 data points, and a sweep width of 8064 Hz. The free induction decays were then multiplied by an exponential factor ranging from 10 to 20 Hz prior to Fourier transform. Proton decoupling was not employed, since spectral line widths greatly exceeded both the field inhomogeneity and the ^{31}P-^{1}H J coupling. Orthophosphoric acid at 85% was used as an external reference, and all spectra were acquired while the instrument was locked on 10% D_2O. The assignment of observed resonances was based on published values (see Jackson, 1989).

3.2.6 Acid-phosphatase Activity in Zooxanthellae

For measurement of acid-phosphatase activity, each algal pellet (obtained as in Section 2.2.2, Chapter 2) was resuspended in 0.45-μ Millipore-filtered sea water (MFSW) and lysed in a French Press. The extract was then centrifuged at 27 000 *g* for 1 h at 4°C. The activity of acid phosphatase in the extract was determined by monitoring the hydrolysis of ρ-nitrophenylphosphate (PNPP) for 30 min at 37°C. The 1-ml reaction mixture consisted of 0.5 ml of 10 m*M* PNPP, 0.3 ml of 0.1 *M* succinate buffer (pH 5.0), and 0.2 ml of enzyme sample (extract). Addition of the enzyme sample initiated the reaction, while addition of 0.8 ml of 1 *M* NaOH terminated it. The release of ρ-nitrophenol was measured at 400 nm. The hydrolysis of substrates other than PNPP was measured by following the release of phosphate (Heinonen and Lahti, 1980) under the same assay conditions. Blanks in both assays contained the same reaction mixture, but NaOH was added prior to the enzyme sample. The molar extinction coefficient used for ρ-nitrophenol under these conditions was 1.75×10^4 $M^{-1}cm^{-1}$ (Patni and Aaronson, 1978). Protein was measured by the microassay procedure of Bradford (1976) using serum albumin (Fraction V) as a standard.

3.2.7 Statistical Analyses of Data

Nutrient treatments were not replicated due to technical limitations. However, measures were taken during the execution of the experiment to

minimise major confounding sources of variation (see previous Section 3.2.1, Experimental Design and Maintenance). Twenty clams per treatment were used to provide enough samples for various physiological and biochemical measurements (this chapter; Belda et al. 1993b) and shell measurements (Chapter 4; Belda et al. 1993a). Equal samples of clams for each of the analytical procedures were then randomly taken to allow for balanced factorial analyses of the data. Since the individual clams represent subsamples, however, they were amalgamated for each tank. To test the hypothesis that elevated levels of ammonium and phosphate have no effect on clam and zooxanthellae growth at $\alpha = 0.05$, data were analysed using two-way ANOVA (fixed factors) without an interaction term, and linear contrast (Underwood, 1981; Zar, 1984). All statistical analyses were performed using the analytical software package, Statistix (Version 3.0). Tests of the assumptions (normality, homoscedasticity, additivity) underlying the analytical procedures were made prior to performing each analysis (Shapiro and Wilk, 1965; Winer 1971, Shapiro and Francia, 1972; Zar, 1984). Except for the MI and T data ($p<0.05$), no serious violations of the assumptions were evident in the different data sets ($p>0.05$). Data for MI and T were log-transformed to conform to the assumptions of normality and homogeneity of variances ($p>0.05$).

3.3 RESULTS

3.3.1 Whole-clam Parameters

Addition of P alone significantly decreased the C:P ratio in the soft tissues (ANOVA, $F = 42.83$, $df = 3,6$, $p < 0.001$) (Fig. 3.2), indicating increased phosphorus content, but it did not affect the C:N ratio (ANOVA, $F = 1.11$, $df = 3,6$, $p > 0.05$) (Fig. 3.2), nor the soft-tissue weight (ANOVA, $F = 0.34$, $df = 3,6$, $p > 0.05$) (Fig. 3.3). Addition of N or N+P, on the other hand, significantly decreased the C:N ratio (ANOVA, $F = 50.87$, $df = 2,6$, $p < 0.001$) (Fig. 3.2), and increased the C:P ratio (ANOVA, $F = 170.26$, $df = 2,6$, $p < 0.0001$) (Fig. 3.2), indicating increased synthesis of nitrogenous materials and carbon skeletons as amino-group acceptors at these nutrient regimes. Such changes in the tissue chemical composition with N-supplementation are compatible with the marked increase observed in the amount of soft tissue (Fig. 3.3). Nevertheless, considerable variability in the measurement of wet weight, even after care was taken to remove most liquid remaining on the clams, did not yield significant results (ANOVA: for N effect, $F = 2.66$, $df = 2,6$, $p > 0.05$; for P effect, $F = 0.34$, $df = 3,6$, $p > 0.05$). It is also interesting that the C:P ratios in soft tissues in N+P nutrient regimes were lower than those in N-only regimes (Fig. 3.2). This could reflect greater assimilation of phosphorus in N+P than in N alone, or alternatively, higher C production in the N-only regimes. A comparable trend is apparent for the C:N ratios.

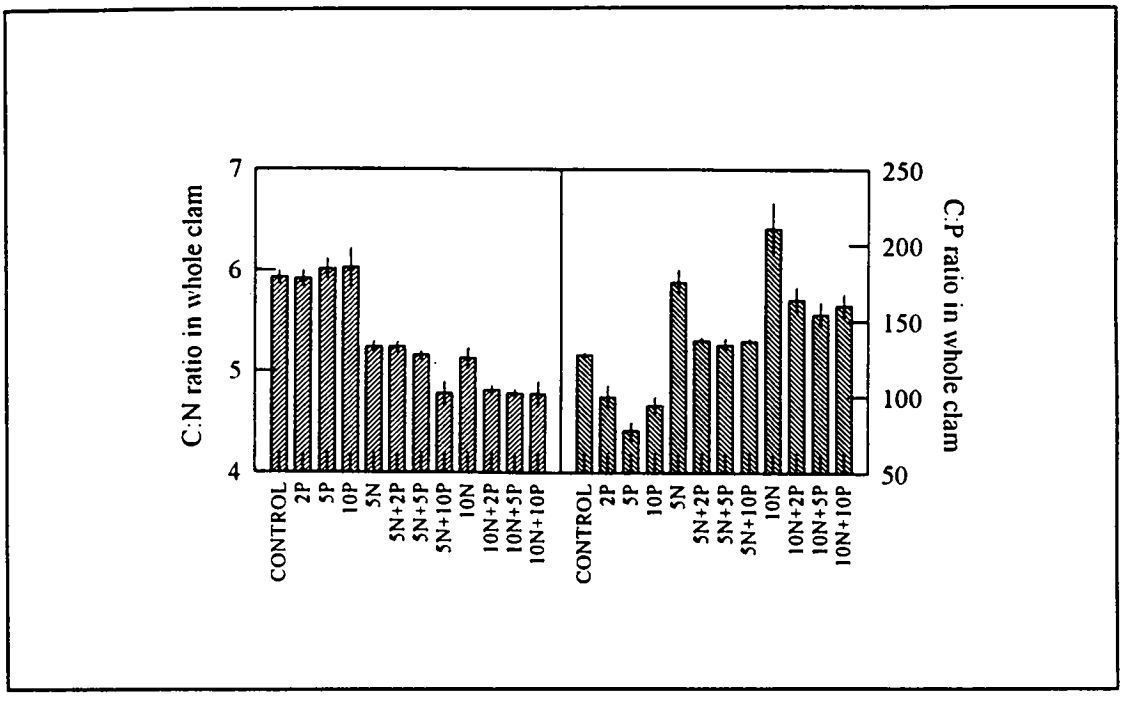


Fig. 3.2. Mean C:N and C:P atomic ratios (± SE) in soft tissues of *Tridacna gigas* supplemented with or without different concentrations (μ*M*) of ammonium (N) and phosphate (P). (*n* = 3).

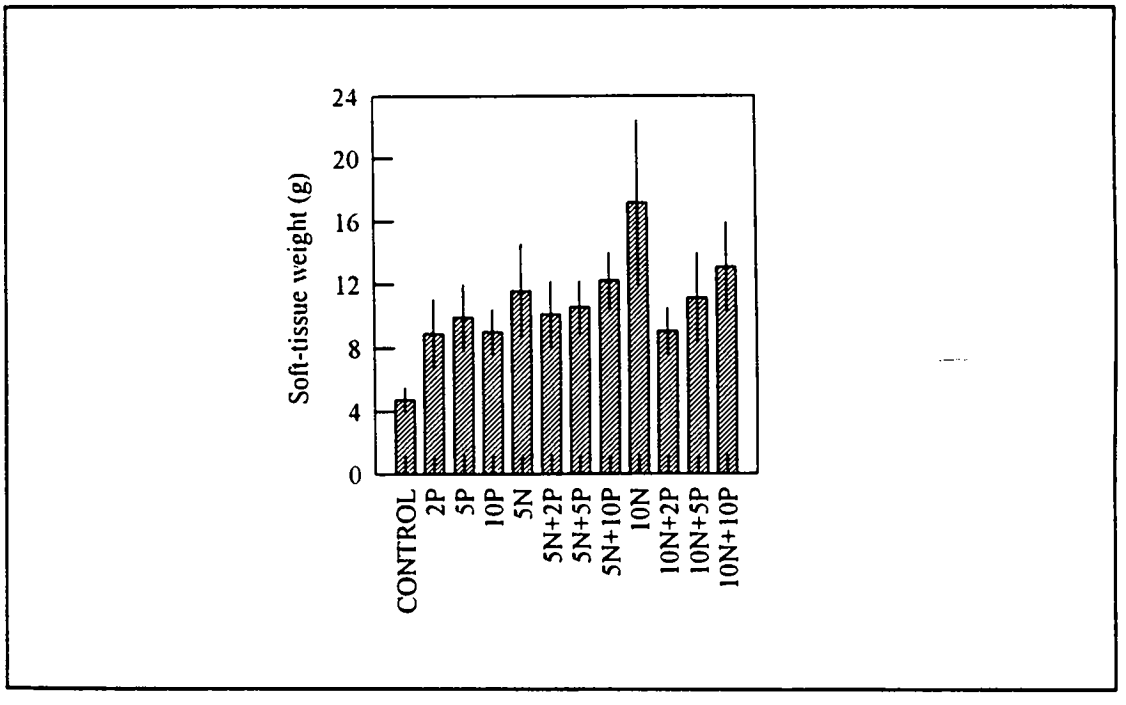


Fig. 3.3. Mean soft-tissue weights (± SE) of *Tridacna gigas* supplemented with or without different concentrations (μ*M*) of ammonium (N) and phosphate (P). (*n* = 3).

3.3.2 Zooxanthellae Parameters

Clams exposed to N or N+P treatments had much darker mantles than those exposed to P alone. Similarly, the zooxanthellae population significantly increased with addition of N (ANOVA, $F = 105.80$, $df = 2,6$, $p < 0.0001$) and P (ANOVA, $F = 15.91$, $df = 3,6$, $p < 0.01$), although with respect to P, only the 10P treatment yielded a distinct increase in zooxanthellae population (Fig. 3.4). The higher the level of N-supplementation, the greater the zooxanthellae numbers. The increase in zooxanthellae population with 10P was rather unexpected but could be related to the markedly reduced ammonium-depletion rate of zooxanthellae from 10P-, N-, and N+P-treated clams (preliminary data not shown, but see also Chapter 5). Furthermore, 10P in combination with N yielded distinctly higher zooxanthellae numbers than any of the lower levels of P in combination with N.

Chl *a* per zooxanthella (Fig. 3.5), on the other hand, significantly decreased with N (ANOVA, $F = 27.03$, $df = 2,6$, $p < 0.001$) and P (ANOVA, $F = 5.73$, $df = 3,6$, $p < 0.05$). The higher the level of either N- or P-supplementation, the lower the Chl *a* content. Fig. 3.6 shows that Chl *a* per clam is directly related to number of zooxanthellae ($r = 0.82$), while Chl *a* per zooxanthella is inversely related to zooxanthellae number ($r = -0.85$).

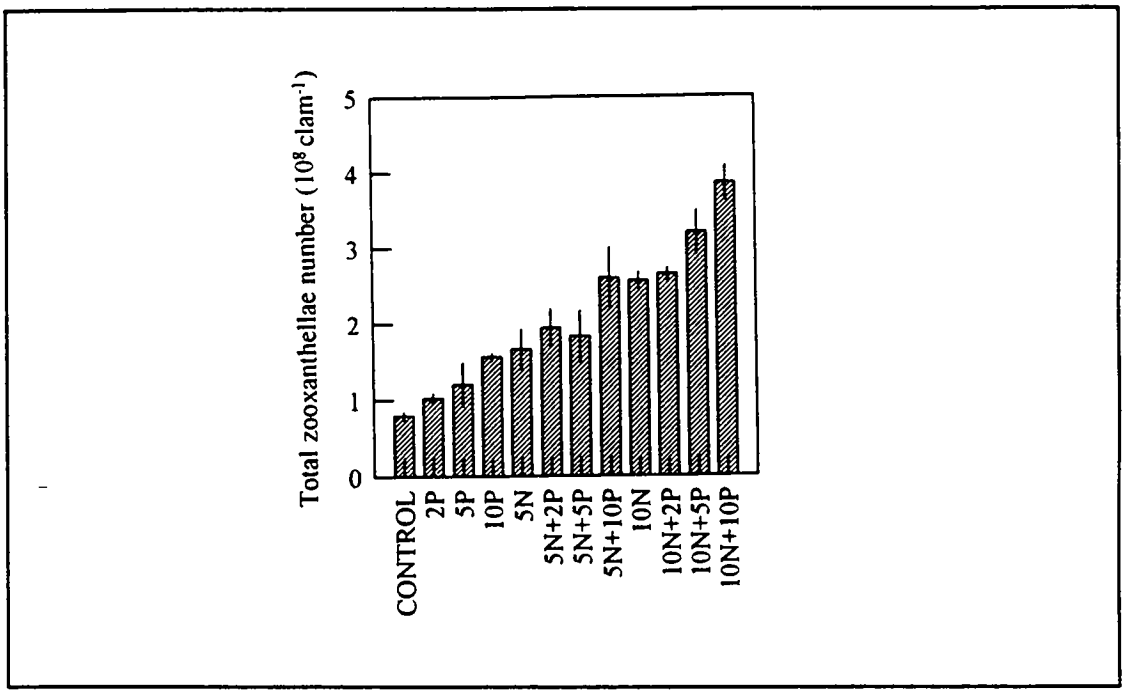


Fig. 3.4. Total zooxanthellae numbers in *Tridacna gigas* supplemented with or without different concentrations (μ*M*) of ammonium (N) and phosphate (P). (n = 2).

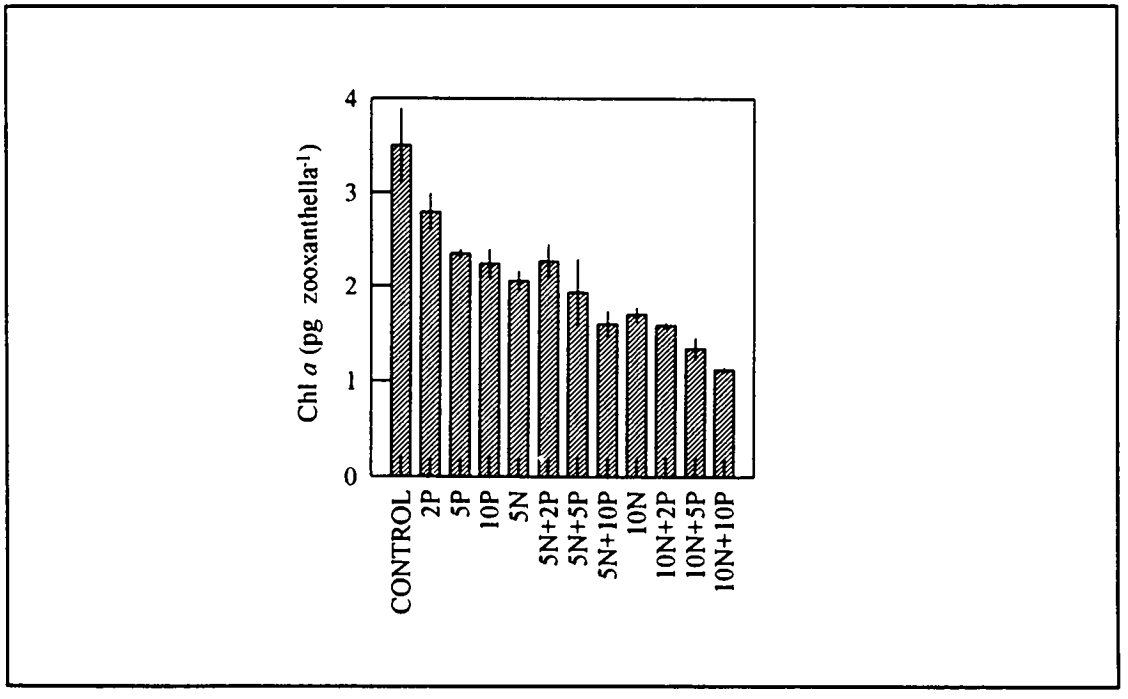


Fig. 3.5. Mean Chl *a* content per zooxanthella in *Tridacna gigas* supplemented with or without different concentrations (μ*M*) of ammonium (N) and phosphate (P). (n = 3).

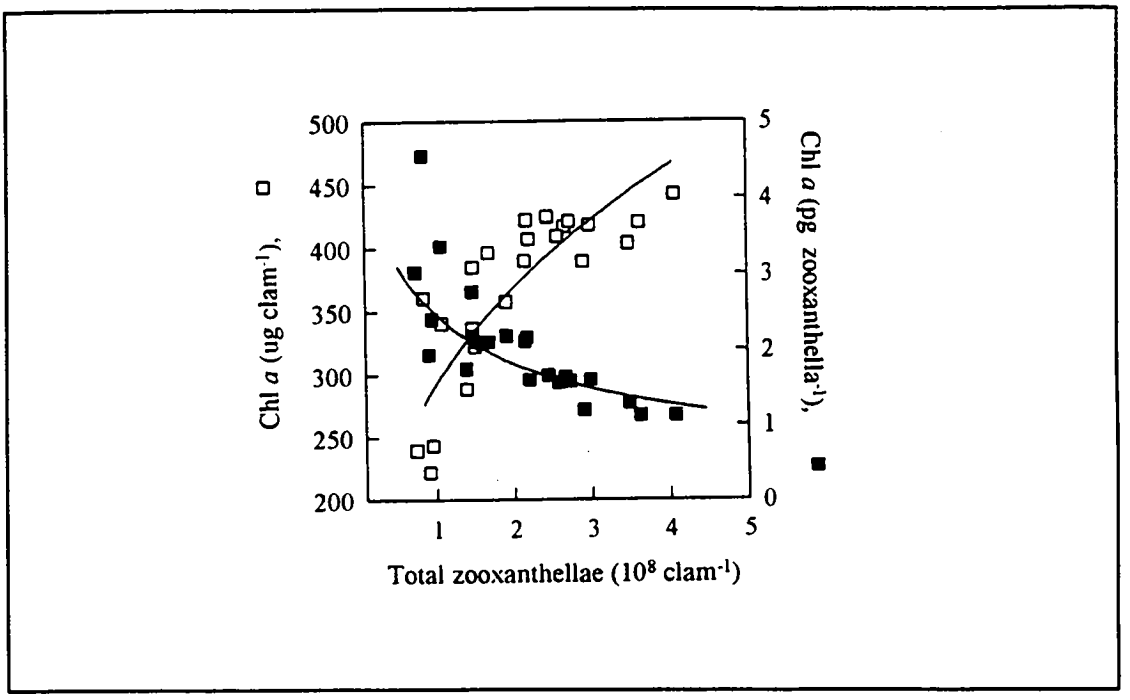


Fig. 3.6. Chl *a* per clam (Chl *a*C) and Chl *a* per zooxanthella (Chl *a*Z) as a function of the total zooxanthellae per clam (Z). Regression lines are Chl $aC = 0.612Z^{3.349}$, r = 0.82 and Chl $aZ = 0.419Z^{-0.602}$, r = -0.85.

The C:N:P ratios of the zooxanthellae are similar in all treatments (ANOVA: for N effect, $F = 1.77$, $df = 2,6$, $p > 0.05$; for P effect, $F = 1.36$, $df = 3,6$, $p > 0.05$) (Table 3.1), with a mean value of 303 ± 16 : 52 ± 2 : 1 (± SE, $n = 36$) across all treatments.

Table 3.1. Mean C:N:P atomic ratios [± SE] in zooxanthellae from clams exposed to different concentrations (μM) of ammonium (N) and phosphate (P) in outdoor tanks on Orpheus Island

Treatment	C:N:P Ratio	(*n*)
Control	278.6 ± 17.6 : 52.2 ± 1.3 : 1	(3)
2P	255.6 ± 14.6 : 45.5 ± 1.1 : 1	(3)
5P	376.3 ± 101.3 : 65.9 ± 13.7 : 1	(3)
10P	237.5 ± 19.8 : 39.4 ± 3.8 : 1	(3)
5N	186.9 ± 31.0 : 33.0 ± 5.7 : 1	(3)
10N	290.7 ± 42.8 : 50.6 ± 7.3 : 1	(3)
5N+2P	302.8 ± 19.2 : 52.7 ± 2.3 : 1	(3)
5N+5P	260.6 ± 59.9 : 41.0 ± 9.6 : 1	(3)
5N+10P	354.0 ± 36.3 : 57.7 ± 5.4 : 1	(3)
10N+2P	365.5 ± 19.8 : 59.9 ± 3.6 : 1	(3)
10N+5P	399.8 ± 49.7 : 66.0 ± 11.1 : 1	(3)
10N+10P	331.2 ± 80.1 : 53.7 ± 11.4 : 1	(3)
Mean	303.3 ± 15.6 : 51.5 ± 2.5 : 1	(36)

Preliminary results showed that the mitotic index of juvenile *T. gigas* varied over a diel cycle, peaking in the pre-dawn to dawn period (0400 to 0700 h) for the control, N-, and P-treated clams (Fig. 3.7). There was no difference in the relative magnitude of the mitotic indices among these treatments.

For the bigger *T. gigas*, statistical analyses gave significant results with addition of N (ANOVA, $F = 12.63$, $df = 2,6$, $p < 0.05$), but not with P (ANOVA, $F = 3.84$, $df = 3,6$, $p > 0.05$). However, as evident in the data (Fig. 3.8), only the N+P combination yielded a distinct increase in mitotic index. The specific growth rate followed a similar trend, with relatively low values of 4 - 10% (Fig. 3.8); while, doubling time ranged from 15-17 days for the control and single-nutrient treatments, to 7-12 days for combination treatments (data not shown).

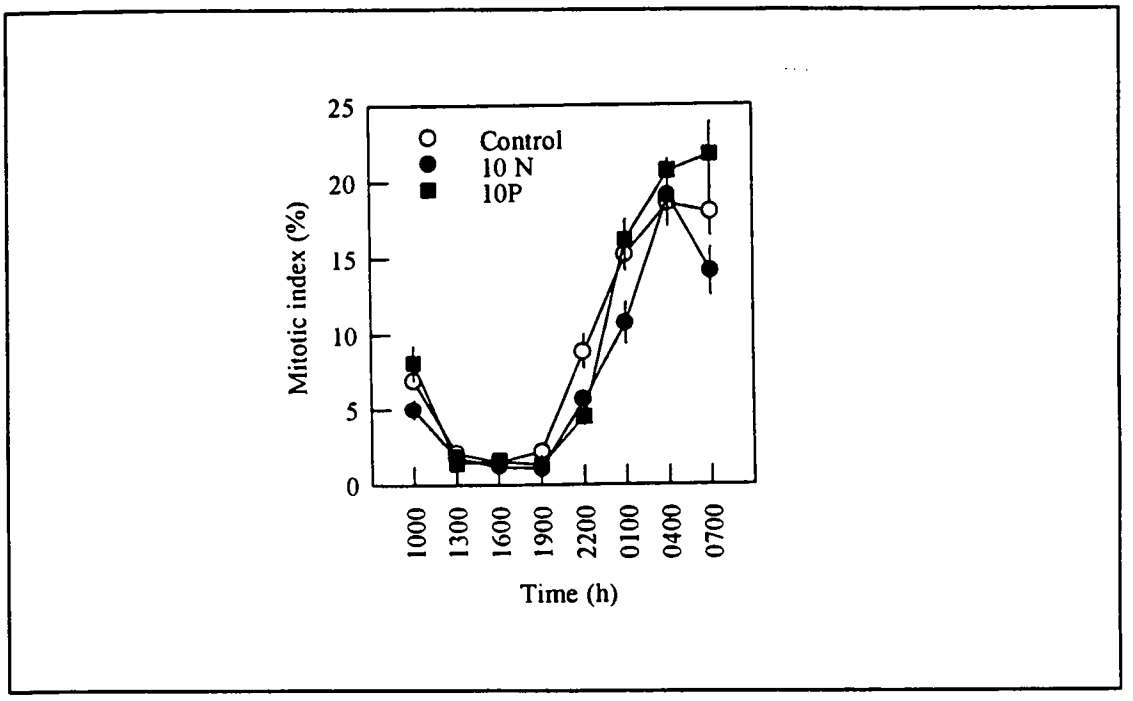


Fig. 3.7. Preliminary data on the mean mitotic index (± SE) of zooxanthellae in *Tridacna gigas* supplemented with or without ammonium (N) and phospate (P) in μ*M* concentrations. (*n* = 10)

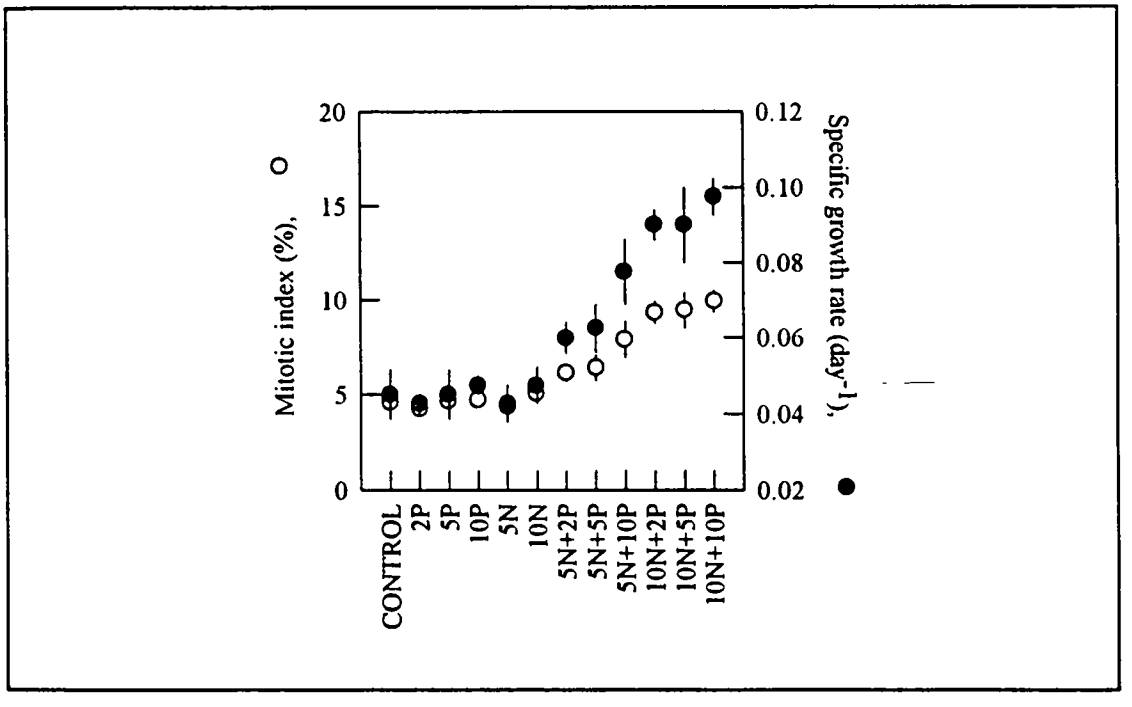


Fig. 3.8. Mean xooxanthellae mitotic index and specific growth rate (± SE) in *Tridacna gigas* supplemented with or without ammonium (N) and phospate (P) in μM concentrations. (*n* = 4).

NMR spectra of zooxanthellae extracts from the control and P-supplemented clams showed that polyphosphate was undetectable in both cases (Fig. 3.9).

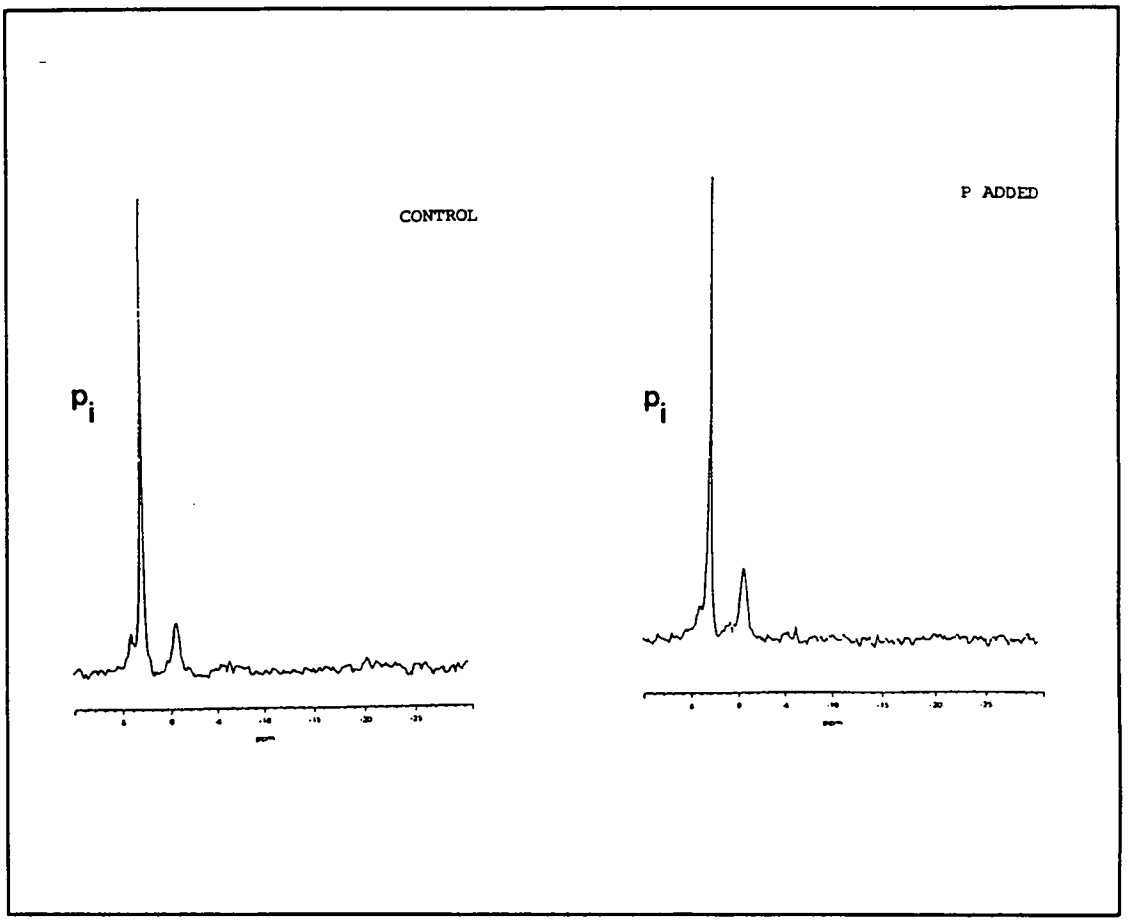


Fig. 3.9. ^{31}P-NMR (nuclear magnetic resonance) spectra of extracts of zooxanthellae from *Tridacna gigas* supplemented with or without phosphate in µM concentrations.

(P_i = inorganic phosphate)

Acid-phosphatase activity did not differ significantly between treatments (ANOVA: for N effect, $F = 0.17$, $df = 2,6$, $p > 0.05$; for P effect, $F = 0.29$, $df = 3,6$, $p > 0.05$), with a mean value of 0.14 ± 0.03 µmol ρ-nitrophenylphosphate hydrolysed min^{-1} mg $protein^{-1}$ ($n = 24$) (Table 3.2).

Table 3.2. Mean [± SE] acid-phosphatase activity (µmol PNPP hydrolysed min^{-1} mg^{-1} protein) in zooxanthellae isolated from *Tridacna gigas* exposed to different concentrations (µ*M*) of ammonium (N) and phosphate (P) in outdoor tanks on Orpheus Island. (PNPP = ρ-nitrophenylphosphate)

Treatment	Acid-phosphatase activity	(*n*)
Control	0.11 ± 0.00	(2)
2P	0.18 ± 0.04	(2)
5P	0.14 ± 0.00	(2)
10P	0.13 ± 0.01	(2)
5N	0.13 ± 0.04	(2)
10N	0.20 ± 0.03	(2)
5N+2P	0.14 ± 0.04	(2)
5N+5P	0.16 ± 0.01	(2)
5N+10P	0.13 ± 0.04	(2)
10N+2P	0.08 ± 0.02	(2)
10N+5P	0.18 ± 0.02	(2)
10N+10P	0.15 ± 0.03	(2)
Mean	0.14 ± 0.03	(24)

3.4 DISCUSSION

3.4.1 Clam Growth

The results of this study clearly show that increased ambient concentrations of inorganic nitrogen and phosphorus substantially affect the clam's soft tissues. Increased N and P in sea water correspondingly led to increased levels of these nutrients in the clam's soft tissues. The observed enhancement in growth of soft tissue with addition of N or N+P, but not with P alone (Fig. 3.3), correlates directly with the increase in shell length (Chapter 4; Belda et al., 1993b).

An increase in shell length was observed by Fitt et al. (1993) in small *T. derasa* (2-3 mm shell length) in response to N-enrichment, and a corresponding tissue-mass increase was documented by Hastie et al. (1992). In contrast to these data for clams, however, there is no evidence that animal biomass in corals increases as a result of nutrient supplementation. Muscatine et al. (1989) concluded from animal protein levels that there was no increase in growth of the host component in the coral *Stylophora pistillata* over a 14-day period following addition of ammonium (20 μ*M*) or phosphate (2 μ*M*) to sea water. Similarly, Hoegh-Guldberg and Smith (1989) showed no significant increase in protein per surface area in *Seriatopora hystrix* and *Stylophora pistillata*, although the mean value for the latter coral was higher in the presence of N than in the control. Furthermore, Stambler et al. (1991) showed that nutrients (N and P) decreased

linear extension in corals and there was no clear evidence for an increase in animal protein per unit surface area.

Although P-supplementation affected the chemical composition of the host's tissue (decreased C:P ratio) (Fig. 3.2), its effect did not translate into enhanced soft-tissue growth (Fig. 3.3) or shell extension (Chapter 4; Belda et al., 1993a). This is not surprising as protein synthesis is probably limited at the translational level by the supply of amino acids (Muscatine et al., 1989), whereas phosphorus plays a more important role in the metabolic energy cycles of the cell.

3.4.2 C:N:P Ratio in Host Tissue

As discussed earlier, C:N:P ratios have been widely used to determine the nutritional status of algae on the basis that either deficiencies in, or, alternatively, an increased availability of, a particular nutrient will be reflected in this ratio. In symbiotic associations such as giant clams and their zooxanthellae, the response of each partner to the increased availability of C, N, or P is complicated by the transfer of metabolites between the two organisms. The response exhibited by each partner will be dependent on the metabolic independence of each organism.

The C:N ratio of the host tissue of the control clams (5.9 ± 0.1, see Fig. 3.2) is greater than the average C:N ratio of their zooxanthellae (5.3 ± 0.3, see Table 3.1). The decrease in C:N ratio of host tissue in response to both N- and

N+P-supplementation (Fig. 3.2) is predictable and in line with the increase in host biomass. This change in host C:N ratio, which has also been demonstrated in corals (Muscatine et al., 1989), may be due to one of two processes: either (a) the assimilation of nitrogen-rich photosynthate from the zooxanthellae, or (b) the fixation of ammonium by the host combined with increased availability of photosynthate (glucose/glycerol) from the zooxanthellae. The latter option is an attractive proposition. High levels of glutamine synthetase present in the clam (Rees et al., in press) support this rather than the alternative that the zooxanthellae are responsible for ammonium assimilation (D'Elia et al., 1983) in zooxanthellate symbioses, with the host as a passive partner. Indeed, the level of this glutamine synthetase in host tissues is regulated by the availability of ammonium (Rees et al., in press).

The addition of P, however, had no effect on the C:N ratio (Fig. 3.2). This again is in agreement with the results of Muscatine et al. (1989), and is consistent with the cellular role of phosphorus in energy status, intermediary metabolism, and replication, rather than tissue production.

While the effect of N+P combinations did not dramatically affect the C:P ratio, N- and P-supplementation had opposite effects (Fig. 3.2). P brought about a significant decrease in the C:P ratio, indicating the capacity for higher levels of P in clam tissues. On the other hand, the dramatic increase in C:P ratio following N-supplementation could be explained by an increase in glycogen or lipid storage,

resulting from a greater flow of photosynthate into the soft-tissue component due to the greater number of zooxanthellae. The increase in soft-tissue weight (Fig. 3.3) supports this, while not eliminating the possibility that there has been little or no increase in the total phosphate.

3.4.3 Zooxanthellae Population

Despite no change in its C:N:P ratio (Table 3.1), the zooxanthellae population increased in response to N and N+P (Fig. 3.4) in agreement with other studies on clams (Fitt et al. 1992) and corals (Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989). However, there was no significant increase in mitotic index (or mean specific growth rate) of the zooxanthellae in the N- or P-supplemented clams following three months' exposure to the nutrients (Fig. 3.8). The increase in zooxanthellae population without an increase in mitotic index in N-supplemented clams may be explained in the following way. Zooxanthellae population numbers may quickly adjust to the nutrient regime and then reach a steady state, reflecting a constant mitotic index (Hoegh-Guldberg and Smith, 1989). Consequently, an increase in mitotic index at this nutrient regime will only be seen during the adjustment period. This is typical of algal cells growing in continuous culture (Goldman, 1977). Additionally, the clam may be able to support a greater retention of zooxanthellae in the presence of increased ammonium, and expel fewer algae in their faecal pellets (Trench et al., 1981). In contrast, the N+P-supplemented clams did show an increase in mitotic index and

mean specific growth rate compared to the controls, even though, for instance, the zooxanthellae populations of the 5N+2P- and 5N+5P-treated clams were less than those of the 10N-treated clams (Fig. 3.4). It seems likely that after three months of exposure to these nutrients, the population numbers of zooxanthellae in the N-supplemented clams and those in the N+P-supplemented clams were already adjusted to a level which can be supported by these nutrient regimes. However, those exposed to N+P could maintain a higher mean specific growth rate, which was apparently a function of the increased availability of P, than those exposed to N alone. Additionally, the lower population numbers at N+P regime could mean that more zooxanthellae were being lost in the faeces at this nutrient regime (pers. obs.).

Viewing the increase in soft-tissue weight, zooxanthellae population, and mitotic index together, a subtle break in this connection appears to have developed as evidenced by a comparison of the response of these parameters to N- and N+10P-supplementation. N-supplementation resulted in an increase in soft-tissue weight comparable to the response with the N+P combination (Fig. 3.3), yet the zooxanthellae population (Fig. 3.4) and specific growth rate (Fig. 3.8) were significantly lower with N-supplementation than with N+P combination. In the presence of N+P-supplementation, it appears that the zooxanthellae are more independent of the host and can support a sustained period of division, and this is presumably coupled with the increased rate at which the animal host expels the zooxanthellae in its faeces in the presence of increased concentrations of both N

and P.

3.4.4 Chl *a* Levels

The significant increase in zooxanthellae population in nutrient-supplemented clams is accompanied by reduced Chl *a* per zooxanthella (Figs. 3.4 and 3.5). Chl *a* content per zooxanthella from field samples of the coral *Seriatopora hystrix* was inversely related to zooxanthellae density, which is consistent with phytoplankton studies suggesting N-limitation of the zooxanthellae (Hoegh-Guldberg and Smith, 1989). In contrast, Chl *a* per zooxanthella from *S. hystrix*, following exposure to 20 μ*M* ammonium, did not change significantly (Hoegh-Guldberg and Smith, 1989). There was also no significant change in the Chl *a* per zooxanthella from the coral *Stylophora pistillata* following exposure to ammonium, while the zooxanthellae density and Chl *a* cm^{-2} increased (Hoegh-Guldberg and Smith, 1989), suggesting no link between these parameters. Likewise, Muscatine et al. (1989) found that Chl *a* per zooxanthella in *S. pistillata* was unaffected by N- and P-supplementation after 14 days. In clams, however, an increase in ammonium resulted in a decrease in Chl *a* content. Such difference in response between clams and corals may be due to their different physiology, zooxanthellae being intracellular in corals and extracellular in clam. Alternatively, it is possibly a function of considerably longer exposure time of clams to ammonium than in similar experiments with corals.

The C:N ratio (refer to Table 3.1) of the zooxanthellae from N-supplemented clams (5N, C:N ratio = 5.7 ± 0.2; 10N = 5.8 ± 0.2) was not significantly different from that of the control (5.3 ± 0.3), indicating that the N-status had not changed with N-supplementation despite an increase in zooxanthellae population and total Chl *a* per clam, and a decrease in ammonium uptake capacity (Chapter 5; see also Rees, 1991). Therefore, N-status would not appear to be the cause of the decrease in Chl *a* content per zooxanthella. Iron-deficiency is known to result in a reduction in pigment concentration in eukaryotic marine algae (Greene et al., 1992). It is possible that the increased zooxanthellae population resulted in iron deficiency in the zooxanthellae. A very likely explanation is a decrease in size of zooxanthellae from clams incubated in the presence of N-supplemented sea water, resulting in a decrease in Chl *a* content per cell. However, zooxanthellae size was not measured in this study (but see Chapter 5). Also, Hoegh-Guldberg and Smith (1989) detected no significant change in zooxanthellae size when intact corals were incubated in elevated ammonium.

Despite the reduced Chl *a* per zooxanthella in this study, there was a significant increase in the total Chl *a* content in the clams' mantle tissues (Fig. 3.6), attributable to the increased numbers of zooxanthellae. An increase in Chl *a* per area reflects an increase in the photosynthetic capacity of the association (Hoegh-Guldberg and Smith, 1989). A resulting increase in the synthesis of organic material translocated to the animal host supports the observed changes in the biomass parameters of clams supplemented with N or N+P. Alternatively, the

increase in host biomass parameters with N- or N+P-supplementation may also be attributed to the increased assimilation of inorganic nitrogen by the host's own ammonium-assimilation enzymes (Rees et al., in press). This latter explanation is more appealing considering that the photosynthetic rate per zooxanthella decreases when the zooxanthellae grow faster, resulting in proportionally less photosynthate being translocated to the host (Falkowski et al., 1993). Nevertheless, a combination of increased total photosynthate translocation and host ammonium-assimilation cannot be discounted.

3.4.5 Host Control Over Zooxanthellae Growth

That the clam host may have some control over the growth of its symbionts is consistent with the finding that, regardless of ambient nutrient concentrations in the sea water, the C:N:P composition of the zooxanthellae in clams remained relatively constant (Table 3.1). Haemolymph, not sea water, is the immediate supply source of nutrients for the clam zooxanthellae. However, the sea water concentration of ammonium influences the haemolymph concentration and is important in determining its availability (Fitt et al., in prep.), particularly as ammonia can diffuse freely across membranes in significant amounts. Inorganic phosphate, however, is always charged and, hence, does not passively cross membranes. In fact, its concentration in the clam haemolymph is extremely low ($< 0.1 \mu M$) (Chapters 5 and 6).

Deviations from the average C:N:P (Redfield) ratio of marine phytoplankton (106:16:1) (see Atkinson and Smith, 1983) are used to infer which particular nutrient is limiting algal growth. The mean C:N ratio of the clam zooxanthellae is 5.9:1, which is below the Redfield C:N ratio of 6.6 and that of N-sufficient zooxanthellae grown in culture (Domotor and D'Elia, 1984). On the other hand, the clam zooxanthellae's mean C:P ratio of 303:1 is about three times as high as the Redfield C:P ratio of 106:1. Stambler et al. (1991) also found that C:P ratios in zooxanthellae from the coral, *Pocillopora damicornis*, although variable, were always greater than the Redfield ratio. Additionally, phytoplankton deprived of P during growth generally have an N:P ratio >30:1, while those deprived of N have an N:P ratio <10:1 (Atkinson and Smith, 1983). Clam zooxanthellae had an N:P ratio of 52:1. On the basis of the Redfield ratio, therefore, clam zooxanthellae are more deficient in P than in N.

Zooxanthellae isolated from both corals and sea anemones show a decrease in C:N ratio following exposure of the host to nutrient-supplementation. For *Stylophora pistillata* (Muscatine et al., 1989) and *Pocillopora damicornis* (Stambler et al., 1991), the C:N ratio of their isolated zooxanthellae decreased following exposure to ammonium, while zooxanthellae isolated from nutrient-replete sea anemones had a lower C:N than those from field-collected animals (Cook et al., 1988). The C:N ratios in these studies were higher than those observed with clam zooxanthellae, and, unlike the results reported here, the C:N ratio of the zooxanthellae from corals and sea anemones was responsive to an

increase of nitrogen availability. The determination of C:N ratios in zooxanthellae derived from cnidarian associations is difficult due to the contamination of algal cells with host protein or membrane. With clams there is negligible, if any, such contamination of the extracellular zooxanthellae. The differences in purity of the zooxanthellae preparations may yet be found to account for the discrepancy between the respective studies.

P-limitation in the clam zooxanthellae is also consistent with absence of polyphosphate and constant acid phosphatase activity in extracts of freshly-isolated cells (Table 3.2) (cf. Jackson et al., 1989). Inorganic polyphosphates are known to act as phosphate reserves for algal cells (Raven, 1984). In phosphate-replete cells, polyphosphates usually accumulate; whereas, in phosphate-deficient cells, polyphosphates are usually low or undetectable (Jackson, 1989; Harold, 1966). The ^{31}P-NMR spectra of zooxanthellae extracts from control and P-supplemented clams did not show any polyphosphate peak. In *Symbiodinium* sp. cultured under phosphate repletion, polyphosphate is present in high concentrations as revealed by *in vivo* NMR spectrum (Jackson, 1989). The same study found low, if not undetectable, polyphosphate levels in zooxanthellae freshly isolated from the coral *Acropora formosa*, an observation consistent with zooxanthellae in the coral existing in a state of phosphate deficiency. Clam zooxanthellae have similarly negligible levels of polyphosphate.

Acid phosphatase levels in zooxanthellae have been shown to be regulated by the availability of phosphate (Jackson et al., 1989). The level of acid phosphatase observed in the zooxanthellae freshly isolated from clams, regardless of the nutrient regime, is similar to that found in algae freshly isolated from the coral *Acropora formosa*, and considerably more than that found in zooxanthellae cultured in non-limiting phosphate concentrations.

The lack of response in the zooxanthellar C:N:P ratio to increased ammonium levels is in contrast to the increase in zooxanthellae population. Fitt et al. (in prep.) observed that an increase in ammonium in sea water was reflected in the clam haemolymph concentration. This, combined with a decrease in the rate of ammonium uptake by zooxanthellae isolated from N-treated clams (see Chapter 6) indicates that the zooxanthellae have access to this nutrient and can rapidly adjust to its increased availability. Zooxanthellae isolated from corals exposed to elevated ammonium also displayed a decreased rate of ammonium uptake (Yellowlees et al., in press). This adaptation to N-supplementation is contrary to the zooxanthellae's response to P-supplementation, where no change in acid phosphatase level was evident, suggesting that P-supply continues to limit growth. Nevertheless, both N- and P-supplementation are necessary for an increased growth rate of the zooxanthellae, and, even then, the zooxanthellae were not growing *in vivo* at or near their maximal rates. Their specific growth rate (4-10%) (Fig. 3.8) is at the lower end of the growth-rate spectrum of algae in general (see Wilkerson et al., 1988).

The results from this study are summarised in Table 3.3. The increase in algal population and the decrease in glutamine synthetase (Rees et al., in press) and ammonium-uptake capacity following N-supplementation are evidence for N-limitation, while the C:N ratio is not typical of N-deficient cells (see Rees, 1991). On the other hand, the evidence for P-limitation is unambiguous, with constant and high acid phosphatase levels, absence of polyphosphate, and high C:P ratio consistent with P-limitation. It appears that zooxanthellae in clams have access to increases in the availability of ammonium and can readily adjust to increased availability of this nutrient, while any increase in ambient phosphate is not accessible to the algae. The lack of decrease in C:P ratio and no change in zooxanthellae density following P-supplementation (2P and 5P) are consistent with this interpretation. N-limitation of the zooxanthellae is, therefore, a function of the availability of ammonium to the symbiosis; while, irrespective of nutrient levels in sea water, clam zooxanthellae exhibit characteristics of P-limitation, possibly through some control by the animal host. Nevertheless, the zooxanthellae are able to sustain a significantly higher mitotic index in the presence of a combination of N and P.

Table 3.3. Summary of responses of the symbiotic partners to nutrient supplementation

Nutrient regimen	Clam host	Zooxanthellae
N	Decrease in C:N; increase in C:P; decrease in glutamine synthetase levels[a]; increased amount of soft tissue	No change in C:N:P; increase in zooxanthellae numbers; decrease in chl *a* content; no change in mitotic index and specific growth rate; decrease in ammonium-uptake capacity
P	No change in C:N; decrease in C:P; no change in amount of soft tissue	No change in C:N:P; no increase in zooxanthellae numbers,except with 10P; decrease in chl *a* content; no change in mitotic index and specific growth rate; polyphosphate absent; no change in acid-phosphatase activity level
N+P	Decrease in C:N; increase in C:P; increased amount of soft tissue	No change in C:N:P; increase in zooxanthellae numbers; decrease in chl *a* content; increase in mitotic index and specific growth rate; no change in acid-phosphatase activity level

[a] Rees et al (in press)

Chapter 4 N- AND P-SUPPLEMENTED *Tridacna gigas* IN TANKS: PERTURBATION OF CALCIFICATION

4.1 INTRODUCTION

The genesis and structural maintenance of coral reefs are dependent on calcifying organisms such as corals and coralline algae. These reef-builders normally flourish in tropical waters of generally low nutrient concentrations, and hence, low phytoplankton levels and good water quality. Ambient nutrient concentrations, however, widely vary in space and time. Open ocean reefs in the Pacific Region have nutrient levels ranging between 0 - 4 μM nitrogen and 0 - 0.6 μM phosphorus (Crossland, 1983; Kinsey, 1991). On fringing and inshore reefs of the Great Barrier Reef, dissolved nitrogen (ammonium, nitrite, and nitrate) concentrations greater than 1 μM (Crossland and Barnes, 1983) and phosphate concentrations greater than 2 μM (Rasmussen, 1988) have been recorded.

Elevated nutrient concentrations can be brought about by natural events such as land run-off, and oceanic upwellings and intrusions. Of increasing relevance, however, are anthropogenic influences such as effluent outfalls and agricultural run-off. Indeed, there has been increasing public and scientific concern about the effects of nutrient elevations on reef health in relation to community structure and calcium-carbonate deposition.

One outstanding documented case is the effect of sewage outfall in Kaneohe Bay, Hawaii. Elevated nutrients brought about a major shift in community structure, with the death of much of the fringing coral reef. Calcification rates were also severely affected (Kinsey, 1988). Similarly, a controlled reef-fertilisation experiment (Kinsey and Davies, 1979) at One Tree Island, Great Barrier Reef, showed > 50% suppression in coral calcification with exposure to phosphate (2 μ*M*) and nitrogen (20 μ*M* urea + ammonium). Such effect was attributed primarily to phosphate.

To date, no similar investigations have been carried out on giant clams, which are important, though less abundant, calcifying constituents of coral reefs. In this study, the effects of N and P on the calcification of *Tridacna gigas* were examined. Tridacnid shells are wholly aragonitic, with an inner complex crossed-lamellar shell layer bounded in extent by a trace of the pallial line, and an outer crossed-lamellar shell layer (Taylor et al., 1973). Here, the internal morphology of the outer layer and the crystal lattice parameters of the aragonite based on x-ray diffraction, along with the shell's extension rate and dry weight, were compared at different nutrient regimens. Giant clams have significant advantages in studies of this nature because of the relative ease with which they can be cultured, and their growth history, as recorded in their shells, can be accurately examined with relatively easy sampling. In addition, as reef-dwelling, symbiotic bivalves, the calcification responses of giant clams have relevant applications for reef corals.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design and Maintenance

See Section 3.2.1 of Chapter 3.

4.2.2 Measurement of Shell Growth

Initial and final shell lengths were measured to the nearest 0.02 mm using vernier calipers. At the termination of the experiment, shells stripped of living tissue were cleaned, air-dried, and weighed to the nearest 0.1 g.

4.2.3 Scanning-electron Microscopy (SEM)

Fractured sections of the outer shell layer above the pallial line were mounted on a stub and vacuum-coated with gold-palladium for 5 min in a Technics gold sputter. The shell samples were then scanned using either a JEOL JXA-840A electron probe microanalyser (500x and 2500x magnification), or a Phillips XL20 scanning electron microscope fitted with a Tracor energy-dispersive analytical system (8500x magnification). Photomicrographs were produced using Ilford HP5 black-and-white film.

4.2.4 X-ray Diffractometry (XRD)

Small fragments were removed from the shell's edge, ground in acetone into a fine powder (ca. 10-20 μm particle size), and smear-mounted on a glass slide. Care was taken to ensure that each smear was uniform and smooth. Each sample was then scanned on a Rigaku 2155D5 horizontal diffractometer, which was fitted with a post-diffraction, curved graphite-crystal monochromator, and which utilised Cu Kα radiation (λ = 0.154178 nm). A 2θ scan was run from 26° to 36° 2θ at a step size of 0.01° 2θ and a counting time of 5 s. Replicate analyses indicated that these conditions were consistent with a precision of ± 0.012° 2θ. The interpretation of the resulting x-ray diffraction patterns entailed measurement of 2θ (the angle between the incident and diffracted rays on a set of parallel planes) for planes with Miller indices of (012) and (200) in the aragonite crystal. These crystal planes were chosen as previous work on corals by Rasmussen et al. (in press a,b) showed that these particular planes are sensitive to changes in ambient nutrient levels. Peak positions in terms of 2θ were measured with reference to a Silicon calibration standard (NBS 640). Using a standard orthorhombic aragonite pattern as a guide (Joint Committee on Powder Diffraction Standards, Swarthmore, Pennsylvania, USA, File No: 5-0453), the degree of shifting of 2θ values, the peak profiles, and the multiplicities of reflections (012) and (200) for nutrient-supplemented shells relative to control shells were then examined for indications of differences in the crystal structure.

4.2.5 Statistical Analyses of Data

For relevant explanation of statistical handling of the data, refer to Section 3.2.7 (Chapter 3) . Equal samples of shell-length measurements (n = 19), shell-weight measurements (n = 16), shells for SEM (n = 3), and shells for XRD (n = 5) were taken randomly to allow for balanced factorial analyses of the data. To test the hypothesis that elevated levels of N and P have no effect on clam calcification at $\alpha = 0.05$, data were analysed using two-way ANOVA (fixed factors) without an interaction term, linear contrast, linear-regression analysis, and analysis of covariance (Underwood 1981, Zar 1984). All statistical analyses were performed using the analytical software package, Statistix (Version 3.0). Tests of the assumptions underlying the analytical procedures were made prior to performing each analysis. In all cases, no serious violations of the assumptions of normality, variance homogeneity, and additivity were evident ($p > 0.05$) (Shapiro and Wilk 1965, Winer 1971, Shapiro and Francia 1972).

4.3 RESULTS

4.3.1 Shell-extension Rate and Shell Weight

The shell-extension rate of the clams significantly increased with addition of N (ANOVA, $F = 7.32$, $df = 2,6$, $p < 0.05$), but not with P (ANOVA, $F = 0.28$, $df = 3,6$, $p > 0.05$) (Fig. 4.1). Furthermore, there is a significant contrast between the control and N- or N+P-supplemented clams (Contrast, $F = 7.29$, $df = 6$, $p < 0.05$). Fig. 4.1 inset shows these effects more clearly.

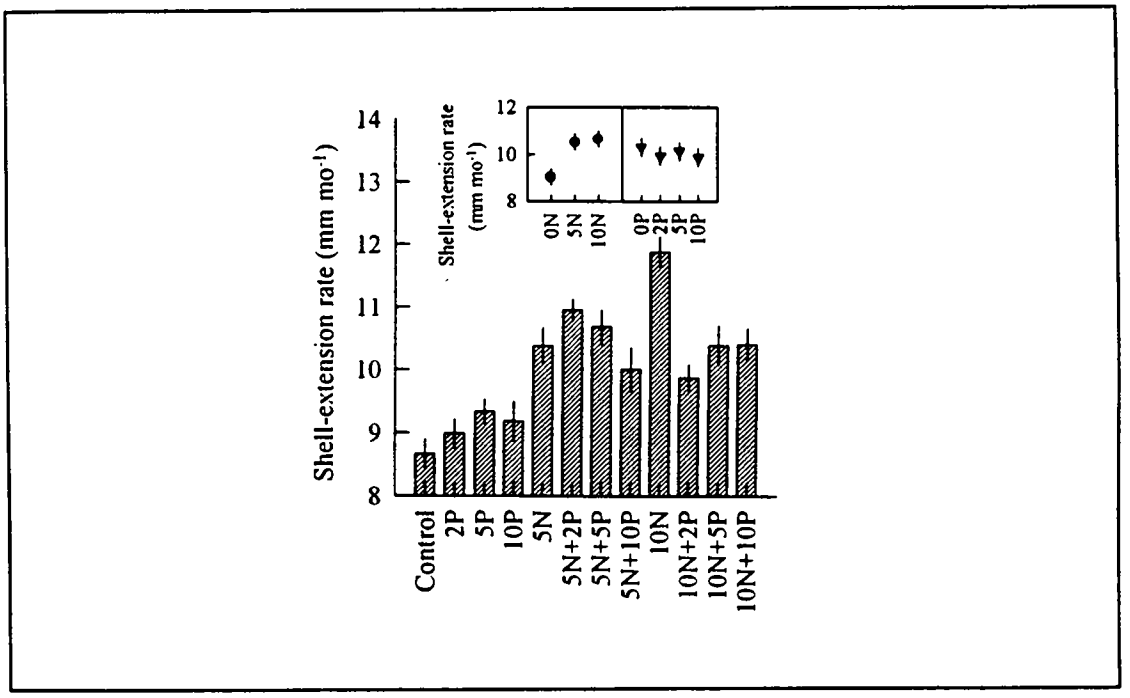


Fig. 4.1. Mean shell-extension rates (± SE) of *Tridacna gigas* supplemented with or without ammonium (N) and phosphate (P) in μ*M* concentrations. ($n = 19$). Inset: Effect of N, averaged across P treatments, and effect of P, averaged across N treatments, on extension rates.

Fig. 4.2 presents a plot of the log-transformed values of shell weight and shell length following three months' exposure to the different nutrient regimes. Correlation between the shell parameters was high. A comparison of the regression lines showed that the growth coefficients are significantly different (ANCOVA, $F = 3.96$, $df = 3,56$, $p < 0.05$), although the difference lies mainly with the intercepts (ANCOVA, $F = 34.67$, $df = 3,59$, $p < 0.0001$). That is, the nutrient-treated shells had intercepts significantly lower than the control.

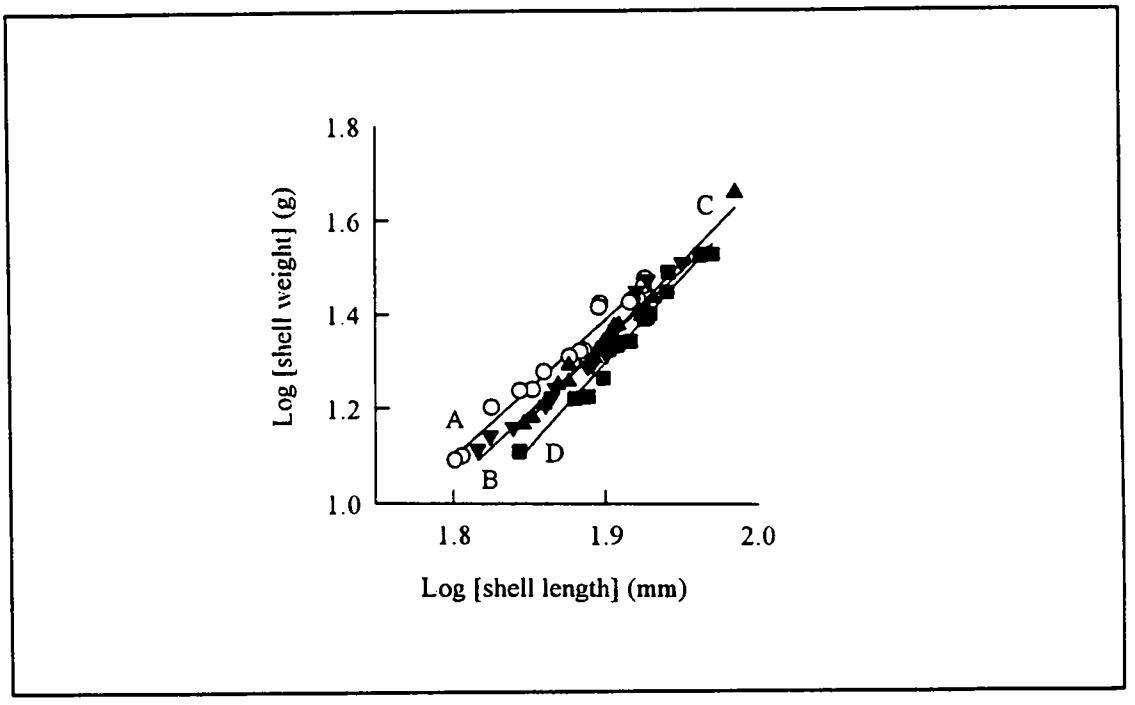


Fig. 4.2. Shell length versus shell weight in *Tridacna gigas* exposed to different nutrient levels for 3 mo (○: Control, A; ▼: 2 μ*M* phosphate, B; ▲: 5 μ*M* ammonium, C; ■: 5 μ*M* ammonium + 2 μ*M* phosphate, D). Regression equations plotted onto log-transformed values are y = 2.92x - 4.16, $r^2 = 0.98$ (A); y = 3.00x - 4.36, $r^2 = 0.96$ (B); y = 3.34x - 5.00, $r^2 = 0.98$ (C); and y = 3.60x - 5.55, $r^2 = 0.98$ (D); $n = 16$. Plots for other nutrient regimens not shown for simplicity

A further analysis of shell weight, after adjustment for differences in shell length using a weighted average of slopes from regression analysis (Packard and Boardman 1988), revealed that shell weight was significantly reduced with addition of N (ANOVA, $F = 33.86$, $df = 2,6$, $p < 0.001$) and P (ANOVA, $F = 9.5$, $df = 2,6$, $p < 0.01$) (Fig. 4.3).

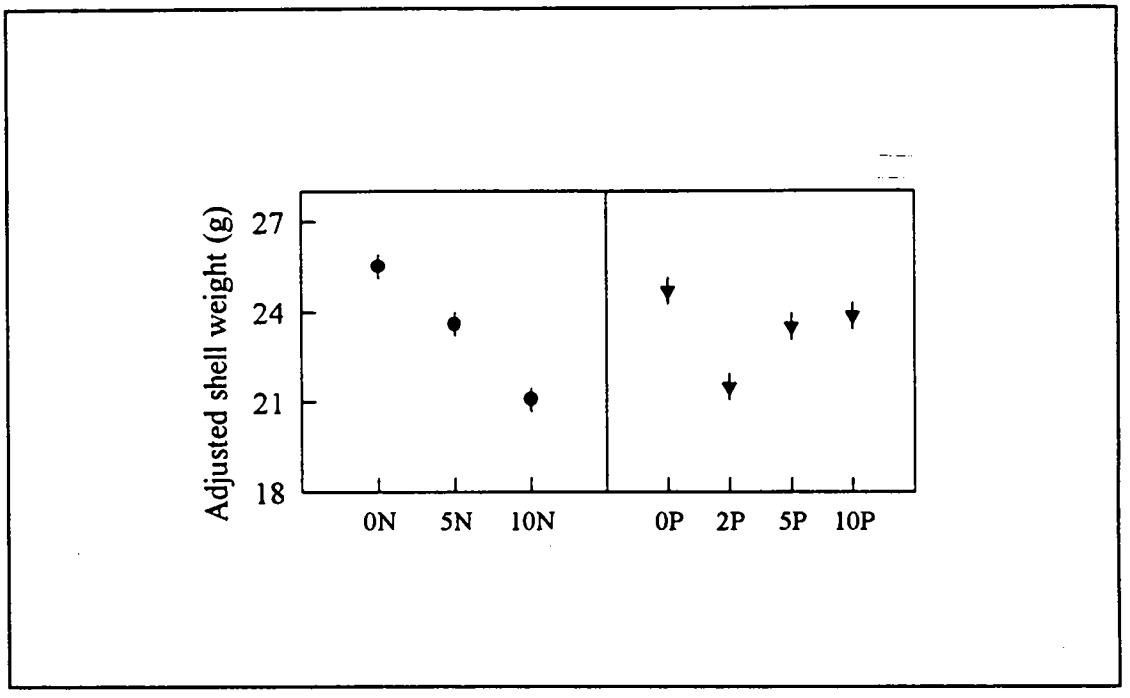


Fig. 4.3. Effect of N, averaged across P treatments, and effect of P, averaged across N treatments, on shell weight adjusted for differences in shell length (± SE) using a weighted average of slopes from regression analysis. Nutrient regimens are different concentrations (μM) of ammonium (N) and phosphate (P). ($n = 3$)

4.3.2 Shell Microstructure Based on SEM

In fractured cross-sections of the growth rim of the clams, the microstructure of the outer layer in each shell was similar at different points parallel to the shell margin. However, striking differences were evident in the crystal morphology and shell fabric of the control and the nutrient-supplemented clams. The outer layer of the control clams consisted of relatively solid and closely-packed, elongate crystals (Fig. 4.4A), which were arranged in a crossed-lamellar fashion typical of a tridacnid outer shell layer (Fig. 4.5A) (Taylor et al., 1973; Moir, 1990). Clams exposed to N, P, and N+P, on the other hand, had misshapen crystals, increased occurrence of cavities, and distorted crossed-lamellar arrangement in their shell fabric (Figs. 4.4B-D, 4.5B-D). In particular, P led to a thinner layering of crystals which formed in plates, while N brought about a chaotic arrangement of spheroidal crystal clusters. The N+P combination also created a chaotic arrangement and combined the perturbations observed following separate exposures to N and P.

Fig. 4.4. Representative SEMs of fractured sections of the outer shell layer, showing shape and packing of aragonite crystals in *Tridacna gigas* grown in normal seawater (A) and sea water supplemented with 2 μ*M* phosphate (B), 5 μ*M* ammonium (C), and 5 μ*M* ammonium + 2 μ*M* phosphate (D). Micrographs for other nutrient regimens not shown; scale bar = 1 μm (high magnification)

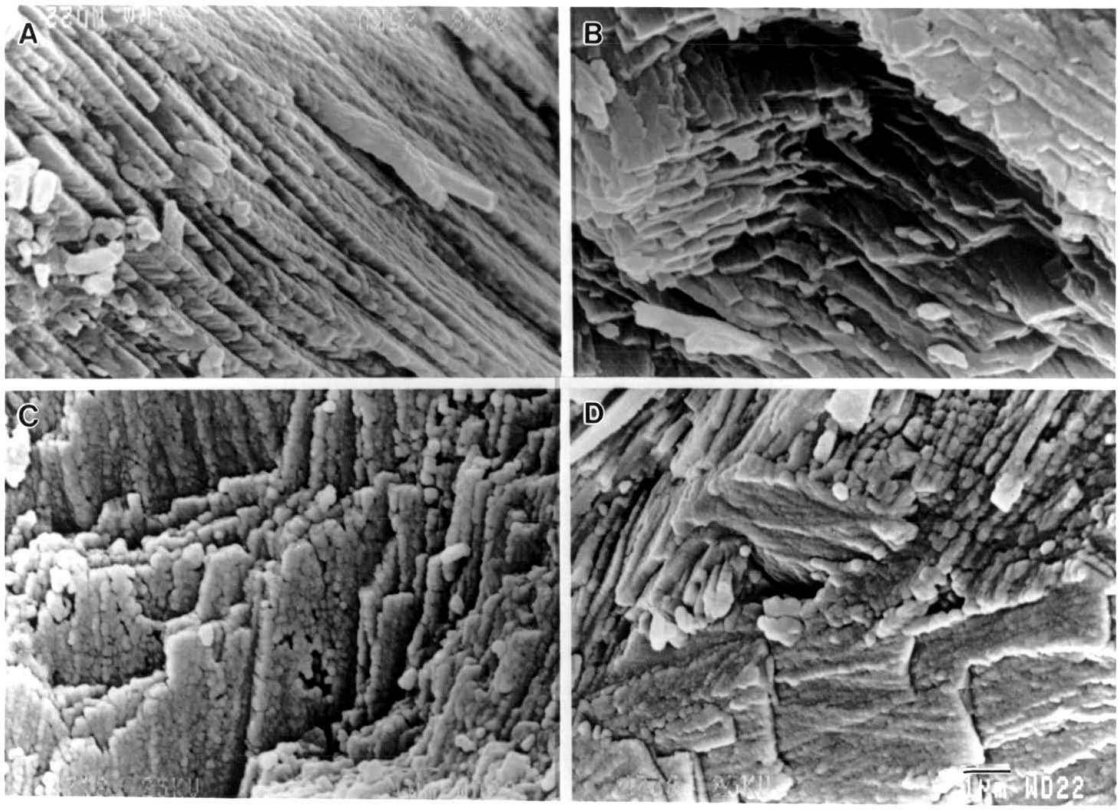
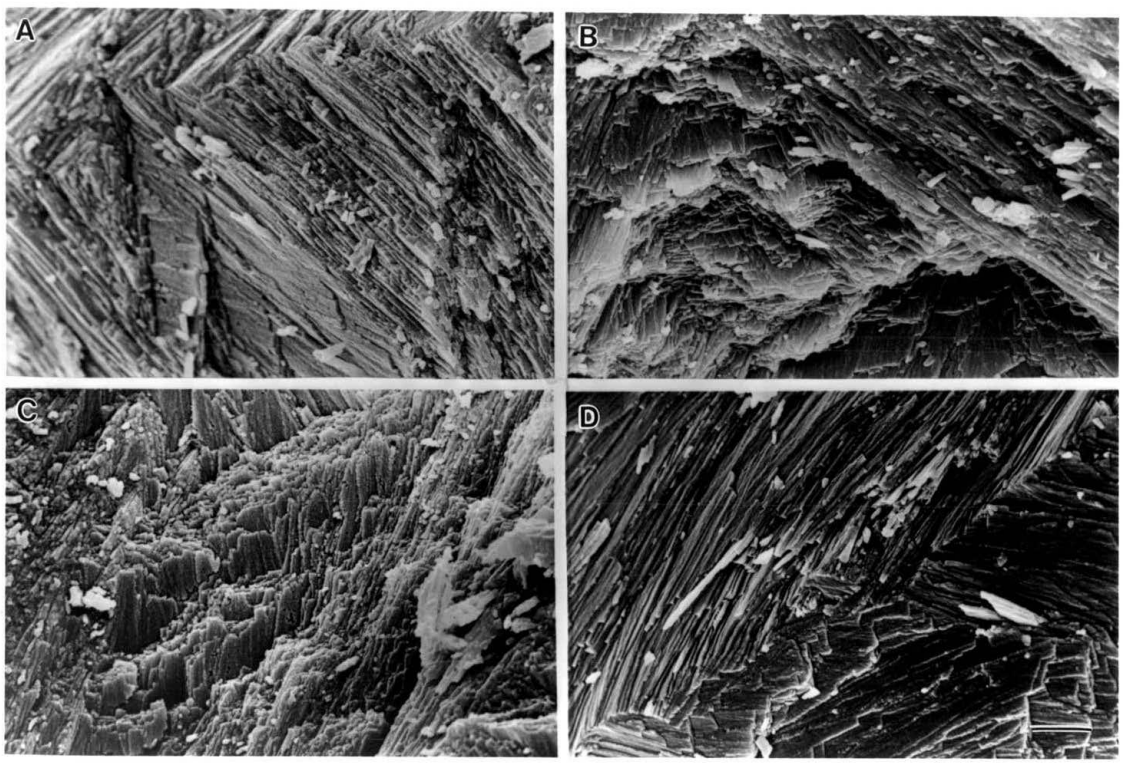

A
B
C
D
ND22

Fig. 4.5. Representative SEMs of fractured sections of the outer shell layer, showing packing and growth orientation of aragonite crystals in *Tridacna gigas* grown in normal seawater (A) and sea water supplemented with 2 μ*M* phosphate (B), 5 μ*M* ammonium (C), and 5 μ*M* ammonium + 2 μ*M* phosphate (D). Micrographs for other nutrient regimens not shown; scale bar = 10 μm (low magnification)


A
B
C
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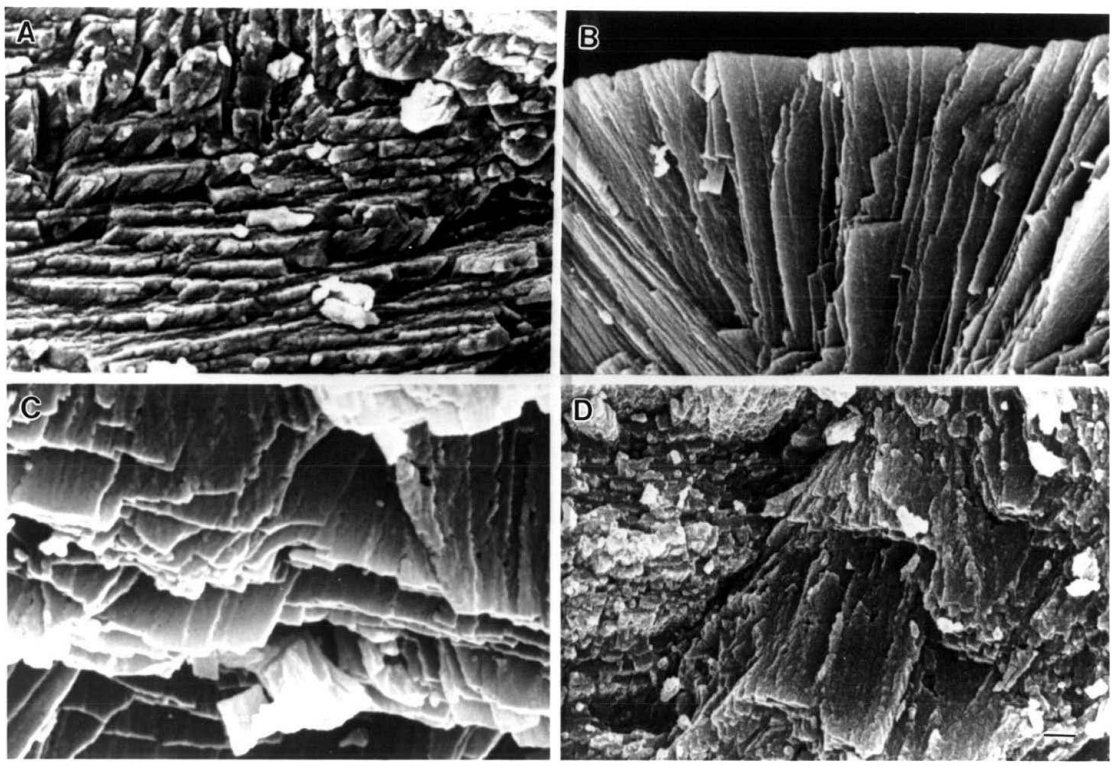
These microstructural changes are presented in greater detail in Fig 4.6. The control sample (Fig. 4.6A) had a regular arrangement of tabular to blocky aragonite crystals with little porosity. The 5P sample (Fig. 4.6B), on the other hand, showed the crystals as extremely thin sheets in a fan-like arrangement, rather than the regular stacking observed in the control. In the 10P sample (Fig. 4.6C), the crystals were still sheet-like but some of them had a well-developed curvature. Additionally, significant porosity was present. The 5N sample (Fig. 4.6D) was similar to the 10P sample, with curved, sheet-like crystals and well-developed porosity. However, if anything, individual crystals and the whole fabric were even more disturbed than in the 10P sample.

Fig. 4.6. Representative SEMs of fractured sections of the outer shell layer, showing, in greater detail, features of crystal morphology and fabric in control *Tridacna gigas* (A) and after addition of 5 μ*M* phosphate (B), 10 μ*M* phosphate (C), and 5 μ*M* ammonium (D). Micrographs for other nutrient regimens not shown; scale bar = 2 μm (high magnification)


A
B
C
D

4.3.3 Crystallographic Structure Based on XRD

A plot of the 2θ values for the (200) and (012) reflection planes is shown in Fig. 4.7. Clustering of the data points for the control clams is evident, as well as a systematic shift of data points to larger values with addition of N, P, and N+P. This shifting of peak positions of the diffracted x-ray beam, however, is not statistically significant (ANOVA, at (012) plane: (a) N effect, $F = 2.17$, $df = 2,6$, $p > 0.05$, (b) P effect, $F = 1.09$, $df = 3,6$, $p > 0.05$; at (200) plane: (a) N effect, $F = 2.04$, $df = 2,6$, $p > 0.05$, (b) P effect, $F = 0.46$, $df = 3,6$, $p > 0.05$). Nevertheless, from Bragg's Law (Sands 1969), a shift to a larger angle of 2θ, as observed here, indicates changes in the interplanar spacings within the crystal structure, deviating from those of the standard orthorhombic aragonite. The presence of these structural irregularities is consistent with the visual modification in the shell fabric's structural integrity, as noted from the SEM study.

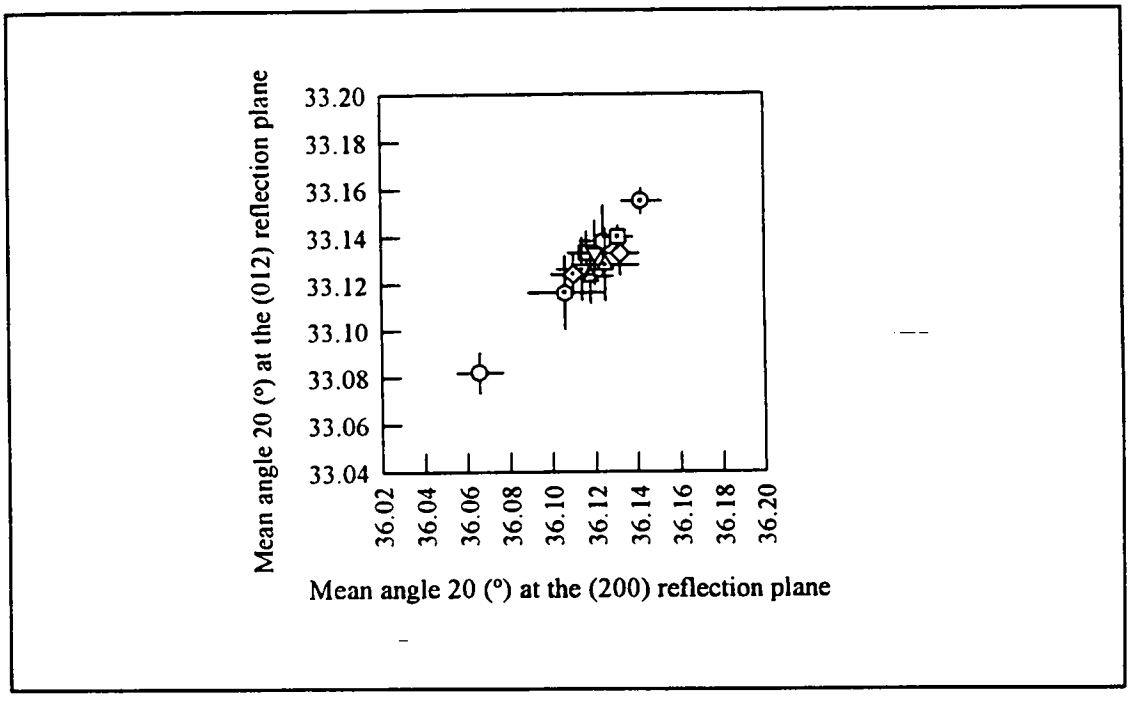


Fig. 4.7. Mean angle 2θ values (± SE) at reflection planes with Miller indices of (012) and (200) in the aragonite crystal of *Tridacna gigas* exposed to different levels of ammonium (N) and phosphate (P); (n = 5) (○: Control, □: 2 μ*M* P, △: 5 μ*M* P, ▽: 10 μ*M* P, ◊: 5 μ*M* N, ◭: 5 μ*M* N + 2 μ*M* P, ⊙: 5 μ*M* N + 5 μ*M* P, ⊡: 5 μ*M* N + 10 μ*M* P, ◬: 10 μ*M* N, ▽: 10 μ*M* N + 2 μ*M* P, ◊: 10 μ*M* N + 5 μ*M* P, ▼: 10 μ*M* N + 10 μ*M* P).

4.4 DISCUSSION

Perhaps the most striking result from this study is that despite the enhanced shell-extension rate observed with nutrient-supplemented clams, they had a reduced amount of deposited shell material relative to the control clams. Indeed, in the case of N-supplemented clams, this was visually apparent as their shells were almost transparent. This is consistent with the observation that during the procedure of mounting on SEM stubs, shells exposed to high nutrient levels were very friable and fragmented upon contact with tweezers and pressure. While the reduction in calcium-carbonate deposition was greatest at mixed concentrations of ammonium and phosphate (Fig. 4.2), both nutrients individually caused a decrease in calcification. The significantly lower regression intercepts and adjusted shell weights demonstrate that at equivalent shell size, clams gained less shell weight in nutrient-supplemented sea water than control clams over three months of nutrient enrichment.

In vitro studies with phosphatic and calcareous skeletons suggest that phosphates can act as crystal poisons that inhibit the deposition of calcium salts in skeletal structures (Simkiss, 1964). Similar suppression of calcification was observed with corals *in situ* (Kinsey and Davies, 1979), following a controlled reef-fertilisation experiment using 2 μ*M* P and 20 μ*M* urea + N. This effect was mainly attributed to phosphate because of the observations of Simkiss (1964) and the lack of previous evidence for suppression of calcification by nitrogen. The results in the present study indicate that ammonium depresses calcification in

clams and the effect of elevated ammonium levels on coral calcification should now be seriously considered.

The nutrient effects on clam shell formation are probably related to the microstructural changes observed in the outer shell layer, being more porous, with loosely-packed aragonite crystals compared to the control clams. In addition, there is general consensus that most shell microgrowth increments reflect variations in the relative proportions of organic matrix and calcium carbonate within the molluscan shell (Lutz and Rhoads 1980, Simkiss and Wilbur 1989). It is, therefore, possible that in clams exposed to nutrient-supplemented sea water, there was increased synthesis of the organic matrix. This could have enhanced skeletal extension, while weakening the skeletal structure due to reduced calcium carbonate.

Microstructural changes in corals, as a consequence of phosphate exposure, have previously been reported to result in a general weakness in the aragonitic skeleton (Rasmussen 1988). Thus, the observed development of porosity with increasing phosphate concentrations found by this study is comparable to the findings of Rasmussen (1989) in her experimental studies of *Acropora formosa.* Rasmussen (1989) also examined coral cores (*Porites* sp.) from the Great Barrier Reef Region, and revealed an erratic calcification history in recent times possibly related to an increase in phosphate levels in the area in modern times. The role of nitrogen sources was not considered.

The possibility that periods of anaerobiosis caused shell dissolution, leading to the microstructural changes observed in this study, can be ruled out. All experimental groups were concurrently held under the same climatic and experimental conditions, thereby eliminating major confounding sources of variation between treatments. Secondly conditions that would have led to reduced availability of dissolved oxygen to the cells were eliminated (Lutz and Rhoads, 1980). For instance, lowered water temperature and prolonged periods of shell closure were avoided by, respectively, conducting the experiment during summer months and keeping the clams well aerated. Furthermore, the XRD study showed changes occurring in crystal structure at elevated nutrient levels. Due to the number of replicates, the results of the statistical analysis of the XRD data were not significant; however, distinct shifts in the positions of reflections (012) and (200) relative to the control were discernible (Fig. 4.7). Indeed, ongoing investigation on the crystal structure of biogenic aragonite from high-nutrient environments using x-ray powder/single-crystal diffraction and convergent-beam electron-microscopy techniques indicates that the true symmetry may not be orthorhombic pnma but monoclinic C2/C, with α, β, and γ all being approximately equal to 90° (Ness, Bevar, and Cuff, pers. comm.).

The effects of phosphate on the precipitation of calcium carbonate have been considered of major importance in the marine environment. For this reason, the role of phosphate in the inhibition of calcium-carbonate nucleation, growth, and crystallisation has been widely studied (Reddy, 1977; Burton and Walter,

1990). Furthermore, other investigations have examined the action of foreign ions in the calcium-carbonate crystallisation, polymorphic transformation, and dissolution processes (Reddy, 1977; Mucci and Morse, 1983).

Polymorphism problems notwithstanding, little detailed work has been carried out regarding the precise nature of the inhibition or promotion mechanisms involved in the calcification process. Based on Langmuir-type adsorption models (Reddy, 1977), it is widely accepted that phosphate inhibits precipitation of calcium carbonate at defined surface sites through adsorption and blockage. The specific phosphate species involved in these processes for aragonite have yet to be identified, although it should be noted that the distance between the negatively-charged oxygen dipoles in phosphate and polyphosphate groups fairly closely matches that between the cation (calcium) sites in the crystal structure (ca. 0.39 nm).

On the other hand, the role of nitrogen-containing ions in calcification has yet to be established. This study is the first to report disrupting effects of nitrogen on calcification.

While more striking quantitative effects were observed with increasing nutrient concentrations (N and P) (Figs. 4.2-4.3), there were major structural effects even at the lower concentrations used (Figs. 4.4-4.6). These lower concentrations are ones which could conceivably be found in coral-reef waters.

Indeed, phosphate levels greater than 2 μ*M* have been reported in several instances, whereas ammonium levels of 5 μ*M* are only likely to be seen transiently since ammonium is rapidly taken up from the environment. Even during the height of pollution in Kaneohe Bay, ammonium levels did not rise above 3 μ*M*, presumably because of the large algal population in the area. Kinsey (1991), however, pointed out that a reduced rate of calcification was evident in Kaneohe Bay at only 1 μ*M* ammonium, and that in the One Tree Island experiment, a 3-4 h daily increase in nutrients was sufficient to affect the calcification rate *in situ*, based on an alkalinity anomaly technique they used (Kinsey and Davies 1979).

In Chapter 3 (Belda et al., 1993b), it has been clearly shown that increased concentrations of inorganic nitrogen and phosphorus in sea water substantially affected various physiological and biochemical parameters in the clam host and its zooxanthellae. Here, the calcification data from the same experiment show that elevated nutrient concentrations also have a significant perturbing influence on calcium-carbonate deposition by a reefal organism (Belda et al., 1993a).

If calcifying organisms, including reef corals, are affected not only by increased P, but also by increased N, in their natural environment, these results have important implications for organisms in reef waters subjected to increasing nutrient levels from anthropogenic sources. That is, any decrease in skeletal strength will result to greater vulnerability to various natural disturbances such as predation--especially on juvenile clams--by crushing organisms (e.g., crab,

octopus, starfish), strong waves, cyclones, and hurricanes. Although the nutrient levels investigated in this study are not typical of reef waters, they are, nevertheless, known to occur in some areas (see Crossland, 1983). It is, thus, important that further research on the effects of nutrients on calcifying organisms and the status of reef-water quality be carried out to help better understand and preserve reef ecosystems.

Chapter 5 N- AND P-SUPPLEMENTED *Tridacna maxima IN SITU*: NUTRIENT-LIMITED GROWTH OF SYMBIOTIC PARTNERS

5.1 INTRODUCTION

In Chapters 3 and 4, it has been clearly demonstrated that a major effect of chronic elevated nutrients on giant clams is a weakening of their shell structure (Belda et al, 1993a), while simultaneously enhancing production of soft tissues (Belda et al., 1993b; see also Braley, 1992; Hastie et al., 1992; Fitt et al., 1993). While the latter effect is attractive to onshore ventures on the commercial mariculture of these bivalves, the effect on calcification has adverse implications for calcifying organisms in reef waters with elevated nutrient concentrations.

Another direct effect of increased nutrient levels in reef waters is enhancement of phytoplankton growth, which can substantially modify benthic communities. This is exemplified by the effect of sewage outfall in Kaneohe Bay, Hawaii, where elevated nutrients brought about a major shift in community structure, killing much of the fringing coral reef (Kinsey, 1988). In addition, the classic experiment of Kinsey and Domm (1974), where nitrogen (mainly urea) and phosphorus were added daily to a patch reef over eight months, showed a 25% enhancement in primary productivity of the reef community, although there was no detectable change in community structure, except for a striking 50% reduction of calcification of the reef system (Kinsey and Davies, 1979; cf. Rasmussen, 1989).

Similarly, the results presented in Chapter 3 (Belda et al., 1993b) clearly showed a substantial influence of increased nutrients on zooxanthellae inside their clam hosts. In particular, the zooxanthellae population increased with increased N (see also Hoegh-Guldberg and Smith, 1989; Fitt et al., 1993), showing that these algae have access to available N in sea water. The study also demonstrated that the zooxanthellae are simultaneously N- and P-limited inside their animal host (cf Falkowski et al., 1993), with N-limitation depending on the external concentration of N, and P-limitation irrespective of ambient P levels in sea water. Host involvement in the availability of P to the zooxanthellae has been clearly implicated (to be addressed in Chapter 6).

While evidence for the disturbing effects of elevated nutrient concentrations on reef organisms has been presented (Kinsey and Domm, 1974; Kinsey and Davies, 1979; Kinsey, 1988; Rasmussen, 1989; Belda et al., 1993a), there is a need to quantify the relationships between nutrient levels and community degradation before any management action can be justified and directed. In response to this concern, Australia's Great Barrier Reef Marine Park Authority (GBRMPA) has initiated a large-scale, manipulative reef fertilisation experiment in the GBR, designed to: (1) provide quantitative evidence for the relative impacts of elevated N and P on reef organisms, and the processes that regulate these responses; and (2) test the usefulness of parameters proposed as sub-lethal indicators of eutrophication. This study was part of GBRMPA's multi-agency project.

In this chapter, the results of an *in situ* experiment on a natural population of *Tridacna maxima* are presented. This study extended the work presented in Chapter 3, where cultured clams were continuously exposed to increased nutrients in outdoor tanks. Here, the effects of discontinuous fertilisation (i.e., twice daily) were investigated, focussing on the growth of the symbiotic partners in terms of N:P ratios, and zooxanthellae density and size. Additionally, the nutrient levels in the clams' haemolymph (i.e., ammonia, phosphate, total phosphorus) were measured to see how haemolymph nutrient composition responds to increased nutrient levels in sea water. Limited time in the field only allowed for investigation of N-uptake by freshly-isolated zooxanthellae, although P-uptake is addressed in the next chapter.

The major aim of this study was to test if clams periodically exposed to increased nutrients in their natural environment behave in a manner similar to that of cultured clams in an artifical environment (i.e., outdoor tanks). This 'realistic' experimental condition will provide supplementary information needed in making definitive conclusions regarding the impact of nutrient enrichment on reef organisms such as giant clams and their zooxanthellae. In turn, this information will hopefully provide a scientific basis for identifying sub-lethal biological indicators of nutrient stress in reef organisms, and developing strategies for water-quality management in reef environments.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Design and Maintenance

This study constituted a small part of a large-scale, manipulative reef fertilisation experiment currently being undertaken on the Great Barrier Reef (GBR), Australia, to document the response of coral reefs to nutrient enrichment. This multi-agency project, called ENCORE (Enrichment of Nutrients on a Coral Reef), is being conducted at the relatively pristine One Tree Island Reef in the Mackay/Capricorn Section of the GBR Marine Park.

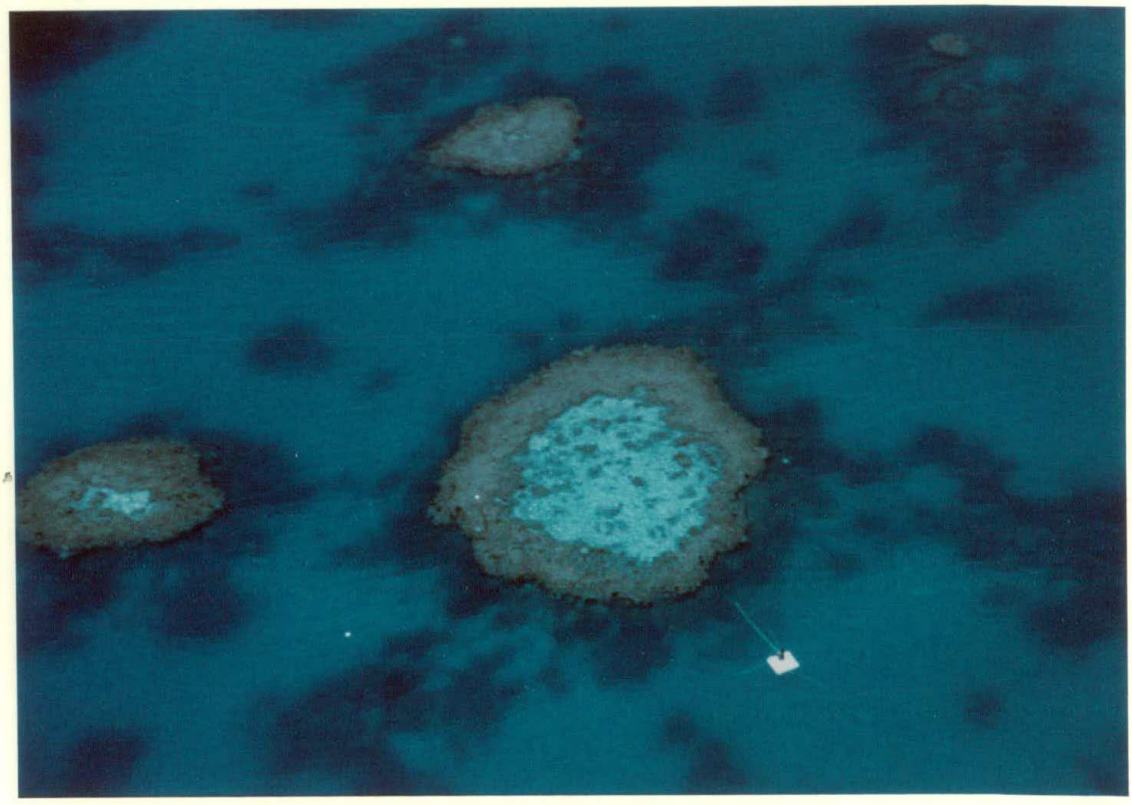
Fertilisation, which commenced in September 1993, will continue for two years. Research by most participants will continue for a further year to assess post-enrichment recovery of the reef. For this study of *T. maxima*, sampling was carried out one month and three months after the fertilisation program began.

The high-ponded lagoon of the One Tree Island Reef has numerous small, circular patch reefs or micro-atolls. Twelve northern micro-atolls, having similar sizes and morphology, have been randomly allocated to one of four nutrient treatments, namely, nitrogen (N), phosphorus (P), N+P, or no nutrient (Control), so that there are three micro-atolls per treatment. Collection of *T. maxima* and random allocation to the micro-atolls (five clams per micro-atoll) were carried out as described in Section 2.1.2 (Chapter 2). NH_4Cl and KH_2PO_4 are added to the

relevant atolls to achieve, at the time of addition, concentrations of 10 μM N l^{-1} and 2 μM P l^{-1}, respectively. These concentrations are at least 10 times the background concentrations presently found on the GBR. Fertilisation occurs twice per 24 h during periods of low tides when the water contained within these micro-atolls is isolated from the surrounding lagoon for about 4 to 5 h.

Simultaneous fertilisation of the nine micro-atolls is achieved through the use of automated nutrient-dispersal units (NDU). These NDU's are moored outside each micro-atoll (Fig. 5.1) and are telemetrically linked to a base station consisting of a computer, a controller, and a radio transmitter unit located at the Research Station (see Steven and Larkum, 1993). Just before low tide, a tidal switch signals the base station, which in turn relays a coded signal to a radio receiver mounted on each NDU. Receipt of the radio signal prompts each NDU to discharge a measured quantity of concentrated nutrient solution from a measuring chamber by admitting compressed air from a SCUBA tank. The concentrated nutrient is discharged along multiple PVC outlet lines into the basins of the micro-atolls, allowing for thorough mixing within each micro-atoll basin. After each discharge cycle, the measuring chamber fills up in preparation for the next discharge. A daily log of operations is stored on computer disk when the base station interrogates each NDU as to the success of the operation. The concentrated nutrient solutions are replenished weekly by the research station personnel.

Fig. 5.1. An aerial view of one of the twelve micro-atolls on One Tree Island Reef. The nutrient-dispersal unit (white object) is shown moored outside the micro-atoll.



5.2.2 Measurement of Biomass Parameters

5.2.2.1 Clam host

Duplicate clams were collected from each micro-atoll and killed (see Section 2.2.1, Chapter 2). Shell length and soft-tissue biomass (i.e., wet weight and N:P ratio) were measured as described previously (Sections 2.2.1.1 to 2.2.1.2, Chapter 2).

5.2.2.2 Freshly-isolated zooxanthellae (FIZ)

FIZ were prepared, stored frozen, and later analysed for density, size, and N:P ratio (limited resources precluded analysis of carbon composition), as described previously (Sections 2.2.2 to 2.2.2.2, Chapter 2).

5.2.2.3 Nutrient Levels in Haemolymph

As described in Section 2.2.1.3 (Chapter 2), the haemolymph from each clam was collected and the volume measured. Ammonia content was immediately analysed, whereas the rest of the haemolymph was stored frozen until analysed for phosphate and total phosphorus content (see Section 2.4.2, Chapter 2).

5.2.3 Measurement of N-uptake by FIZ

N-uptake by FIZ was investigated as described in Section 2.3 (Chapter 2).

5.2.4 Statistical Analyses of Data

To test the hypothesis that elevated levels of N and P have no effect on the various clam and zooxanthellae parameters at $\alpha = 0.05$, data were analysed using ANOVA for two orthogonally-arranged fixed factors (N and P), with a third random factor (micro-atoll) nested within each treatment combination (Underwood 1981). In cases where there were uneven number of replicates, equal sample sizes were randlomly taken to allow for balanced factorial analyses of the data. All statistical analyses were performed using the analytical software package, Statistix (Version 3.0). Tests of the assumptions (normality, homoscedasticity, additivity) underlying the analytical procedures were made prior to performing each analysis (Shapiro and Wilk 1965, Winer 1971, Shapiro and Francia 1972, Zar 1984). Except for the FIZ N-uptake data ($p < 0.05$), no serious violations of the assumptions were evident in the different data sets ($p > 0.05$). Data for N-uptake by FIZ were log-transformed to conform to the assumptions of normality and homogeneity of variances ($p > 0.05$).

5.3 RESULTS

Significant results were obtained only from the second sampling, that is, three months after fertilisation of the micro-atolls. Measurements taken after only one month of fertilisation did not yield clear trends, although the N:P ratio of the symbiotic partners and the nutrient levels in the haemolymph showed trends consistent with those observed after three months of fertilisation.

5.3.1 N:P Ratios in Clam Viscera and Zooxanthellae

After three months of fertilisation, addition of P alone significantly decreased the N:P ratio in the soft tissues of *T. maxima* (ANOVA, $F = 6.57$, $df = 1,8$, $p < 0.05$), indicating increased phosphorus content (Fig. 5.2). Addition of N alone, on the other hand, significantly increased the N:P ratio (ANOVA, $F = 8.13$, $df = 1,8$, $p < 0.05$), indicating increased nitrogen content. Curiously, addition of N+P did not appear to affect the N:P ratio of *T. maxima*, with no significant interaction between N and P (ANOVA, $F = 1.00$, $df = 1,8$, $p > 0.05$). It is likely that the effect of one nutrient cancelled that of the other. There was no significant variation in N:P ratios due to the different micro-atolls (ANOVA, $p > 0.05$).

Most of the viscera samples taken after only one month of fertilisation were accidentally lost (spoiled) when the freeze-drier malfunctioned overnight. Only four samples were not included in the lost batch, and these samples gave the

following N:P ratios: (a) Control = 28:1, (b) N = 36 ± 2:1, and (c) P = 24:1. The trend, though based on just four samples, was similar to the trend after three months of fertilisation.

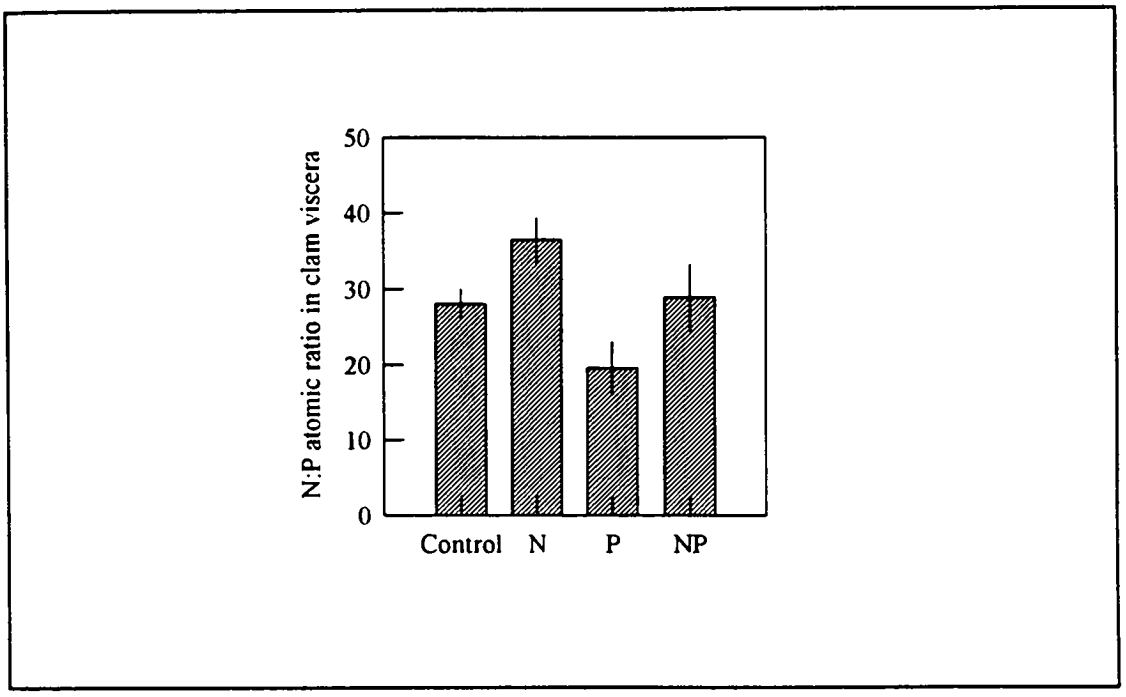


Fig. 5.2. Mean N:P atomic ratios (± SE) of the visceral tissues of *Tridacna maxima* supplemented with or without ammonium (N) and phosphate (P). (n = 6)

In contrast to the animal tissue, the N:P ratios of the zooxanthellae after three months of fertilisation were similar in all treatments (ANOVA: for N effect, $F = 5.11$, $df = 1,8$, $p > 0.05$; for P effect, $F = 2.38$, $df = 1,8$, $p > 0.05$), with a mean value of 34.2 ± 0.9 across all treatments (Table 5.1). A similar trend was observed only one month after fertilisation (Table 5.1).

Table 5.1. Mean N:P atomic ratios [± SE] of zooxanthellae isolated from *Tridacna maxima* supplemented with or without ammonium (N) and phosphate (P) in micro-atolls on One Tree Island Reef

Treatment	One month	(*n*)	Three months	(*n*)
Control	48.7 ± 7.8	(6)	33.1 ± 2.0	(6)
N	45.9 ± 3.0	(6)	38.0 ± 3.8	(4)
P	48.0 ± 3.4	(6)	33.0 ± 0.6	(6)
N+P	44.3 ± 3.0	(6)	34.0 ± 2.2	(6)
Mean	46.7 ± 2.2	(24)	34.2 ± 0.9	(22)

5.3.2 N-uptake By FIZ

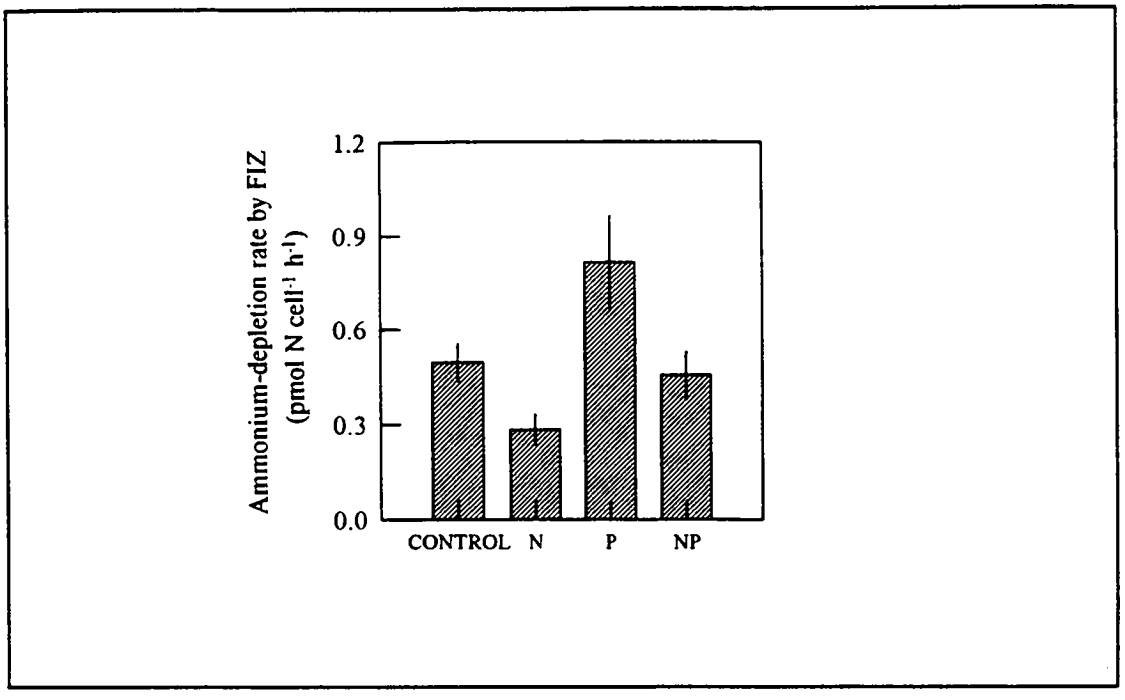


Fig. 5.3. Mean ammonium-uptake rates of zooxanthellae freshly isolated from *Tridacna maxima* supplemented with or without ammonium (N) and phosphate (P). (n = 6)

After three months of fertilisation, zooxanthellae from N-treated clams took up significantly less ammonia (ANOVA, F = 7.82, df = 1,8, $p < 0.05$), and zooxanthellae from P-treated clams took up significantly more ammonia (ANOVA, F = 5.70, df = 1,8, $p < 0.05$), than those from control clams (Fig. 5.3). However, zooxanthellae from N+P-treated clams had N-uptake rates similar to those from control clams (ANOVA, F = 0.53, df = 1,8, $p > 0.05$). Again, any effect of one nutrient cancelled that of the other when present in combination. There was no variation due to the different micro-atolls (ANOVA, $p > 0.05$). The trend after only one month of fertilisation was not significant (ANOVA, $p > 0.05$).

5.3.3 Zooxanthellae Density and Size

After three months of fertilisation, statistical analysis of the effects of N and P on zooxanthellae density was not significant (ANOVA: for N effect, F = 2.77, df = 1,8, $p > 0.05$; for P effect, F = 0.02, df = 1,8, $p > 0.05$). However, it is evident from Fig. 5.4 that addition of N and N+P increased the density, but addition of P did not. The statistical significance of the effects due to the treatments was likely masked by the highly significant variation found due to the different micro-atolls (ANOVA, F = 7.91, df = 8,12, $p < 0.001$). Such variation is attributed to differing efficiency in extracting zooxanthellae from individual *T. maxima* (see Discussion for further explanation). Zooxanthellae densities after only one month of fertilisation were not significantly different between treatments (ANOVA, $p > 0.05$).

Similarly, statistical analysis of the effects of N and P on zooxanthellae diameter was not significant (ANOVA: for N effect, F = 4.31, df = 1,8, $p > 0.05$; for P effect, F = 0.59, df = 1,8, $p > 0.05$), after three months of fertilisation. As evident in Fig. 5.5, however, addition of N and N+P decreased the zooxanthellae diameter. The significance of the effects of N and N+P was likely masked by the large variability of measurements within the P treatment. No significant differences in zooxanthellae size were discernible after only one month of fertilisation (ANOVA, $p > 0.05$).

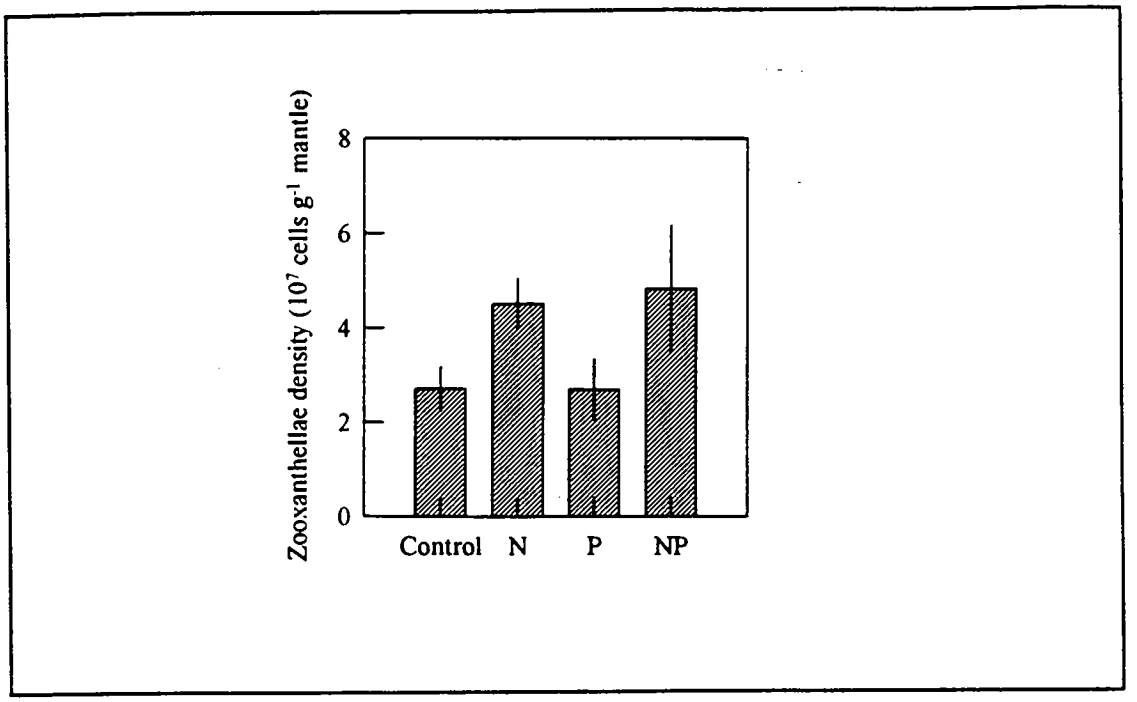


Fig. 5.4. Mean density (± SE) of zooxanthellae from *Tridacna maxima* supplemented with or without ammonia (N) and phosphate (P). (n = 6)

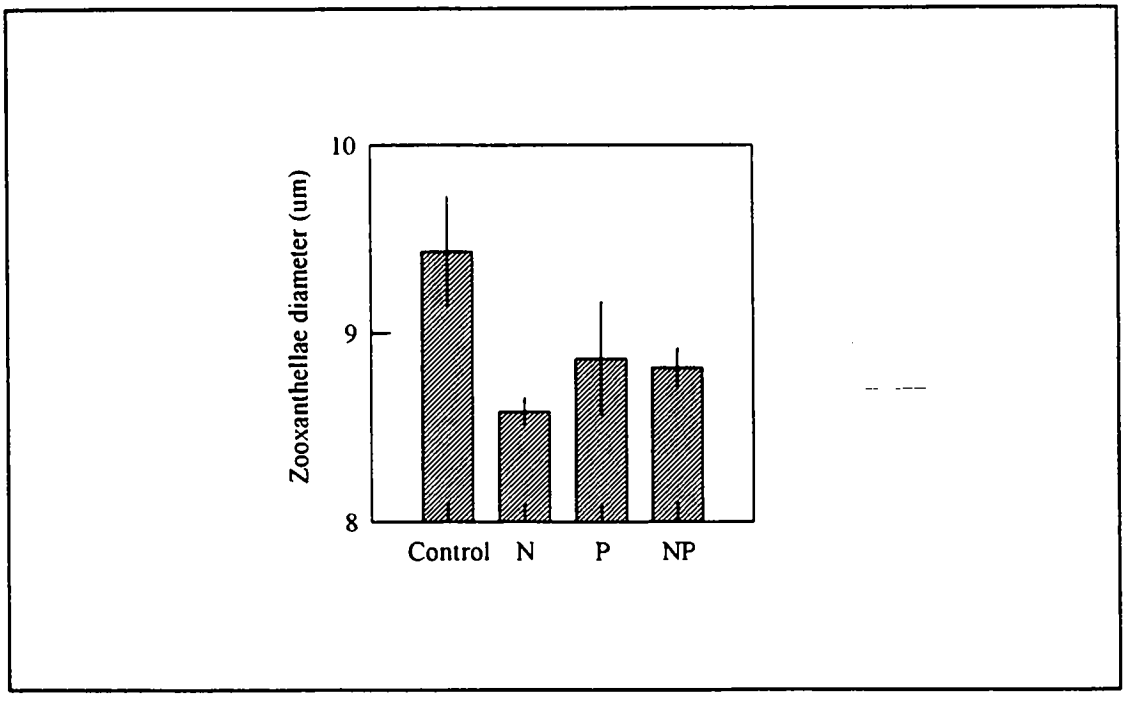


Fig. 5.5. Mean diameter (± SE) of zooxanthellae from *Tridacna maxima* supplemented with or without ammonia (N) and phosphate (P). (n = 6)

5.3.4 Ammonia, Phosphate, and Total Phosphorus Levels in the Haemolymph

In all treatments, there were no significant differences in the levels of phosphate, total phosphorus, and ammonia in the haemolymph (ANOVA, $p > 0.05$) (Table 5.2). Ammonia levels were surprisingly high, while phosphate concentrations were very low, < 0.1 μM or undetectable. Similar trends were observed only one month after fertilisation (Table 5.2).

Table 5.2. Mean concentrations [μM ± SE] of inorganic phosphate (P_i), total phosphorus (P_t), and ammonia in the haemolymph of *Tridacna maxima* supplemented with or without ammonia (N) and phosphate (P) in micro-atolls on One Tree Island Reef. (n = 6; nd = no data, due to some technical error in assay)

Treatment	One month			Three months		
	P_i	P_t	ammonia	P_i	P_t	ammonia
Control	< 0.1	35.8 ± 2.3	43.5 ± 4.2	< 0.1	nd	22.4 ± 3.2
N	< 0.1	37.3 ± 4.7	35.7 ± 2.7	< 0.1	nd	23.0 ± 2.4
P	< 0.1	34.9 ± 3.1	40.9 ± 3.8	< 0.1	nd	19.4 ± 1.1
N+P	< 0.1	32.8 ± 2.6	41.8 ± 2.9	< 0.1	nd	22.8 ± 2.0

5.4 DISCUSSION

5.4.1 N:P Ratios; Zooxanthellae Density and Size

The results of this field investigation on *T. maxima* confirm some major responses observed from the outdoor study on *T. gigas* (Belda et al., 1993b). That is, changes in ambient concentrations of inorganic nitrogen and phosphorus in sea water are reflected in the N:P ratios of the animal host, but not in the zooxanthellae. The N:P ratios of the zooxanthellae were generally >30:1 (Table 5.1), confirming that growth of the zooxanthellae is P-limited *in situ* (see Atkinson and Smith, 1983; Belda et al., 1993b).

The limited availability of P to the zooxanthellae is consistent with the trends observed in zooxanthellae density, which are comparable to previous findings (Belda et al., 1993b). Zooxanthellae density *in situ* increased with addition of N or N+P, but not with P (Fig. 5.4). It should be pointed out that, compared to *T. gigas*, zooxanthellae from *T. maxima* were generally difficult to extract. The considerable numbers of zooxanthellae lost in the process of extraction from some clams likely led to significant variations due to the different micro-atolls and, thus, a lack of a clear-cut significance of the effects of the treatments. Nevertheless, the trend in density is clear (Fig. 5.4).

It is also quite interesting that zooxanthellae from the N or N+P-treated clams were generally smaller in size than zooxanthellae from control or P-treated clams (Fig. 5.5). Inasmuch as fast-growing phytoplankton are usually smaller than slow-growing ones (Fitt, pers. comm.), the trends observed here support the idea of free access of zooxanthellae *in situ* to increased N concentrations in sea water, but not to P (Belda et al., 1993b).

5.4.2 N-uptake of Freshly-isolated Zooxanthellae (FIZ)

The N-uptake activity of freshly-isolated zooxanthellae provides further evidence. Zooxanthellae from clams exposed to N had significantly lower N-depletion rates than zooxanthellae from control clams (Fig. 5.3), confirming that the zooxanthellae from N-treated clams were exposed to increased ammonia *in situ* at increased N concentrations in sea water. In contrast, zooxanthellae from clams exposed to P had significantly higher N-depletion rates than zooxanthellae from control clams (Fig. 5.3), suggesting that the zooxanthellae managed to acquire some of the available P, which increased their demand for N. This result is consistent with the observation that addition of N+P did not affect the N-uptake rates of FIZ. P in the N+P treatment had likely increased the zooxanthellae's demand for, and uptake of, N; while, N in the N+P treatment down-regulated the zooxanthellae's N-uptake rate, resulting in a net effect of similar N-uptake rates of zooxanthellae from control and N+P-treated clams.

The down regulation of the zooxanthellae's ammonium-uptake activity confirms their access to increased N concentrations in sea water. The results also suggest that zooxanthellae *in situ* do get some P, which increases their demand for N, albeit clearly insufficient to significantly enhance their growth. This compares with the increased, yet still low, mitotic index of zooxanthellae from clams exposed to N+P (Belda et al., 1993b).

5.4.3 Nutrient Concentrations in the Haemolymph

The levels of ammonia in the haemolymph of *T. maxima* (Table 5.2), although not significant for the different treatments, were much higher than those found in *T. gigas* (see Chapter 6, Fitt et al., in prep.). These high levels are not understood, although haemolymph nutrient composition may constitute an interspecific variability among tridacnids. In addition, the results obtained here directly contradict the findings of Deane and O'Brien (1980) of undetectable ammonia and very low total phosphorus (0.27 μM) in the haemolymph of *T. maxima*. On the other hand, inorganic phosphate was consistently low for both species (i.e., < 0.1 μM) (see also Deane and O'Brien, 1980). This low concentration of inorganic phosphate in the haemolymph, which surrounds the zooxanthellal tube system, further illustrates the poor P environment of zooxanthellae *in situ* (Belda et al., 1993b).

5.4.4 Conclusions

This study has clearly demonstrated free access of zooxanthellae *in situ* to increased N concentrations in sea water, but only a very limited access to available P. This confirms previous conclusions in Chapter 3 (Belda et al., 1993b) that N-limitation of the zooxanthellae is a function of the availability of ammonium to the symbiosis, while P-limitation is irrespective of nutrient levels in sea water.

The agreement of the results between this field investigation and those of the previous outdoor study (Chapter 3), despite the fact that different species of clams were investigated in each case, is quite lucid. This has direct implications for tridacnids and other symbiotic associations in their natural environment.

Reef organisms symbiotic with zooxanthellae are adapted to the poor nutrient conditions of reef waters. Consequently, any chronic increase in nutrient levels substantially affects the symbiotic partners. In particular, elevated N increases the zooxanthellae population inside the animal host (Fig. 5.4); while, changes in both N and P levels are reflected in the zooxanthellae's N-uptake processes (Fig. 5.3) and the animal host's N:P ratio (Fig. 5.2).

Although these parameters mainly reflect positive growth responses of the symbiotic partners to elevated nutrients in sea water, the probable concurrent

negative impact on calcification is of greater concern (Belda et al., 1993a; see also Kinsey and Davies, 1979; Rasmussen, 1989), considering that the association's structural maintenance has a direct bearing on its survival. In the event of an acute stress, such as a storm or predation by crushing organisms, the impact on the skeletal structure may be fatal. Therefore, the above growth parameters could be useful as indirect indicators of nutrient stress on the skeletal structure of tridacnids, and possibly, reef corals.

The time it took to see discernible effects of elevated nutrient concentrations on clams and their zooxanthellae was three months. One month of fertilisation of the micro-atolls did not yield clear trends, although the N:P ratios of the symbiotic partners and the haemolymph nutrient composition showed trends consistent with those observed much later. It appears that the first two months of chronic nutrient elevations constitute an adjustment period for tridacnids, after which measurable biological changes become evident.

It is not known whether reef corals show similar changes within the same time frame, although trends in N-uptake by their zooxanthellae similar to those of clams were observed only one month after fertilisation (Belda and Yellowlees, unpublished results).

Overall, this study has provided valuable insights on organism-level responses to nutrient enrichment in reef waters. These, along with forthcoming information on coral responses and nutrient consequences on population and

community structure and function, will be useful in developing strategies for the management of water quality in reef environments.

Chapter 6 ZOOXANTHELLAE *IN VITRO* AND *IN VIVO*: ROLE OF THE ANIMAL HOST IN THE SUPPLY OF P

6.1 INTRODUCTION

The individual roles of algal symbionts and their animal host in the metabolism of inorganic nutrients are of significant interest as they impact on two diverse but inter-connected problems: (1) What effects do elevated ambient nutrients have on the symbiotic association? (2) How does the host regulate algal numbers? Current thinking centres on nutrient limitation as a means by which the animal host controls the population size of its zooxanthellae (Falkowski et al., 1993). As to the nutrient which limits zooxanthellae growth, both nitrogen (N) and phosphorus (P) are attractive candidates.

To date, all available evidence shows that zooxanthellae inside their invertebrate hosts (e.g., tridacnids, corals, anemones) have access to increased supply of ammonia from sea water, as demonstrated by a consequent stimulation of zooxanthellae numbers and growth (e.g., Wilkerson and Trench, 1986; Cook and D'Elia, 1987; Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Fitt et al., 1993; Belda, et al., 1993b). On the other hand, zooxanthellae have very limited access to available phosphate, as demonstrated for tridacnids in Chapters 3 (Belda et al. 1993b) and 5 (Belda and Yellowlees, in prep). For instance, it has been shown that increased P in sea water does not enhance growth of the zooxanthellae, and their N:P ratios are typical of P-starved algal cells (Belda et al.,

1993b). Similarly, zooxanthellae from other symbioses, such as corals, exhibit a lack of response to increased P in sea water (e.g., Snidvongs, 1988; Muscatine et al., 1989; Stambler et al., 1991).

It is hypothesised that zooxanthellae in clams are both N- and P-limited, with N-limitation being dependent on ambient N concentrations in sea water, while P-limitation involving some intervention by the animal host. Host control of zooxanthellae's access to phosphate, for instance, is possible in that phosphate cannot passively diffuse across biological membranes, owing to its charged form regardless of the pH of the medium (see Miller and Yellowlees, 1989). Ammonia, on the other hand, can passively diffuse across membranes, depending on its concentration gradient across the membrane barrier (see Fitt et al, in prep.). Therefore, transport of phosphate, but not ammonia, may be controlled physiologically. Additionally, the typical millimolar concentrations of inorganic phosphate inside cells (McGilvery, 1983), which is in stark contrast to the submicromolar phosphate concentrations in sea water (see Crossland, 1983), further precludes any passive diffusion of this nutrient across biological membranes.

In this chapter, results from outdoor and laboratory experiments on *Tridacna gigas* and its zooxanthellae are presented. The study focussed on the effects of increased P in sea water on the individual symbiotic partners. In particular, the behaviour of cultured zooxanthellae and zooxanthellae freshly-isolated from clams, in the presence of increased P, were compared to elucidate

the role of the animal host in the availability of this nutrient to the zooxanthellae.

6.2 MATERIALS AND METHODS

6.2.1 The Clam Host

6.2.1.1 Collection and maintenance of clams

Thirty-two *Tridacna gigas*, ranging in shell length from 22 to 26 cm, were collected from cultured stocks maintained on the intertidal reef flat in Pioneer Bay, opposite the Orpheus Island Research Station, northern Great Barrier Reef, Australia. These clams were carefully scrubbed to remove epiphytic algae from their shells, and then randomly allocated into four 1700-l raceways (500 cm long x 85 cm wide x 40 cm deep). Dissolved inorganic phosphate (P) was added to two raceways by letting 0.1 *M* P solution constantly drip (1.0 ml min^{-1}) from suspended buckets into the inflowing filtered sea water (10 l min^{-1}, filtered to 80-100 µm, FSW). The final concentration in the two P-treated raceways was approximately 10 µM, while ambient P concentration in the two control raceways was < 0.1 µM. The clams were acclimated in these raceways for at least a week before experimentation and analyses. P concentrations in the sea water were determined using the method described in Section 2.4.1 (Chapter 2). Flow rates of sea water (2.8 h turnover time) and concentrated P solutions into the raceways

were checked three times daily. Light intensity, water temperature, and salinity were checked daily.

6.2.1.2 P-depletion by clams

Depletion of P from sea water by intact clams was investigated as follows. Ten 14-l buckets, each containing 10 l of 10 μ*M* P solution, were partly immersed in a raceway with running sea water to control temperature. Two clams from each of the P-treated raceways and two from each of the control raceways were placed separately in eight buckets; two buckets without clams served as controls. Duplicate water samples (10 ml) were collected at regular intervals from each bucket, with thorough stirring of the water prior to each sample collection. The sea water samples were immediately analysed for phosphate as described in Section 2.4.1 (Chapter 2). To account for any significant amounts of phosphate that may have adsorbed onto the shells of the clams, a similar experiment was carried out using empty shells of clams previously incubated in each of the raceways. These shells were completely cleaned of any animal tissues prior to use.

After 8 h of incubation, the clams were killed. The haemolymph was collected from each clam and its volume measured. The haemolymph samples were then centrifuged at 2000 *g* for 10 min and the inorganic phosphate and total phosphorus in the supernatant measured as described in Section 2.4.2 (Chapter 2).

The haemolymph of two clams, which remained in each of the control raceways, was also collected and analysed for comparison.

6.2.1.3 Clam biomass

Two clams from each raceway were killed and the kidneys separated from the rest of the visceral tissues. Both tissue samples were frozen, lyophilised, and then weighed prior to the determination of N:P ratio, as described previously (Sections 2.2.1 to 2.2.1.2, Chapter 2).

6.2.2 The Zooxanthellae

6.2.2.1 Collection and maintenance of zooxanthellae

Zooxanthellae were isolated from the mantle tissues of two clams from each raceway, and prepared as described previously (Section 2.2.2, Chapter 2). The resulting algal pellet was resuspended in 0.45 µm-Millipore filtered sea water (MFSW) to obtain a suspension of 10^8 cells ml^{-1}. Zooxanthellae densities were measured using a haemocytometer (Section 2.2.2.1, Chapter 2).

Cultured zooxanthellae (*Symbiodinium* sp. CS-163), which had originally been isolated from *T. gigas*, were obtained from the CSIRO Culture Collection of Micro-algae in Hobart, Tasmania. Cells were grown in f/2 media (Guillard and

Ryther, 1962) in Fernbach flasks at a constant temperature of 28°C, irradiance level of 150 μE m^{-2} s^{-1}, and photoperiod of 12 h: 12 h. Samples were taken from duplicate cultures at two-day intervals to determine the growth curve. Growth was exponential during the week following inoculation, slowing down and plateauing during the second week, and declining thereafter. Since growth of the cultures was at its peak at about day 7 (Fig. 6.1), all experiments were carried out using one week-old cultures. Zooxanthellae were harvested by centrifugation at 1000 *g* for 5 min, and washed three times with sterile MFSW. Sterile f/2 media containing 0, 5, and 10 μ*M* P in duplicates were inoculated with 1.5 ml of the thoroughly-mixed zooxanthellae suspension and incubated for seven days under the above conditions of light and temperature.

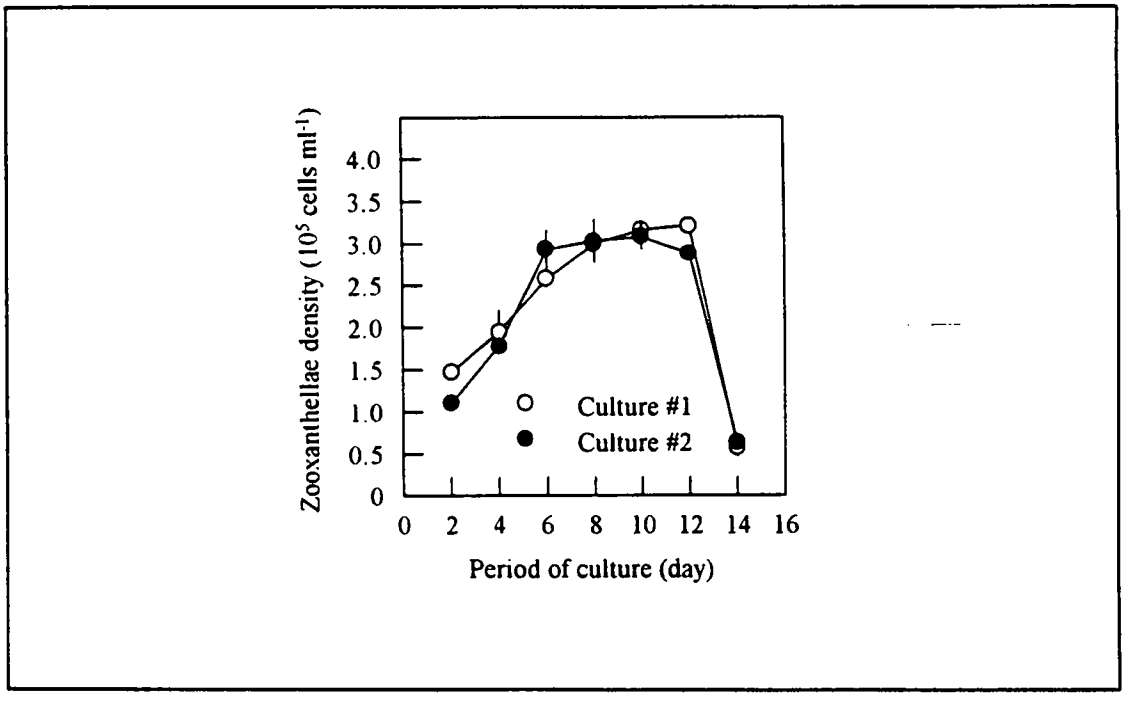


Fig. 6.1. Growth curves of cultured zooxanthellae originally isolated from *Tridacna gigas*.

6.2.2.2 P-depletion by zooxanthellae

The experiment on P-uptake by FIZ was carried out as described previously (Section 2.3, Chapter 2).

Duplicate cultures of zooxanthellae grown in 0, 5 and 10 μ*M* phosphate were harvested and washed in MFSW. One ml of the thoroughly-mixed resuspended algae, previously sampled for density determination, was added to 20 ml of a sterile 10 μ*M* P solution in MFSW. A control, to account for any bacterial uptake of phosphate, consisted of 1 ml of a filtrate, obtained by passing the zooxanthellae suspension through a 5 μm filter, in place of 1 ml of the resuspended algae. Triplicate samples were incubated at a constant temperature of 28°C for up to 6 h, either in the dark or under a 150 μE m^{-2} s^{-1} light regimen. Phosphate depletion was measured following removal of zooxanthellae from the incubation medium by filtration of the suspension on Whatman GF/C filters (see Section 2.4.1, Chapter 2).

6.2.2.3 Zooxanthellae biomass

Two clams from each raceway were killed and the zooxanthellae extracted for measurement of density and N:P ratio, as described in Sections 2.2.2 to 2.2.2.2 (Chapter 2).

For cultured zooxanthellae, duplicate cultures of zooxanthellae grown in 0, 5 and 10 μ*M* phosphate were harvested and washed in MFSW (see Section 6.2.2.1). Samples were then taken for measurement of density of the cultures (see Section 2.2.2.1, Chapter 2). Another set of duplicate cultures of zooxanthellae grown in 0, 5 and 10 μ*M* phosphate were harvested and washed in MFSW. Algal pellets were frozen, freeze-dried, and weighed prior to the determinations of N:P ratios (see Section 2.2.2.2, Chapter 2).

6.2.3 Statistical Analyses of Data

To test the hypothesis that elevated levels of phosphate have no effect on the different clam and zooxanthellae parameters at $\alpha = 0.05$, data were analysed using simple nested or one-way ANOVA, whichever was appropriate (Underwood 1981). All statistical analyses were performed using the analytical software package, Statistix (Version 3.0). Tests of the assumptions (normality, homoscedasticity, additivity) underlying the analytical procedures were made prior to performing each analysis (Shapiro and Wilk 1965, Winer 1971, Shapiro and Francia 1972, Zar 1984). In all cases, no serious violations of the assumptions were evident ($p > 0.05$).

6.3 RESULTS

6.3.1 The Animal Host

Clams acclimated in either the control or phosphate-supplemented (10 μ*M*) raceways took up significant amounts of phosphate (Fig. 6.2) (ANOVA, F = 76.11, df = 2,9, $p < 0.0001$), with the former depleting phosphate at a much faster rate (11.5 ± 0.7 μmol P h^{-1}) than the latter (4.3 ± 0.9 μmol P h^{-1}) (Tukey, $p <$ 0.05). The empty shells did not significantly adsorb phosphate from the sea water (ANOVA, $p > 0.05$).

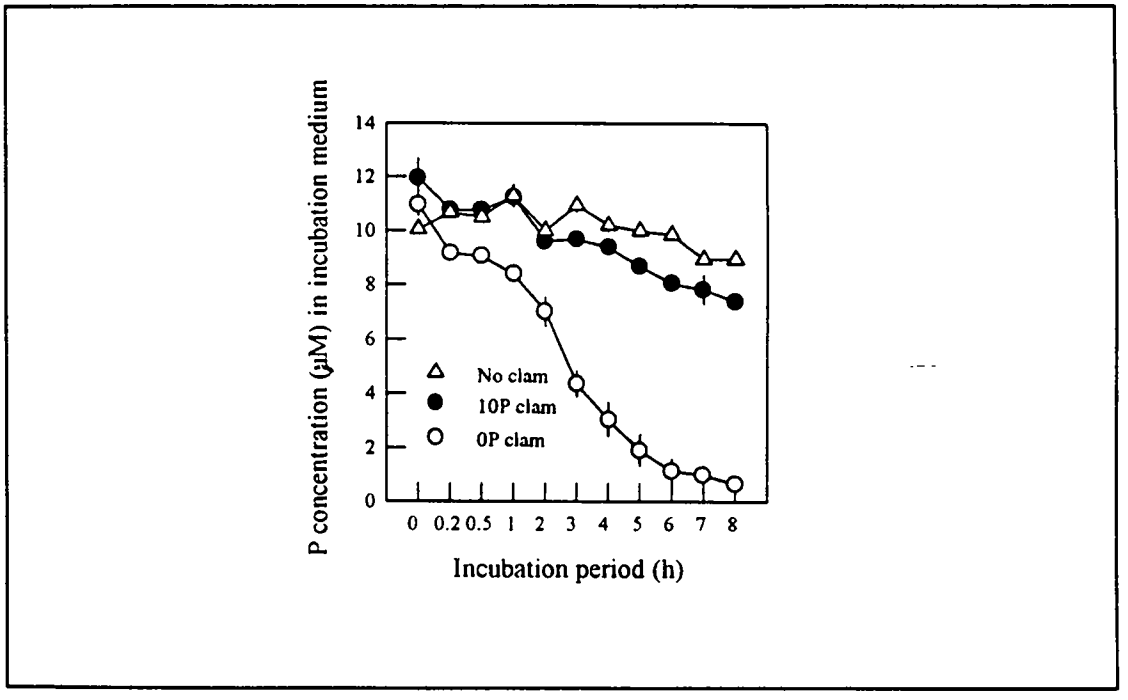


Fig. 6.2. Mean phosphate-depletion rates (± SE) of *Tridacna gigas* previously incubated with or without phosphate (P) in μ*M* concentrations. (n = 4)

Inorganic phosphate was only present at very low concentrations (< 0.1 μM or undetectable) in the haemolymph of both the control and P-treated clams (Table 6.1). Total phosphorus was high in both groups, but did not significantly differ (ANOVA, $F = 0.20$, $df = 1,2$, $p > 0.05$). Similar results were obtained for control clams which remained in the raceways (ANOVA, $p > 0.05$).

Table 6.1. Mean concentrations ($\mu M \pm$ SE) of inorganic phosphate and total phosphorus in the haemolymph of *Tridacna gigas* supplemented with or without phosphate in raceways on Orpheus Island. ($n = 4$)

Treatment	Inorganic phosphate	Total phosphorus
Control	< 0.1	20.2 ± 2.0
P-treated	< 0.1	19.8 ± 1.8

The N:P ratio of the viscera (minus kidneys) from P-treated clams was lower than the N:P ratio of the viscera from control clams, although the difference was not significant (ANOVA, $F = 0.99$, $df = 1,2$, $p > 0.05$) (Table 6.2). Similarly, the N:P ratios of the kidneys did not vary between the control and P-treated clams (ANOVA, $F = 1.27$, $df = 1,2$, $p > 0.05$). Comparing the elemental composition of the viscera and the kidneys (Table 6.3), however, a stark difference was evident. That is, the kidneys had a phosphorus content seven times higher than the

phosphorus content of the viscera. Also, the kidneys had a nitrogen content twice as low as the nitrogen content of the viscera.

Table 6.2. Mean N:P atomic ratios (± SE) of viscera (minus kidneys) and kidneys from *Tridacna gigas* supplemented with or without phosphate in raceways on Orpheus Island. (n = 2)

Treatment	Viscera (minus kidneys)	Kidneys
Control	42.1 ± 1.6	3.0 ± 0.3
P-treated	39.1 ± 1.4	2.6 ± 0

Table 6.3. Mean N and P composition (% ± SE) of viscera (minus kidneys) and kidneys from *Tridacna gigas* supplemented with or without phosphate in raceways on Orpheus Island. (n = 4)

Treatment	Viscera (minus kidneys)	Kidneys
% P	0.4 ± 0.01	2.7 ± 0
% N	7.7 ± 0.3	3.3 ± 0.1

6.3.2 The Zooxanthellae

The densities of the zooxanthellae isolated from control (0 μ*M* P) and P-treated (10 μ*M* P) clams did not significantly differ from each other (Fig. 6.3) (ANOVA $F = 0.36$, $df = 1,2$, $p > 0.05$). In the case of the cultured algae, however, addition of phosphate to the culture medium significantly increased zooxanthellae density (ANOVA, $F = 59.70$, $df = 2,3$, $p < 0.01$), with cultures in 10 μ*M* P being significantly denser than those in 5 μ*M* P (Tukey, $p < 0.05$) (Fig. 6.4).

Similarly, the N:P ratios of zooxanthellae isolated from control and P-treated clams did not significantly differ from one another (Fig. 6.5) (ANOVA, $F = 0.67$, $df = 1,2$, $p > 0.05$); whereas, addition of phosphate to the culture medium significantly decreased the zooxanthellae's N:P ratios (ANOVA, $F = 120.38$, $df = 2,3$, $p < 0.01$) (Fig. 6.6). The mean N:P ratio of freshly-isolated zooxanthellae (36.3 ± 1.2) is comparable with the mean N:P ratio of P-starved cultured zooxanthellae (33.1 ± 1.1). In contrast, zooxanthellae cultured in 5 and 10 μ*M* P regimens had much lower N:P ratios of 17.2 ± 0.9 and 14.0 ± 0.7, respectively, than either freshly-isolated or P-starved cultured zooxanthellae.

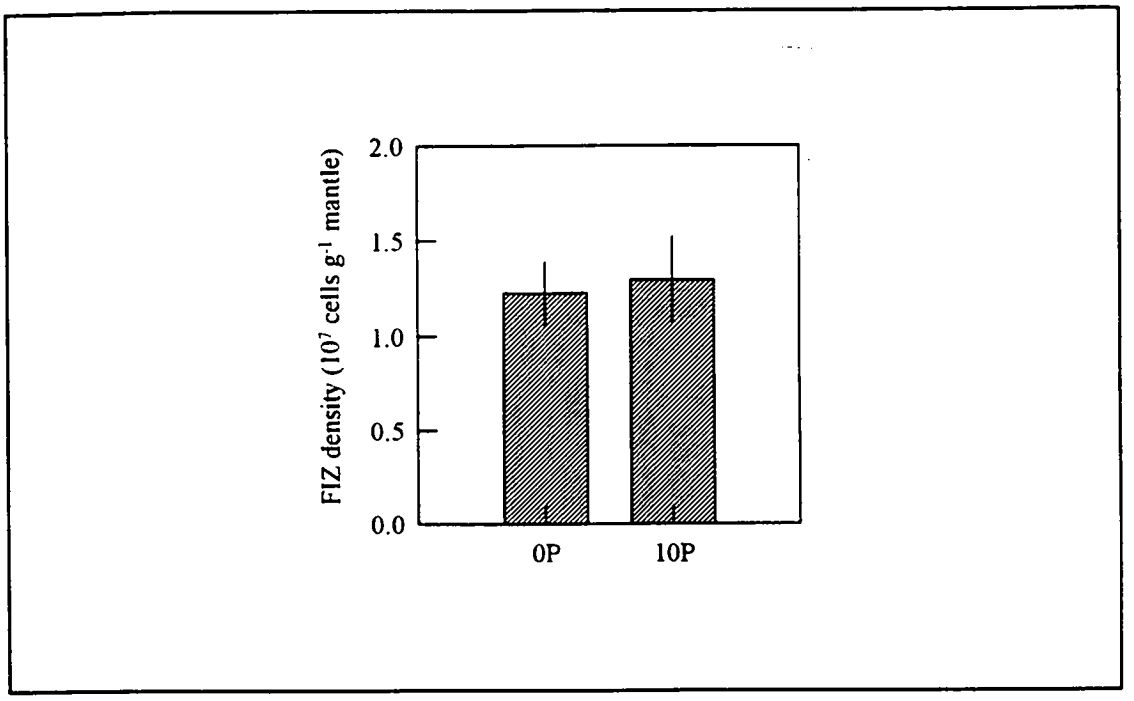


Fig. 6.3. Mean densities (± SE) of zooxanthellae in *Tridacna gigas* supplemented with or without phosphate (P) in μ*M* concentrations. (*n* = 4)

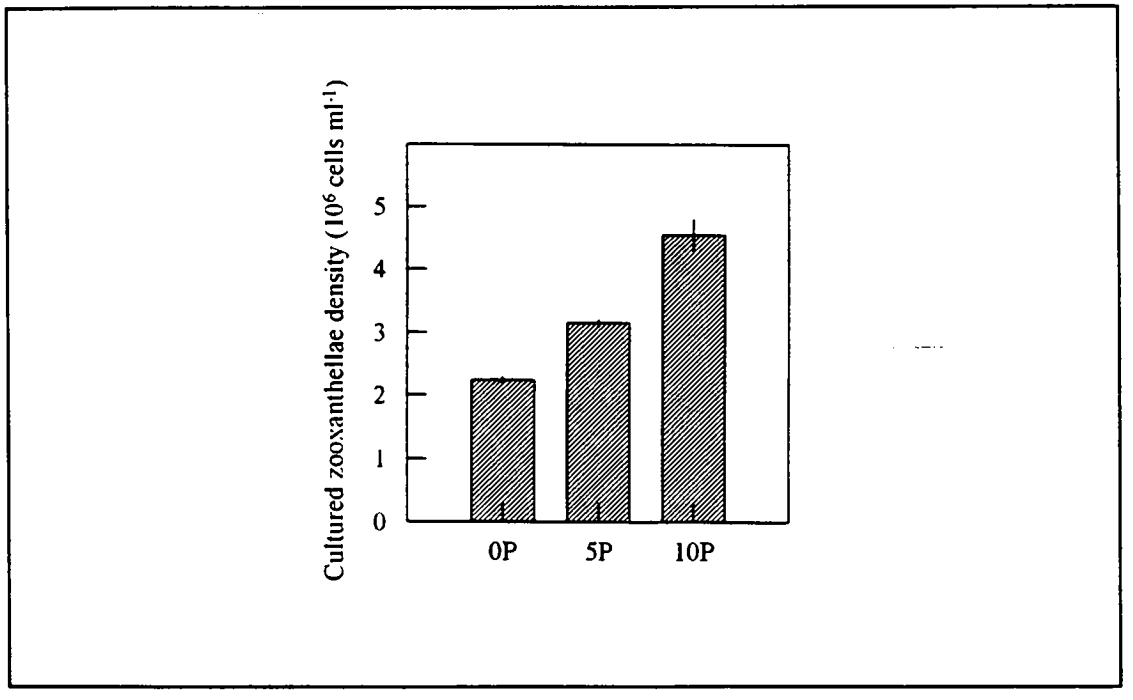


Fig. 6.4. Mean densities (± SE) of zooxanthellae cultured with or without phosphate (P) in μ*M* concentrations. (*n* = 2)

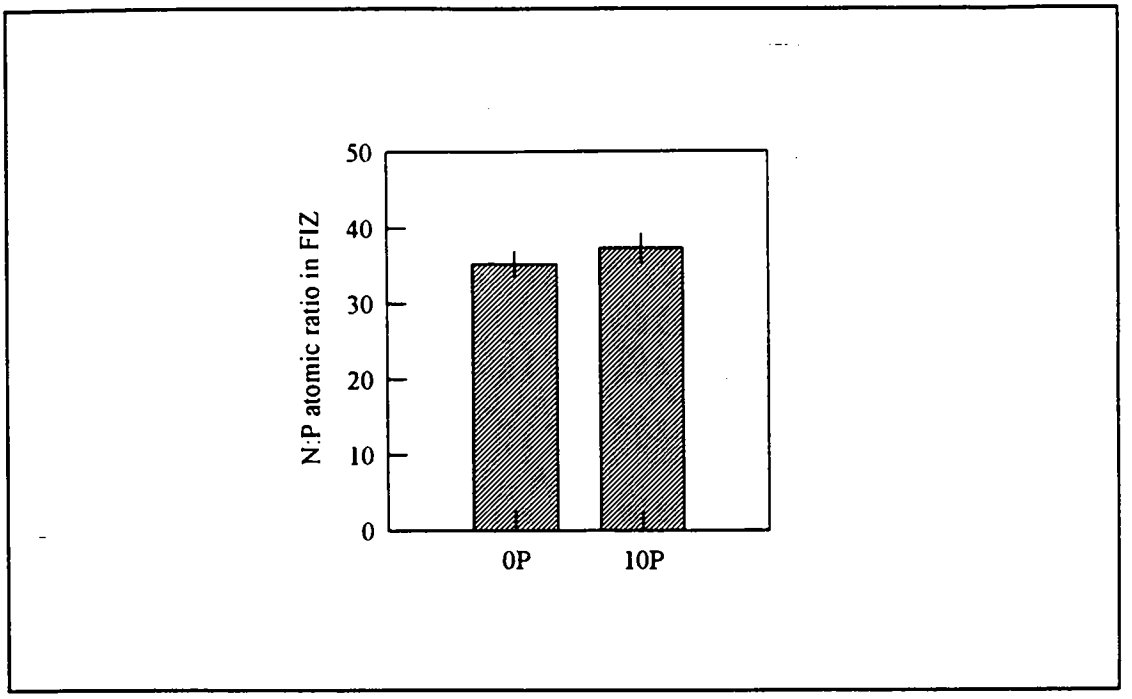


Fig. 6.5. Mean N:P atomic ratios (± SE) of zooxanthellae from *Tridacna gigas* supplemented with or without phosphate (P) in μ*M* concentrations. (*n* = 4)

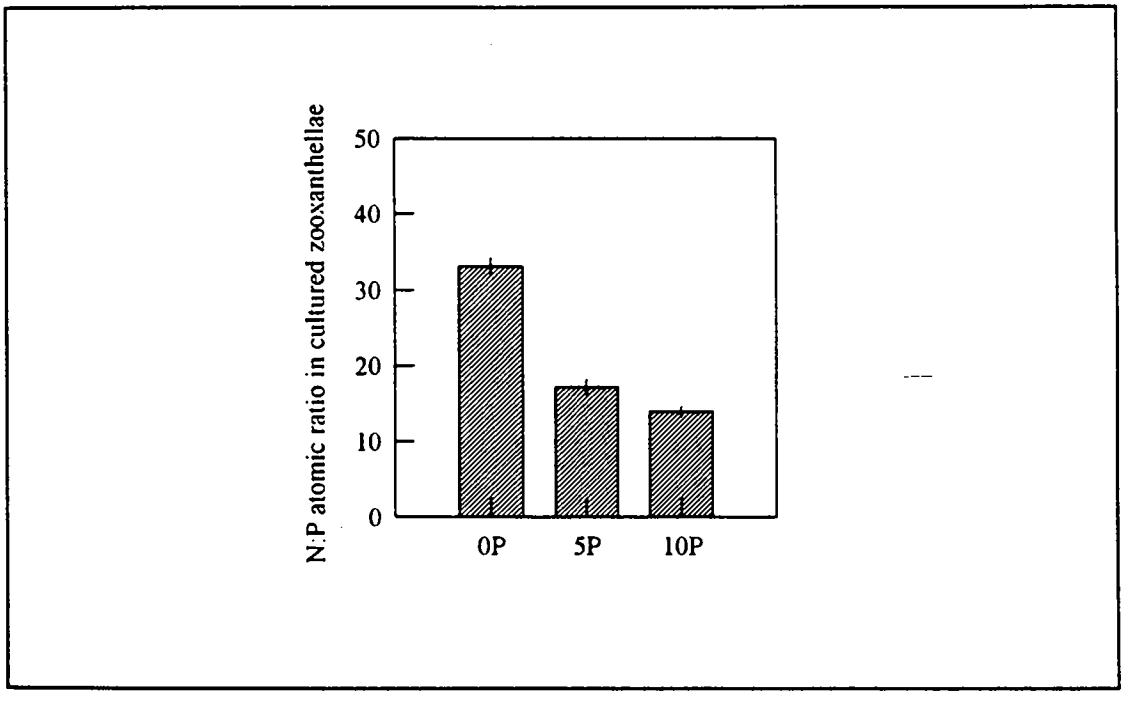


Fig. 6.6. Mean N:P atomic ratios (± SE) of zooxanthellae cultured with or without phosphate (P) in μ*M* concentrations. (*n* = 2)

The contrasting responses of freshly-isolated and cultured zooxanthellae were also evident in their P-depletion rates. The P-depletion rates by zooxanthellae isolated from control and P-treated clams did not significantly differ (ANOVA, $F = 3.60$, $df = 1,2$, $p > 0.05$) (Fig. 6.7). In contrast, after 6 h of light incubation in 10 μ*M* P, zooxanthellae from control flasks (0 μ*M* P) took up significantly greater amounts of phosphate than those previously cultured in either 5 or 10 μ*M* P (ANOVA, $F = 64.55$, $df = 2,3$, $p < 0.01$) (Fig. 6.8). Furthermore, uptake rates significantly decreased with previous exposure to increasing amounts of phosphate (Tukey, $p > 0.05$) (Fig. 6.8). Comparable results were obtained with dark incubation of cultured zooxanthellae (ANOVA, $F = 561.50$ $df = 2,3$, $p < 0.001$). In addition, uptake rates were generally lower in the dark than in the light, and were similar for zooxanthellae previously cultured in 5 or 10 μ*M* P (Tukey, $p > 0.05$) (Fig. 6.8).

In the light, the mean P-depletion of freshly-isolated zooxanthellae (8.0 ± 0.2 fmol P cell^{-1} h^{-1}) is comparable with the mean P-depletion rate of P-starved cultured zooxanthellae (7.4 ± 0 fmol P cell^{-1} h^{-1}). In contrast, zooxanthellae cultured in 5 and 10 μ*M* P regimens had lower P-depletion rates of 4.0 ± 0.1 fmol P cell^{-1} h^{-1} and 1.4 ± 0.1 fmol P cell^{-1} h^{-1}, respectively, than either freshly-isolated or P-starved cultured zooxanthellae.

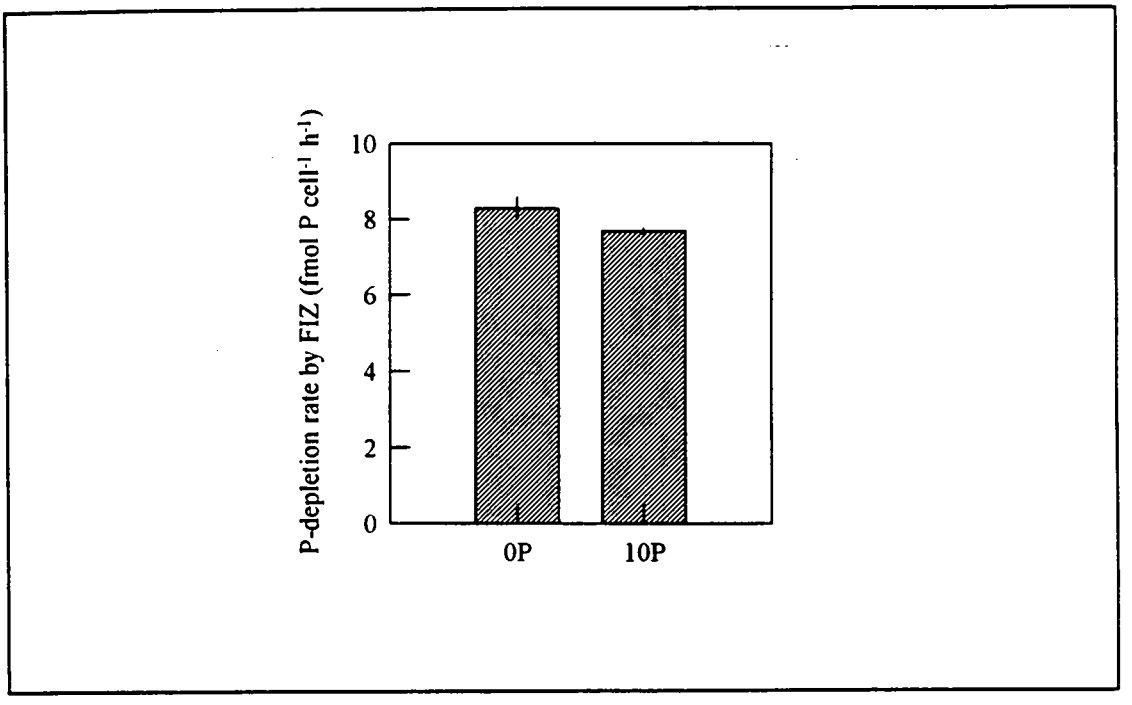


Fig. 6.7. Mean P-depletion rates (± SE) of zooxanthellae freshly isolated from *Tridacna gigas* supplemented with or without phosphate (P) in μ*M* concentrations. (*n* = 2)

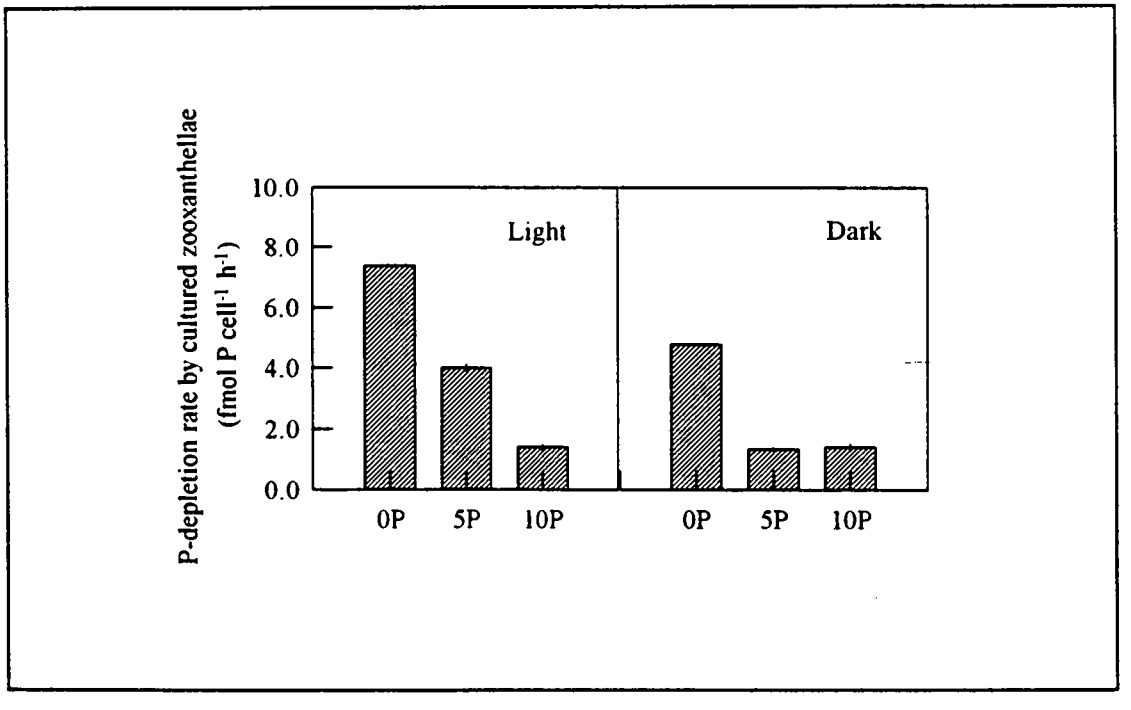


Fig. 6.8. Mean P-depletion rates (± SE) in light and in dark of zooxanthellae previously cultured with or without phosphate (P) in μ*M* concentrations. (*n* = 2)

6.4 DISCUSSION

One significant outcome of this investigation is the clear demonstration of a distinct similarity between the attributes of zooxanthellae *in vivo* and zooxanthellae grown under P-deficient conditions. Regardless of the ambient P concentrations of sea water surrounding the clams, their algal symbionts had N:P ratios (36.3 ± 1.2) (Fig. 6.5) which conform with those found for P-starved zooxanthellae in culture (33.1 ± 1.1) (Fig. 6.6). These values compare favourably with the general N:P ratio of P-starved phytoplankton, namely, >30:1 (see Atkinson and Smith, 1983). That zooxanthellae isolated from both control and P-treated clams depleted P from sea water at a rate (8.0 ± 0.2 fmol P $cell^{-1}$ h^{-1}) (Fig. 6.7) not different from that measured for cultured zooxanthellae deprived of P (7.4 ± 0 fmol P $cell^{-1}$ h^{-1}) (Fig. 6.8) further substantiates these findings. Therefore, these results confirm previous conclusions that clam zooxanthellae are P-limited *in vivo* (Belda et al., 1993b; Belda and Yellowlees, in prep.).

The consistent differences observed between the responses of zooxanthellae *in vivo* and zooxanthellae *in vitro* to elevated ambient P concentrations are equally remarkable. Exposure of clams to increased P in sea water had no effect on the density (Fig. 6.3) and N:P ratio (Fig. 6.5) of their zooxanthellae, indicating limited access by the zooxanthellae to this nutrient. This lack of response is further corroborated by the similar P-depletion rates displayed by zooxanthellae from control and P-treated clams (Fig. 6.7), suggesting that the environment of the

zooxanthellae inside their animal host is P-deficient, irrespective of P concentrations in sea water. However, outside the animal host, zooxanthellae grown in culture displayed a clear ability to take up and assimilate available phosphate from sea water. Addition of P to the culture medium stimulated an increase in the density of the cultures (Fig. 6.4), and a decrease in the N:P ratio (Fig. 6.6) of the zooxanthellae. This responsiveness of cultured zooxanthellae to available P is further demonstrated by the behaviour of P-supplemented zooxanthellae, which showed increasing density (Fig. 6.4), decreasing N:P ratios (Fig. 6.6), and decreasing P-uptake rate (Fig. 6.8) with increasing P-supplementation.

It is clear from these observations that the animal host affects the P environment of the zooxanthellae *in vivo* (see also Belda et al., 1993b; Belda and Yellowlees, in prep). Indeed, clams took up P from sea water in significant amounts (Fig. 6.2), but inorganic phosphate in the haemolymph, which surrounds the zooxanthellal tube system, remained very low (< 0.1 or undetectable) (Table 6.1) (see also Belda and Yellowlees, in prep). The unaffected levels of total phosphorus in the haemolymph (Table 6.1) (see also Belda and Yellowlees, in prep) precludes any significant amounts of phosphate from sea water being incorporated into organic phosphates or other phosphate compounds in the haemolymph.

This raises the question of the role the animal host plays in the availability of phosphate to its zooxanthellae. Two hypotheses are proposed: (1) the limited availability of P to the zooxanthellae *in vivo* may simply be a natural consequence of their morphological and spatial relationship with their animal host, which has the first access to available P from sea water; or (2) the animal host may actively restrict its zooxanthellae's access to P, possibly to control their growth.

The first hypothesis is plausible in that the morphological and spatial arrangement of the symbiotic partners relative to one another imposes that phosphate from sea water is first taken up by the animal cells, and may be kept there for the animal's benefit. As a consequence, only a very limited amount of phosphate can reach the zooxanthellae. Phosphate is an integral requirement for cellular existence, owing to its role in many cellular metabolic functions, the most important of which are: (1) synthesis and breakdown of adenosine triphosphate (ATP); and (2) synthesis of nucleic acids (McGilvery, 1983). Conservation of phosphate, therefore, is important for the cells' maintenance and survival. Interestingly, control clams practically depleted supplied phosphate in sea water, while previously P-treated clams took up much lesser amounts of phosphate (Fig. 6.2). This may be a display of "luxury" phosphate consumption by clams when occasionally exposed to elevated phosphate.

The alternative hypothesis, on the other hand, is equally appealing in that phosphate, being a charged molecule, can only be transported across cell

membranes via active transport or facilitated diffusion, which are energy-requiring processes (see Miller and Yellowlees, 1989). Such energy-requiring transport of phosphate is supported by the observed light stimulation of P-uptake by algal cells in culture, with uptake in the dark being likely supported by the cells' carbon reserves (Fig. 6.8). The animal host can, thus, control transport of phosphate across cell membranes by regulating the activity of their membranes' transport systems. By restricting the availability of this nutrient to the zooxanthellae, the animal host can control the growth of its zooxanthellae. In contrast, such control is not possible with ammonia, as ammonia diffuses across cell membranes, depending on its concentration gradient, which, in turn is affected by the pH of the media separated by the membrane barrier (Miller and Yellowlees, 1989; Fitt et al., in prep.). The only probable way the animal host could limit the availability of ammonia to its zooxanthellae is by assimilating this nutrient itself, and this is possible due to the high ammonium-assimilation enzymes (glutamine synthetase) present in clams (Rees et al., in press). Ammonia will only then be available to the zooxanthellae after these host enzymes have been saturated.

Whichever is the case, the question of where phosphate from sea water ends up in the animal host is raised. Indeed, the N:P ratios of the animal tissue in *T. gigas* and *T. maxima* significantly decreased with increased P in sea water (Belda et al., 1993b, Belda and Yellowlees, in prep). In this study, however, there was only a slight decrease in the N:P ratio of *T. gigas* viscera (minus the kidneys) (Table 6.2), although this is probably not surprising considering the short exposure

time (8 h) of the clams to increased P. The observed concentration of phosphate in the kidneys (Table 6.3) may have a significant relevance, but it is not understood at this stage.

In summary, this study confirms previous findings that zooxanthellae in clams are not only N-limited, but also P-limited, and the animal host plays an active role in the availability of phosphate to its zooxanthellae (see also Belda et al., 1993b; Belda and Yellowlees, in prep.).

Chapter 7 GENERAL CONCLUSIONS

A significant body of evidence indicates that zooxanthellae are N-limited inside their animal hosts (Summons et al., 1986; Wilkerson and Trench, 1986; Cook and D'Elia, 1987; D'Elia and Cook, 1988; Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Braley, 1992; Hastie et al., 1992; Falkowski et al., 1993; Fitt et al., 1993). However, available evidence for P-limitation of the zooxanthellae, although equally compelling, is limited (see Yonge, 1936; Jackson et al., 1989). That both N and P may be limiting the growth of the zooxanthellae *in vivo* in their various hosts has only been alluded to in the scientific literature (Miller and Yellowlees, 1989). This study clearly demonstrates that both types of limitation operate simultaneously in clam-zooxanthellae symbiosis.

Both N and P are present at low concentrations in reef waters (see Crossland, 1983). At increased ambient levels, this study and others (e.g., Yonge, 1936; Wilkerson and Trench, 1986) have clearly shown that clams are capable of depleting these nutrients from sea water. The significant responses to increased N by both the animal host (i.e., tissue growth, N:P ratio) and zooxanthellae (i.e., density, N-uptake) (Chapters 3 and 5) indicate that both symbiotic partners have access to exogenously-supplied N.

On the other hand, while clams can deplete P from sea water and correspondingly decrease their N:P ratio (Chapter 6), the zooxanthellae do not appear to have free access to this nutrient. This has been clearly shown by the

zooxanthellae's lack of response in their density, N:P ratio, and P-uptake rate when clams were supplied with increased P (Chapters 3, 5, and 6). Outside the animal host, however, zooxanthellae can respond to increased P levels (Chapter 6). That is, zooxanthellae cultured in the presence of P had increased growth rate, decreased N:P ratio, and decreased P-uptake rate, while those grown under P-starvation had properties similar to those of zooxanthellae *in vivo*. These results strongly implicate host involvement in the availability of P to its zooxanthellae.

Access of the zooxanthellae to N and P inside the animal host largely depends on these nutrients' chemical properties. Ammonia, being an uncharged molecule, diffuses across cell membranes (see Miller and Yellowlees, 1989), maintaining equilibrium concentrations between compartments (e.g,, sea water, animal cells, haemolymph, zooxanthellal tubes). However, within each compartment, ammonia is protonated to form ammonium or *vice versa*, with the relative abundance of each molecular species being dependent on the pH of the medium (see Miller and Yellowlees, 1989). Therefore, while the concentrations of ammonia in the different compartments remain similar, the concentrations of ammonium vary, depending on the differences in the pH of these compartments.

The pH of the clam's haemolymph is similar to that of sea water during daytime, or lower than that of sea water during nighttime, depending on the zooxanthellae's light-dependent photosynthetic activity (Fitt et al., in prep.). When haemolymph pH decreases, there is a corresponding decrease in the

concentration of ammonia in the haemolymph due to increased conversion of ammonia into ammonium. Therefore, ammonia diffuses from sea water into the haemolymph (Fitt et al., in prep.), leading to an accumulation of ammonium as ammonia is protonated, until ammonia in the haemolymph is again in equilibrium concentration with ammonia in sea water. At increased ambient N levels of ammonia in sea water, more ammonia diffuses and more ammonium accumulates in the animal host. Therefore, at a low pH, the haemolymph acts as an acid trap for ammonium, which cannot cannot diffuse back from the haemolymph to the sea water. The same processes presumably occur in the zooxanthellal tube system. Thus, ammonium trapped inside the animal host will be available for assimilation by either the animal host or the zooxanthellae. Availability of exogenous N (ammonia/ammonium) to the zooxanthellae, therefore, is a function of the ambient sea water concentration of ammonia and the photosynthesis-dependent pH of the haemolymph and the zooxanthellal tubes. In particular, zooxanthellae growth *in vivo* is limited mainly by the naturally-low ambient concentrations of N in reef waters.

P, on the other hand, is always present as a charged molecule regardless of the pH of the medium (see Miller and Yellowlees, 1989). As such, it cannot passively cross membranes. Additionally, inorganic phosphate is typically present in cells in millimolar concentrations (McGilvery, 1983), which are orders of magnitude higher than this nutrient's submicromolar concentrations in sea water (see Crossland, 1983). Uptake of P from sea water against a concentration

gradient is only possible through active transport or facilitated diffusion (Miller and Yellowlees, 1989). Thus, unlike N-limitation, P-limitation of the zooxanthellae is irrespective of the ambient concentrations of phosphate in sea water.

P-limitation of zooxanthellae in clams may be explained in two ways: (1) P availability to the zooxanthellae is limited as a natural consequence of the morphological and spatial relationships between the symbiotic partners; or (2) P availability to the zooxanthellae is actively regulated by the animal host for the latter's benefit.

The morphological and spatial arrangement between the animal host and its zooxanthellae dictates that phosphate from sea water is first taken up by host cells. Since animal cells actively conserve phosphate, it is possible that the limited availability of phosphate to the zooxanthellae is just a natural consequence of this, unless the symbiotic relationship has evolved a mechanism for the host to selectively release phosphate to its zooxanthellae. In the case of N, the morphological and spatial relationship between the symbiotic partners has less bearing on the availability of this nutrient to the zooxanthellae, owing to ammonia's capacity to diffuse across membranes down its pH-dependent concentration gradient.

Unlike ammonia, phosphate is not a waste product of metabolism. While phosphate is involved in many aspects of metabolism, its two most important functions are in: (1) metabolic energy conversions, through ATP (adenosine triphosphate) synthesis and breakdown; and (2) nucleic-acid synthesis (McGilvery, 1983). Conservation of phosphate is, therefore, important to the cells' existence.

The amount of phosphate that supports the low specific growth rates of zooxanthellae *in vivo* (Chapters 3, 5, and 6) is likely very limited. The zooxanthellae's potential sources of P would include: (1) recycled P from senescent cells in the tube system; (2) low P concentrations in the haemolymph (Chapters 5, and 6); and (3) dissolved organic phosphates derived from the host (see Taylor, 1973).

On the other hand, the alternative explanation for P-limitation of zooxanthellae is equally appealing. As discussed previously, for phosphate to reach the zooxanthellae, it must first cross the animal host's membranes either by active transport or facilitated diffusion. By controlling the activity of the transport systems in these membranes, the host can restrict availability of P to its zooxanthellae. This control is possible owing to the charged nature and high cytoplasmic concentrations of phosphate, preventing passive diffusion from sea water across host cell membranes.

That the animal host would restrict phosphate availability to its algal symbionts can be explained in terms of the benefit it derives from the photosynthate translocated from its zooxanthellae. If the zooxanthellae grow fast as a result of exposure to increased nutrients, they translocate photosynthate to their host at a reduced rate (e.g., Stimson and Kinzie, 1991; Falkowski et al., 1993). That is, the zooxanthellae expend more energy on cell division when they grow fast than on synthesis of energy-rich compounds that can otherwise be translocated to the host. Increased expulsion of zooxanthellae in the clam's faeces at increased nutrient levels (e.g., Stimson and Kinzie, 1991) would reduce the zooxanthellae's population with little increase in the rate of translocation of photosynthate, as the zooxanthellae would still be growing at a rapid rate. Therefore, by allowing a limited amount of phosphate to the zooxanthellae, the clam host can maintain a particular level of algal population dividing at a rate at which the clam host can derive maximum amounts of photosynthate.

Since ammonia diffuses freely across membranes, it would be hard for the animal host to control the zooxanthellae's access to this nutrient. The only way it could limit ammonia availability is by assimilating the nutrient itself. This is possible as the clam host has high amounts of glutamine synthetase in its gill and mantle tissues (Rees et al., in press). In addition, Rees et al. (in press) noted that glutamine synthetase levels in mantle tissues decreased following incubation of *T. gigas* in 20 μM ammonia. Such high levels of glutamine synthetase and a capacity for down regulation of the enzyme is indicative of a major functional role

of this enzyme in ammonia metabolism in the clam.

Zooxanthellae appear to have some access to P when N is not limiting, as suggested by their increased division rates *in vivo* only with addition of N+P, and their increased demand for, and uptake of, N when clams were exposed to P (Chapters 3 and 5). Nevertheless, specific growth rates of the zooxanthellae were still generally low compared to phytoplankton (see Wilkerson et al., 1988). Additionally, the zooxanthellae's generally high N:P ratios, which were similar to the N:P ratios of P-starved zooxanthellae in culture, strongly indicates P limitation regardless of N availability (Chapters 3, 5, 6).

Based on this study, the question of host control over zooxanthellae growth via regulation of the zooxanthellae's access to inorganic nutrients can be explained. The clam host could actively control their zooxanthellae population either through: (1) a possible restriction of phosphate availability to them, and/or (2) assimilation of ammonium itself. The availability of nutrients to the zooxanthellae, however, may not only hinge on active involvement by the host, but also on the morphological and spatial relationships between the symbiotic partners, coupled with the chemical properties of ammonia and phosphate. Additionally, clams likely expel any surplus zooxanthellae resulting from an increase in zooxanthellae numbers after occasional exposure to increased nutrients *in situ* (see also Stimson and Kinzie, 1991). This in itself will affect nutrient demand by the symbiont population. Whatever is the case, the zooxanthellae

seem to have adapted well to a naturally nutrient-limited environment inside their host.

Such simultaneous N and P limitation of zooxanthellae may presumably apply, with minor deviations, to marine symbioses other than tridacnids. Further research is necessary to demonstrate that such is the case. For tridacnids, in particular, there is a need to clarify the details of the mechanisms involved in P-limitation of their zooxanthellae.

With respect to calcification, while the addition of N on its own had dramatic effects on the growth of the animal host and its zooxanthellae, both N and P, alone or in combination, affected the shell formation of the clams (Chapter 4). There have been previous reports of phosphate 'poisoning' of skeletal formation in corals (Kinsey and Davies, 1989; Rasmussen, 1989), but none directly attributed to increased inorganic nitrogen. This study is the first to report the perturbing effect of both P and N, with N having an even more striking effect than P. The weakening effect of elevated N concentrations on clam shells could possibly be a consequence of organic-matrix synthesis which is too rapid for normal accretion of calcium carbonate to occur. This is suggested by the increased shell length of the clams, but reduced shell weight at equivalent size (Chapter 4).

These perturbations at elevated ambient nutrient concentrations would appear to present a major threat to the structural integrity of clams. Weakened shell structure, particularly in juvenile clams, implies greater vulnerability to predation by crushing organisms (e.g., crab, octopus, starfish). If nitrogen has a comparable effect on corals, then, along with the recognised influence of phosphate, similar consequences may be expected. In the event of physical disturbances such as bioerosion and wave damage, a major cause of coral-reef destruction in areas where tropical cyclones and hurricanes occur (Woodley et al. 1981, Hughes 1989), any decrease in skeletal strength would result in greater susceptibility. More importantly, weakening of the skeletal structure could give way to coral-reef damage over a wider radius or by storms of even moderate intensity.

To date, a lot of emphasis has been placed on the more conspicuous consequences of nutrient elevations, namely, algal blooms, which compete with reef corals for space and light. This study describes a less evident, insidious effect on calcification, which may also become a serious concern in the long term. The organism-level responses investigated here provide useful indicators of stress, especially with respect to the skeletal structure, at increased nutrient concentrations in reef waters. While the nutrient levels used in this study are not common, they are not unknown in reefal waters. Hence, it is imperative that nutrient levels and their effects on calcification be both monitored and controlled for the future of the diminishing number of healthy coral reefs.

Overall, the approaches used to address the objectives outlined for this thesis proved fruitful. Controlled outdoor experiments on clams and their zooxanthellae generated information which was tested and largely verified in a natural situation in the field. Laboratory experiments on the zooxanthellae further clarified the responses of these algae to increased ambient nutrient levels, when living inside and outside the animal host. In brief, this thesis has brought forth valuable insights on tridacnid inorganic nutrition, which may not only resolve contentious issues of nutrient-limitation in marine symbioses, but also lay the groundwork for serious recognition of the detrimental effects of nutrient enrichment on calcifying reef organisms.

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APPENDICES

(Publications)

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Modification of shell formation in the giant clam *Tridacna gigas* t elevated nutrient levels in sea water

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Nutrient limitation in the giant clam–zooxanthellae symbiosis: effects of nutrient supplements on growth of the symbiotic partners

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