STRATEGIES FOR GROWTH MANAGEMENT OF *PORPHYRA YEZOENSIS*(UEDA) BLADES IN SUSPENSION CULTURES: A STEP TOWARDS LAND-BASED MARICULTURE

by

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ABSTRACT

Porphyra yezoensis has been cultivated for centuries in Asia. Ocean-based operations, where the blade phase is grown attached to synthetic nets, and placed in the ocean for grow-out, are the norm. There are many problems associated with this type of cultivation, many of which could potentially be overcome by using land-based tanks for grow-out of blades. However before land-based mariculture can begin, research is needed into techniques for the production of blade suspension cultures, as well as into the nutritional requirements of free-floating blades. This thesis reports on experiments investigating these areas of research, and represents a step towards land-based cultivation.

Techniques for propagating *Porphyra yezoensis* (strain U-51) blades using both conchospores and monospores in laboratory scale land-based tank culture were investigated. Suspension cultures of *P. yezoensis* blades were produced by seeding conchospores onto various fixed and suspended substrata. The use of $CaCO_3$ suspended substrata (particle size 74 - 212 μm) resulted in the highest early growth rates (10% per day) because of their rough surface topography, which was ideal for conchospore settlement. Suspended blades were significantly more lanceolate than fixed blades, as they increased their L:W in response to higher water velocities. When detached, the more ovoid fixed blades grew more slowly (4% per day) than the more lanceolate suspended blades (13% per day) because their shape was not initially

adapted to the higher water velocities in suspension. By remaining in suspension, blades were exposed equally to light and nutrients when suspended substrata were used. Cheap sources of $CaCO_3$ could be exploited (ground bivalve shells), and suspended substrate techniques are not as labour intensive as fixed substrate methods may be, reducing production costs.

Once blade suspension cultures were produced from conchospores, a vegetative method for the propagation of blades via monospores was investigated. Blades of various sizes were cut into tissue sections of various diameters. These tissue sections were cultured under 15°C, 8L:16D photoperiod, 200 µmol photons m^{-2} s^{-1}, and moderate aeration in 200 ml of f/2 medium. After an initial period of growth (10 days), the tissue sections began to disintegrate as they released monospores. The best early growth of new blades resulted with large tissue sections (93.8 mm^2) from small blades (5 - 7 cm in length), but all sections from all blade sizes tested released monospores, and new suspension cultures resulted. This technique represents an easy, low-techology method for blade propagation that has great potential for application on a commercial scale. It allows the conchocelis to be by-passed during cultivation, and allows the propagation of desirable clones.

The effect of tissue N and P on growth of *Porphyra yezoensis* (strain U-51) blades in suspension cultures was investigated. Before these experiments began, culture conditions such as photoperiod, temperature, and stocking density had to be

investigated so that they would be optimal for growth during experiments. Blades grew best at 15°C, and 1.0 g fresh wt per 3L of medium. A range of photoperiods between 8 - 12 h of light per day had no effect on growth. Because blades are typically grown commercially during temperate winters, a photoperiod of 8L:16D was used.

Blades had the ability to store N in excess of requirements. The critical (0.40% fresh wt) and subsistence (0.15% fresh wt) levels were constant regardless of N source (NO_3^- or NH_4^+) or light level. Blades did not have the ability to store excess P, within the range of P concentrations tested here. The subsistence quota for P was higher when blades were grown on NH_4^+, suggesting a decreased ability to utilize tissue P for growth.Therefore, NO_3^- was considered a better N source than NH_4^+. Blades became bright green in colour when they were N-limited, suggesting a link between phycoerythrin and tissue N. The optimal molar N:P of 13 - 15 was constant regardless of N source (NO_3^- or NH_4^+) or light level. N:P < 13 -15 indicated N limitation, while N:P > 13 - 15 indicated P limitation. P-limited and light-limited blades could store more N when NH_4^+ was given, than when NO_3^- was the N source, suggesting physiological mechanisms for taking advantage of this usually ephemeral N source, even when growth was limited. N and P reserves were used up relatively quickly (5 days), a characteristic of opportunistic species. Tissue analysis of N and P was shown to be a very useful technique in determining nutrient status of *P. yezoensis* blades in land-based tanks. As long as tissue N > 0.40% fresh wt and molar N:P = 13 - 15, blades grew unlimited by N or P.

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To my wife Paula, thanks for the patience and love. This work was done for you.

CHAPTER ONE

INTRODUCTION

Porphyra cultivation, the single largest near-shore fishery world-wide, is centred in Japan, Korea, and China. *Porphyra* is used extensively for food, and is known as nori in Japan, zicai in China, and purple laver in Great Britain. It is eaten by coastal peoples in southeast Asia and the Pacific Ocean basin, including the Maoris of New Zealand and B.C. First Nations. Cultivation began in the 1650s in Tokyo Bay, using bamboo twigs for blade attachment (Chihara, 1974). Before cultivation, wild harvests had been taken since 3000 B.C. in China. The production of nori is now the single largest aquacultural industry world-wide, with 25 000 dry metric tonnes worth US $2 billion produced in 1990 ($1.25 billion in Japan, $0.5 billion in Korea, and $0.25 billion in China (Egan, 1990)). Research on this important alga has been extensive, and has long been recognized as having room for establishment and expansion on North American shores.

There are over 70 species in the genus *Porphyra* (25 species in Japan, 17 species native to B.C.). All of the most important commercial species in Asia (*P. yezoensis, P. tenera, P. haitanensis, P. kuniedai, P. pseudolinearis, P. akasakai,* and *P. seriata)* are monostromatic because the processing of this type of thallus results in high quality product. Based on selection criteria for blade thickness, size, shape, color, means of reproduction, tidal zone, and substrate, five native B.C. species show

promise for cultivation: *P. abbottae, P. nereocystis, P. perforata, P. pseudolanceolata*, and *P. torta* (Bergdahl, 1990; Conway et al., 1975; Garbary et al., 1980).

LIFE HISTORY

Any discussion of *Porphyra* mariculture must begin with an account of the complex life history of this genus. All facets of *Porphyra* cultivation are intimately linked to its life history. There is much variation among species in this genus (Kornmann, 1994) and a general overview is presented here. *Porphyra* alternates between a filamentous (dipliod, sporophyte), and a blade-like (haploid, gametophyte) phase during its life cycle (Fig. 1.1). There are many species in which the chromosome number of the foliose and conchocelis phases are equal, and fertilization and meiosis have never been recorded (South and Whittick, 1987). *Porphyra* taxonomy is based on the morphology, reproductive features and chromosome number of the leafy thallus or blade phase (Campbell and Cole, 1984). In many respects the sporophytes more closely resemble members of the Florideophyceae than they do gametophytes of their own life histories. The conchocelis stage is characterized by long and cylindrical vegetative cells that contain elongate plastids, with peripheral encircling thylakoids. Adjacent cells of the filament are joined by pit connections. The gametophyte is characterized by isodiametric cells with central, stellate plastids, which do not have the encircling thylakoids. There are no pit connections between cells in the gametophyte, and the cell walls also differ between the two alternate hetermorphic generations. Cellulose is present in the walls of the conchocelis, whereas xylan is found in blade cell

walls (Mukai et al., 1981; Baldan et al., 1995). Most of the above mentioned differences between the two phases develop during spore germination (Pueschel and Cole, 1985).

Porphyra can be found in the littoral and upper sublittoral zones, attached to rocks and to other algae. The parenchymatous blade can be either di- or monostromatic. *Porphyra nereocystis* is an epiphytic species on *Nereocystis* stipes and is the largest known species, growing up to 2 - 3 m in length. The blade is annual whereas the conchocelis is perennial. It is thought that the thick-walled conchosporangial branches perenniate from September to March while the rest of the filament eventually dies out and is regenerated before conchospore release (Conway and Cole, 1977). Blades are attached by rhizoidal projections from cells in the holdfast region, and cell division is diffuse, occurring in all portions of the blade (i.e., no meristematic region) (Pueschel and Cole, 1985).

Conchocelis was considered to be a separate algal genus until Drew (1949) was able to show that it is in fact the alternate stage in the life cycle of *Porphyra*. Drew found that the carpospores of *P. umbilicalis* germinated in enriched seawater, and formed a filamentous mat. The cells of the filaments were abnormal in appearance, and suggested that a specific host or substratum was needed for normal growth. In the presence of several types of molluscan shells (and even eggs shells), these filaments were identical to *Conchocelis rosea*. This information revolutionized the *Porphyra* cultivation industry (Baker, 1965; Doty, 1977).

We know little about the conchocelis in nature because its size and morphology make it a difficult subject to study. The conchocelis-phase is a filamentous shell-boring alga that has been found in the shells of various organisms, such as razor clams dredged from six to eight fathoms, in the calcareous matrix of bivalve shells, inside the living shells of the stalked barnacle *Pollicipes,* inside the hydroid *Obelia,* and in the plates of high level barnacles (Conway and Cole, 1977). Most of the records of conchocelis in nature are from shells, where the fine, well pigmented filaments can be readily seen. It has been suggested that in some species, conchocelis may float free and lodge in crevices until conchospores are shed (Conway and Cole, 1977). Some more recent work (Martinez, 1990), suggests that conchocelis distribution extends into the intertidal.

Work by Campbell and Cole (1984) may make working with, and identifying conchocelis of various species, easier. They demonstrated that substrate boreholes made by various species of conchocelis have characteristic structures, and resin casts of these boreholes can be made and analyzed, conserving their 3-D orientations. In this way conchocelis can be taxonomically identified by its borehole morphology, which is easier and more precise than traditional techniques (which involve culturing the conchocelis to produce the blade phase which is then identified), allowing the study of the distribution and abundance of conchocelis in nature. Only eight species have been investigated so far with this technique.

Of the various types of spores released by the different phases of *Porphyra* (Fig. 1.1), none are motile. Spermatia are liberated and fuse with projections (in some species) from the carpogonium (called trichogynes) on the thallus surface. The carpogonium then divides to produce 4 - 16 or more carpospores, which germinate to produce the conchocelis (South and Whittick, 1987). The thallus phase disintegrates following carpospore release (Iwasaki, 1961). Carpospores are released in the spring (beginning in March), giving rise to a summer conchocelis in most species (Suto, 1954), however this is quite variable. Kornmann (1994), and Magne (1991) have proposed some changes in the terminology related to spores. New proposed names describe the spores more accurately than the old names. Carpospores are renamed zygotospores, and monospores are to be called archeospores. This paper refers to these spores by the older, more well known terminology.

In most Asian species such as *P. yezoensis*, conchospores are liberated from the conchocelis in the fall (September to November), and give rise to winter blades. In some species, seasonality of sporophyte and gametophyte may be reversed, with summer blades and winter conchocelis. Meiosis may occur during the development of the conchospores within the conchosporangial branches, or during germination (Burzycki and Waaland, 1987). Conchospores of *P. yezoensis* are shed periodically, in the morning, mainly between 7 - 10 am. Shedding is retarded in the dark, and is most vigourous at 12 - 22^{o}C (Suto et al., 1954). During *Porphyra* cultivation, up to 200 - 1000 conchospores may be shed per cm^2 of shell (Takeuchi et al., 1954).

Conchocelis can be long-lived despite continuous sporulation (Bird et al., 1972). Conchospores attach to the substratum with a mucilage that surrounds the spore and fills a deep invagination on the ventral side of the spore (Pueschel and Cole, 1985). Germination is accomplished by the emergence of a germ tube that penetrates the mucilage. Growth is driven by vacuolation, photosynthetic activity of the spore, and mobilization of starch reserves. Experimental determination of the relative contributions of these mechanisms is yet to be done (Pueschel and Cole, 1985).

Monospores are produced by blades as a means of asexual reproduction by 13 of the 33 Asian species, while only 1 (*P. gardneri*) of the 17 north-east Pacific species produce them. All species that produce monospores are monostromatic with one chloroplast per cell (but not all monostromatic, one chloroplast species release monospores). Monospores are characterized by the presence of fibrous vesicles, which are similar to those found in other red algal spermatia, carpospores, and tetraspores. It is thought that these vesicles play a role in spore release and adhesion. It has also been suggested that fibrous vesicles may release enzymes that break down the thallus cell walls at the time of release. Monospores are usually released from blades only a few millimetres long. In a few species (eg. *P. gardneri, P. kuniedai, P. suborbiculata, P. yezoensis,* and *P. tanegashimensis*) older thalli that are several hundred millimetres long can release them (Hawkes, 1980). Factors affecting production and release have not been well investigated, and this area of research could be very fruitful.

Photoperiodic control of spore release

During *Porphyra* cultivation the entire life history is manipulated, and to some extent controlled. Therefore this alga is truly farmed, unlike the cultivation of most marine animals that still rely on wild stocks of young adults with eggs (Mathieson, 1975). The ability to control the timing of spore release has obvious advantages in aquaculture, and as a result there has been much research in this area. Typically the timing of spore release is controlled by response to seasonal variation in photoperiod (i.e., the number of hours of light per day). However, there is much variation among species in regard to environmental signals that induce spore production.

Iwasaki (1961) found that a daily photoperiod of 8 - 11 hours induced formation of conchospores in free living (i.e., not grown in shells) conchocelis of *P. tenera*, and that these conchospores germinated into blades in 3 - 8 weeks. He also found that there were no significant effects of temperature or irradiance on conchospore development and release. However, the growth of young germlings was retarded or prevented by reduced irradiance. Under continuous light, the conchocelis cultures failed to develop conchosporangia, but growth of the conchocelis may be favoured by continuous light. When these continuously illuminated cultures were transferred to a photoperiod of 8L:16D (i.e., 8 hours of light followed by 16 hours of dark per day), thallus germlings began growing after 5 weeks. Conditions that favour growth will inhibit spore production, and vice versa.

A summary of responses to environmental stimuli in inducing spore release will be useful here to illustrate the variation among species, and the unifying theme of responses to photoperiod. A daily photoperiod of 10 - 12 hours induced conchospore release in *P. suborbiculata*. With this species there is an effect of temperature and photon flux density (PFD) on conchospore release, with maximum spore production at 15°C and 55 µmol photons m^{-2} s^{-1} (Iwasaki and Sasaki, 1971). *Porphyra torta* produces conchosporangia under a wide range of PFDs and temperature conditions, but conchospores will mature and release only when exposed to a short day photoperiod of just shorter than 12 hours. A photoperiod of 8 hours almost always promoted spore release while a 16 hour photoperiod was almost always inhibitory (Waaland et al., 1987). *Porphyra leucosticta* releases conchospores in response to photoperiod and PFD, with optimal conditions of 10 hour photoperiod and 31 - 44 µmol photons m^{-2} s^{-1} (Sidirelli-Wolff, 1992). *Porphyra miniata* releases conchospores when the photoperiod is reduced to 8 hours, and the temperature is between 3 - 7°C. *Porphyra columbina* from central Chile produces conchosporangia under short day conditions, but only a temperature drop from 15 to 10°C triggers conchospore release (Waaland et al., 1987). The Japanese species, *P. yezoensis,* responds to both temperature and photoperiod (Miura, 1975).

In contrast to the conchocelis phase of *P. tenera*, Iwasaki (1961) found that blades of this species die quickly, when grown under continuous light. Growth is highest with daily photoperiods of 8 - 10 hours. Carpospores were produced and

released after 40 days when illuminated for 13 hours per day. At this photoperiod, blade growth is inhibited. Again, the suggestion is that the thalli, like the conchocelis, cannot allocate resources to both growth and reproduction. The different responses to photoperiod by the two phases (i.e., carpospore production induced by long photoperiods, conchospore production induced by short photoperiods) is expected because the two phases in the life history correspond very sharply with the seasons, the blades growing in the winter (short day season), and the conchocelis growing in the summer (long day season) in most species. There is a great disparity in the amount of information on thallus responses to photoperiod, when compared to the literature on conchocelis responses. This is because it is the conchocelis that is usually grown in settings where the photoperiod can be manipulated. There has been little investigation into blade responses because one cannot manipulate the photoperiod in large areas of the ocean where blades are grown commercially.

Porphyra linearis seems to represent an exception to the rule of photoperiodic control of conchospore release. Temperature is the factor most responsible for the induction of conchospore production, with the optimal temperature at 13 ^{o}C. Even in continuous darkness, at 13^{o}C, conchospores continued to be released, until the conchocelis eventually became unhealthy and died (Bird et al., 1972).

COMMERCIAL PRODUCTS

In Japan the traditional diet consists largely of rice, fish and nori, a dried *Porphyra* product that resembles paper. In the preparation of nori, raw *Porphyra* is chopped and poured as a slurry into rectangular frames. The slurry is dried and a thin rectangular (19 x 21 cm) sheet weighing about 3 g is produced (Merrill, 1990). Toasted and seasoned nori, which is treated with soy sauce and other seasonings after toasting, account for most processed nori in Japan (Oohusa, 1993a). Nori is usually sold in packages of 5 - 10 sheets (Merrill, 1993).

North American indigenous peoples from Washington to Southeastern Alaska collect and consume *P. perforata.* It is dried or chewed and then fermented and partially dried before consumption. Hawaiians reserved the consumption of *Porphyra* for royalty, and the Maoris of New Zealand use *P. columbina* as a food called karengo. In New Zealand today, *Porphyra* is boiled and the resulting mush is fried in butte. In Great Britain, *P. laciniata* is collected and consumed especially in those areas with strong Celtic roots. It is fried with butter, or made into jelly or laver bread (Mumford, 1988).

A pigment called R-phycoerythrin (Ogawa et al., 1991) can be extracted and used to make fluorescent-tagged antibodies. These are used widely in the medical industry as fluorophores on immunofluorescent probes for biological macromolecules (Mizuno et al., 1982). R-phycoerythrin from *P. yezoensis* is very valuable because of its

stability and high fluorescence. This pigment can make up as much as 8% dry weight and sells for US $15 - 50 per mg. Purification costs and the small market currently limit production (Mumford, 1988).

Except for *Laminaria* cultivation, no other seaweed system is more productive, so biomass production for fertilizer or fodder could be a possible objective of farms (Waaland, 1981). The cell walls of thalli can be processed into a low quality agar suitable for food use. This agar can be used as a gelling agent in bakery products, confectionery making, and in puddings, creams, and jellied products (Waaland, 1981). Commercially produced species have been selected for thin cell walls (better quality when dried), so agar yields and quality would be low. However, low grade surplus thalli could be used to produce this agar.

Nutritional properties

It is not surprising that *Porphyra* has been used by so many different cultures world-wide when its food value is considered. Many essential vitamins and minerals are present in blades (Noda, 1971). Vitamin C content is similar to that found in citrus fruits such as lemons (Waaland, 1981), and it is also rich in the B vitamins (Lobban and Harrison, 1994). Minerals present include calcium (4.7 mg / g dry wt.), iron (0.23 mg / g dry wt.), and iodine (0.005 mg / g dry wt.). Also relatively high levels of Zn, Cu, Mn, and Se have been found (Noda, 1993). The total protein content ranges from 30 - 50% dry weight. The nutritional value of the amino acids that make up the bulk of the protein is

about the same as that for whole eggs. Sodium content is low (1% dry weight) because most salt is washed from blades during processing.

With the rise in popularity of healthy diets, the use of *Porphyra* could become widespread. One sheet of high grade nori contains 27% of the recommended daily allowance of vitamin A as β-carotene. A diet of *Porphyra* has also been found to lower the blood cholesterol levels in rats (Mumford, 1988). The high levels of taurine (≥ 1.2% dry weight) are notable as this compound aids enterohepatic circulation of bile acid, thus preventing gallstone production through controlling blood-cholesterol levels. A sulfated galactan (similar to agar) occurs in relatively large amounts, and this dietary fibre has important functional activities such as an antiblood coagulant, antihypercholesterolemia and shows antitumour activity (Noda, 1993).

In Japan the quality of nori depends on a number of factors (McLachlan et al., 1971): 1. Dark colour and good lustre results from the persistence of pigments during the toasting process; 2. Superior flavour is found in nori containing high levels of gyanylic acid (0.01 mg / g dry wt) and inosinic acid (0.8 mg / g dry wt), and the amino acids taurine (4.8 mg / g dry wt), asparagine (2.5 mg / g dry wt), glutamic acid (10 mg / g dry wt), and alanine (18 mg / g dry wt) (Note: numbers given are minimum amounts for good taste); 3. Softness in the mouth which results from moderate amounts of freshwater, siltation, calm conditions, and early harvesting; 4. Sweetness which depends on the levels of free sugars such as

floridoside, iso-floridoside, glucose, galactose, mannose, arabinose, xylose, and ribose (Mumford, 1988).

In order to preserve the quality of nori after processing, it is necessary to dry it to at least 5% water content as quickly as possible and to store at temperatures as low as possible. If this is not done, proteins and fats in nori products may be degraded. Nori should be packaged in air tight plastics. In an oxygen-free atmosphere, pigments and vitamin C content do not degrade, even after 6 months of storage. Nitrogen gas has recently be used in the preservation of nori (Oohusa, 1984).

Product markets

The U.S. market for nori is the largest outside Asia, accounting for 95% of North American imports. Retail prices in 1992 were between U.S. $0.20 - 0.60 per sheet, depending on the quality and the number of levels in the distribution chain that the final product must pass through before public sale in North America. At each level in the distribution chain, markups of 20 - 40% are common, so the final retail price may be 2 - 4 times the landed value (Merrill, 1993).

Japanese exports to the U.S. experienced strong growth during the period 1976 - 1985, with annual growth estimated at over 23%. This growth is attributed to the increased awareness of non-Japanese Americans to Japanese restaurants. Since 1985, Japanese exports have been steady at 200 tonnes per year dry weight. This

stabilization has occurred because of the increased exports from China and Korea. Japanese producers invested heavily into the Chinese and Korean industries in the mid 1980s, allowing these farms to produce Japanese style nori (Oohusa, 1993b). The Chinese and Korean products are typically of lower quality than Japanese grown nori, but North Americans do not have the stringent, high quality standards found in Japan. As a result, Chinese and Korean grown nori has begun to dominate the North American market. In 1992 market shares for Japan, China, and Korea were estimated at 30%, 60%, and 10% respectively of a total market of about 660 tonnes per year dry weight. Total landed value of U.S. imports from Japan, China, and Korea was estimated at U.S. $20 - 25 million in 1991 (Merrill, 1993). Market growth of >20% annually has remained, despite stabilization of Japanese exports.

The U.S. consumes about 0.9 sheets per person annually. In Japan, 70 - 90 sheets per capita is consumed (Freeman, 1985). Modest gains in per capita consumption in North America can translate into significant growth in sales. The market is predicted to continue growth at 15 - 20% annually (Merrill, 1993). This depends upon strong marketing efforts. Nori products have not been marketed well outside of Asia. The market in North America has remained mostly in restaurants. Potential is great for expansion to home use. With the spread and popularity of Japanese restaurants, it was expected that consumers would begin to experiment with home preparation of nori recipies. This has not occurred because most consumers consider this type of food to be exotic and difficult to prepare. Marketing should include teaching simple traditional

recipes, developing western-style dishes that use nori (Merrill, 1993), and publishing cook books including recipes that emphasize the terrific health benefits that a diet including nori can give.

Currently the market size is large enough to support several farms in North America. The establishment of a nori industry here could generate public interest that may stimulate market growth. Market growth has been strong and is expected to remain that way (Merrill, 1993). The largest obstacle in the way of nori industry establishment and expansion in North America has been the difficulty in obtaining necessary permits (Lindstrom, 1990). Because of the large area needed for ocean-based farms, there are many resource use conflicts with other user groups such as upland owners, and recreational and commercial boaters. British Columbia shows the greatest potential for expansion because of the presence of large uninhabited areas of coastline (Merrill, 1990). Egan (1990) notes an enthusiastic welcome by current producers, of new producers in B.C. because they are not likely to affect the world nori market, and therefore will not really be competing against each other. Because of its favourable geographic location, and natural resources, British Columbia could make a major contribution to the world's seaweed industry (Lindstrom, 1990). Investment in this industry is attractive because of *Porphyra's* rapid growth, high nutrient value, farmability, and large market size (Bergdahl, 1990).

NUTRIENTS

There has been little published research in the area of *Porphyra* nutrition. Researchers have focused on the three main macronutrients important for blade and conchocelis growth: carbon, nitrogen, and phosphorus. Fewer studies exist that concern themselves with micronutrition, and the evidence from these studies suggests that micronutrients such as zinc or vitamins may be more important than once thought. The availability of nutrients is one of the primary factors regulating the growth of *Porphyra.* In many areas where *Porphyra* is cultivated, inorganic fertilizers are used to enhance crop production. At least 56 elements have been reported to be present in *Porphyra.* However, the presence of a particular element should not be taken as evidence that the element is essential because certain elements are absorbed in excess of requirements and others are absorbed but not utilized. There is evidence that C, H, O, P, N, Mg, Fe, Mn, Zn, and Mo are all required for growth (DeBoer, 1981).

The three main nutrients that can potentially limit growth in an aquaculture setting are carbon, nitrogen, and phosphorus (McLachlan, 1973). Liebig's law of the minimum states that when other factors such as light and temperature are favourable, the nutrient available in the smallest quantity, with respect to the requirements of the plant, will limit its rate of growth (DeBoer, 1981). Seaweed growth will be limited by the availability of nutrients in the system. This problem is especially intense in tank cultivation of seaweeds, since dense cultures in smaller volumes result in faster nutrient depletion than in traditional ocean-based net cultivation. Fertilization can be

more effective in tank cultivation since nutrients do not become as diluted or washed away by ocean currents. Carbon is rarely limiting to growth in the ocean, but during tank-cultivation, carbon may be reduced to levels that become limiting (Lapointe et al., 1976) unless a pH-stat device is used (explained later). Nitrogen and phosphorus are also required for rapid blade growth. Tank culture poses an interesting technical problem when nutrient enrichment for growth enhancement is managed. Successful *Porphyra* mariculture depends upon knowledge about the relationships of nutrients to growth (Hanisak, 1990).

It would seem at first that continuous nutrient enhancement that keeps uptake saturated would result in the highest yields possible. Most seaweeds can take in nutrients and store them in excess of growth requirements, so enhancements are only necessary when internal nutrient concentrations fall to near the critical level. The critical level is the minimal internal nutrient concentration required for maximal growth. Therefore continuous fertilization that constantly saturates uptake is wasteful, and can lead to reduced yields due to epiphyte enhancement (Lapointe and Ryther, 1978). For commercial farms, pulses of nutrients are used because this is the most convenient and economical method of nutrient enhancement (Hanisak, 1990). During tank cultivation, all nutrients should ideally be assimilated in algal tissue, leaving nothing behind for epiphytes and other contaminants.

Saturating uptake rate of a limiting nutrient can be related to its concentration by a rectangular hyperbola. This relationship is described by the following equation known as the Michaelis-Menton equation: $V = V_{max} [S / (K_S + S)]$, where V is the uptake rate, V_{max} is the maximal uptake rate, S is the substrate concentration and K_s is the half-saturation constant (i.e., the substrate concentration where $V = V_{max} / 2$). V_{max} and K_s describe the uptake kinetics of the nutrient of interest (Harrison and Druehl, 1982). However these "constants" are not constant at all. They are influenced by many factors such as the nutritional past history of the plant, culture conditions, and the experimental method. These kinetic parameters are of great physiological significance, but their use in *Porphyra* mariculture is limited because this seaweed may take in nutrients in excess of growth requirements and can store these nutrients until needed (Hanisak, 1990).

Growth rate has been related to external nutrient concentrations of the medium. However it has been shown that growth rate can be estimated more accurately from nutrient concentrations within phytoplankton cells (Harrison et al., 1989; Thomas and Harrison, 1985). A better method than the one above for investigating nutrient relationships with growth involves monitoring the internal nutrient status of blades, and relating this to growth rate. The growth rate can be related to the internal tissue nutrient concentration (or cell quota) by using the Droop equation: $\mu = \mu_{max} [1 - (Q_0 / Q)]$, where Q is the tissue concentration of the nutrient within the algal cells, Q_0 is the lowest level of Q at which the algae can grow (the subsistence level), and μ_{max} is the maximal growth rate (South and Whittick, 1987). The level of Q where growth just attains its

maximum is defined as the critical tissue concentration. An algal species may not have just one critical tissue level. It may be influenced by light levels, age of the alga, and the nutrient source. Photoadaptation leads to increased internal nitrogen levels (assimilated into pigments), without a concurrent increase in growth rate. Young thalli will usually store less nutrients as they begin to grow rapidly than older, established thalli. Very little of this type of work has been done with *Porphyra.*

Optimal management of nutrients occurs when enough nutrients are applied to give maximal yields, but without excesses that result in wasted fertilizer and/or epiphyte problems. An example of this optimal management comes from *Gracilaria tikvahiae* (Ryther et al., 1981). This seaweed was cultivated in ponds where high levels of nutrients were applied every two weeks, at which time the flow of water into the pools was shut off for two days. This allows rapid uptake of the nutrients by *G. tikvahiae*, and then non-nutrient limited growth for two weeks as it utilizes internal nutrient storage pools. This pulsing strategy resulted in non-nutrient limited growth at all times without fertilizer wastage or epiphyte enhancement. Pulses of nitrogen, and phosphorus resulting in internal nutrient concentrations equal to the critical nutrient levels, will result in non-nutrient limited growth of *Porphyra* blades. Blade tissue analysis for nitrogen, and phosphorus can act as a management tool for nutrient enhancement schedules. Concentrations that are higher or lower than the critical concentration indicate nutrient storage or deficiency, respectively (Hanisak, 1990). With this

knowledge a nutrient enrichment regime can be developed that will result in maximum yields without wastage of fertilizer or enhancement of epiphytes (Lapointe, 1985).

Porphyra has been cultured in the laboratory using a number of artificial and enriched seawater media. The media of choice seems to reflect the individual researcher's personal preference. Almost all of the media listed in table 2-5 and 2-6 in McLachlan (1973, pp. 43-44) have been used at one time. For example, Hannach and Waaland (1989) used f media, Iwasaki (1961, 1967) used a modified ASP media, Gao et al. (1991) used PES media, and Oohusa (1980) used Suto's artificial seawater. All media used gave good growth of thalli and conchocelis. A comparison of these media on the basis of maintaining healthy, growing blades and conchocelis would be extremely useful.

Nitrogen

Inorganic nitrogen is available to algae in three forms: ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-). Because both nitrite and nitrate must be further reduced to ammonium before assimilation, ammonium is usually the preferred source of nitrogen for seaweeds. High ambient ammonium concentrations can inhibit nitrate uptake, but when nitrogen-starved, *P. perforata* showed no preferential uptake (Thomas and Harrison, 1985). Nitrogen gas is not available to *Porphyra,* as it cannot fix this form. Organic sources of nitrogen include amino acids, urea, and purines and occur in seawater at concentrations of less than 20 µM. After carbon, oxygen, and hydrogen,

nitrogen is the most abundant element in *Porphyra* tissues (Atkinson and Smith, 1983). It is assimilated into amino acids, purines, pyrimidines, porphyrins, amino sugars, and amines. Nitrogen is also the most likely nutrient to be limiting growth in the temperate oceans, because it is rapidly utilized but supplied at relatively low concentrations (Thomas and Harrison, 1985).

Iwasaki (1967) found that the conchocelis of *P. tenera* shows the highest growth rate with NO_3^-. *Porphyra* clearly shows typical uptake preference for ammonium (Thomas and Harrison, 1985). However growth rate is not well estimated by high ammonium uptake (Iwasaki, 1967). The conchocelis grew well with low concentrations of ammonium, urea, asparagine, and lysine, but highest growth was obtained with high concentrations of nitrate (the highest concentrations of nitrate used were toxic to the conchocelis). Chao-yuan et al. (1983) found that ammonium was the best source of nitrogen for the blade-phase of *P. yezoensis*. The highest growth (11.6% per day frond area) was obtained with intermittent application of 5 ppm NH_4^+ at 5 day intervals. This suggests that the blades may not be as sensitive to the toxic effects of high ammonium concentrations.

Carbon

Very little work has been done on carbon nutrition of *Porphyra* because carbon is rarely (if ever) limiting in the ocean. There is a large reservoir of various forms fo inorganic carbon in the oceans that can be used during photosynthesis. Inorganic

carbon is present in seawater as carbon dioxide (CO_2), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}). These three forms are in equilibrium with each other, and the relative proportion of each form depends on the pH. Carbonate is usually present in trace amounts at the normal pH of seawater. In seawater CO_2 and HCO_3^- are present in concentrations of 0.01mM, and 2.4 mM respectively. Both CO_2 and HCO_3^- can be used during photosynthesis (Lobban and Harrison, 1994).

Recently there has been much investigation into the effects that elevated CO_2 levels in the atmosphere (the "greenhouse effect") will have on the growth of marine macroalgae, and how to make efficient use of this CO_2 (Bidwell and McLachlan, 1984). It has been found that the higher the CO_2 concentration in water aerated with high CO_2 air, the faster the growth of *P. yezoensis* blades (Gao et al., 1991). CO_2 dissolved in water ($[CO_2]aq$) can reach an equilibrium with that in bubbling gas (PCO_2) during aeration with high CO_2 air. Carbonic acid forms and dissociates to bicarbonate, and then to carbonate as follows (Gao et al., 1991):

1. $PCO_2 \leftrightharpoons [CO_2]aq$
2. $[CO_2]aq + H_2O \leftrightharpoons H_2CO_3$
3. $H_2CO_3 \leftrightharpoons H^+ + HCO_3^-$
4. $HCO_3^- \leftrightharpoons H^+ + CO_3^{2-}$

When CO_2 concentrations in the air increase, the above reactions proceed toward the right, which gives an increase of H^+ ions in the water. Therefore aeration with high CO_2 air can reduce pH of the culture water. In daylight, the pH of the water

rises as photosynthesis proceeds and results in the fixation of carbon. Since bicarbonate is utilized by this red alga, and is converted to CO_2 by carbonic anhydrase before it is assimilated, a lot of OH^- ions are produced and raise the pH. This conversion proceeds (Gao et al., 1991) as follows (PS = photosynthesis):

5. $HCO_3^- \rightleftharpoons CO_2 + OH^-$ 6. $CO_2 + H_2O \rightarrow PS \rightarrow [H_2CO] + O_2$

This diurnal response of pH to CO_2 has obvious applications for large scale tank culture of *Porphyra*. A useful system has been developed for use in tank cultivation (Bidwell et al., 1985). A pH sensitive device is installed, which is attached to a CO_2 gas cylinder. When the pH increases during the day, the device triggers the release of CO_2, which decreases the pH back to about 8.0, and aleviates any carbon limitation that may be occurring. Once the pH is returned to 8.0, the device shuts off CO_2 injection. In this way carbon levels are kept high during the day when photosynthesis demands the utilization of carbon, and are lowered at night when photosynthesis does not occur. All that needs to be known is the optimum pH level for growth. This type of system is analogous to demand feeders, used extensively by fish farms.

Phosphorus

Phosphorus is available to *Porphyra* in the form of orthophosphate ions (PO_4^{3-}). Phosphorus is not generally considered to be a limiting nutrient in temperate seawater. However, in carbonate-rich tropical waters, phosphorus is generally more limiting than

nitrogen to macroalgae (Lapointe, 1987; Lapointe et al., 1992). Growth of *Porphyra* is stimulated by concentrations of up to 320 µM, but is inhibited by a concentration of 1600 µM which is toxic (DeBoer, 1981). There have been comparatively few studies done on *Porphyra* phosphorus nutrition, probably due to the fact that it rarely limits growth in temperate marine waters (Lobban and Harrison, 1994).

Micronutrients

There have been very few studies on the effects of trace elements and vitamins on the growth of *Porphyra*. The principle roles of micronutrients such as Mn, Cu, Zn, Se, Ni, and Mo are as enzyme cofactors. These cofactors are used in such small quantities that sufficient amounts to sustain *Porphyra* in culture can usually be found as contaminants in the medium (Lobban and Harrison, 1994).

Zinc is important for algal nutrition because it acts as an activator of many dehydrogenase enzymes. Noda and Horiguchi (1971) found that the optimum concentration of zinc for growth and protein synthesis is 0.03 ppm for *P. tenera*. This is four times the concentration found in seawater, because the medium (ASP) contained chelators that reduce the amount of free ions of trace metals (Kain and Norton, 1990). In the absence of zinc, both chlorophyll and phycobilin synthesis were inhibited, and photosynthesis was retarded. Typical rapid uptake kinetics by zinc-starved cells was observed. As a result of their work, zinc is now recognized as one of the most important nutrients in terms of commercial cultivation of *Porphyra*. This is due to the positive

correlation found between the final product quality and the zinc content of the blade. Quality is dependent upon carbohydrate, protein, total nitrogen, and zinc content. Superior grades of nori are low in carbohydrate and high in protein, total nitrogen, and zinc. Also, there is a positive correlation between zinc and protein, and the total nitrogen content of the blade. Zinc is an essential element for growth and formation of chlorophyll, phycobilin, and proteins in *P. tenera* (Noda and Horiguchi, 1972).

Vitamins are important for algae because they act as enzyme cofactors. Many seaweeds, when grown under axenic conditions, develop abnormal morphology. In the absence of an accompanying microflora, some seaweeds can develop only filamentous or callus growth. This may be due to the absence of trace metals, organic chelators, vitamins or other growth promoting substances from the media when bacteria, fungi, and microalgae are not present. In several cases it appears that vitamins required by some algae may come directly from the microorganisms living on seaweed surfaces (DeBoer, 1981). The three main vitamins that are important to algal nutrition are biotin, thiamine, and B_{12}. No requirement for biotin or thiamine has ever been recorded for any members of the Rhodophyceae. *Porphyra tenera* has been found to require B_{12} for growth in bacteria-free cultures (Kain and Norton, 1990).

Trace elements are very important in *Porphyra* nutrition, yet there have been few studies concerned with micronutrition. In addition to zinc and B_{12}, copper (Kain and Norton, 1990), and Fe-EDTA (Iwasaki and Matsudaira, 1958) have also been found to

be essential for growth and photosynthesis of *P. tenera.* More investigation into this area will certainly uncover more micronutrient requirements for *Porphyra.*

CULTIVATION METHODS

History

Porphyra cultivation began with the casual collection of wild blades in China and Japan. In China, there are records dating from A.D. 533 to 544 showing that a highly valued *Porphyra* species was collected from intertidal rocks (Mumford, 1988). About 1000 years ago the Chinese began to consider *Porphyra* a delicacy, and during the Sung Dynasty (A.D. 960 - 1279), *Porphyra* was selected annually for presentation to the emperor (Waaland, 1981). In Japan, cultivation began in Tokyo Bay, near the estuary of the Sumida River, in the 1650s (Mathieson, 1975). The method used included the placement of bamboo twigs and branches at the correct tidal height. Plants grew on the branches in the fall and early winter, and were hand-picked. This method of seeding twigs was common until the twentieth century when methods that made use of nets became popular (Mumford, 1988).

During the 1910s and 1920s, methods were developed that sought to replace the branches with more productive and efficient mats or nets. Initially the mats were made from thin bamboo shoots, and later natural fibres replaced bamboo. In the 1960s, synthetic fibres were used for the first time. Today netting is made from a mixture of polypropylene fibres for strength and Vinylon or Cremona fibres to which spores attach

readily. In Japan, the netting is a standard size and mesh (18 x 1.5 m net with 30 cm stretched mesh) (Mumford, 1988).

Originally, sites that were valued as good growing areas were located near the mouths of rivers, where nutrient levels were high. When nets and mats began to be used, they were suspended horizontally from poles driven into the bottom, so deeper waters could be exploited. This technique allows blades to be subjected to naturally occurring fluctuations in temperature, and desiccation time. The heights of the nets are adjusted to allow a period of desiccation that kills competing organisms, but does not harm *Porphyra* (usually 3 - 4 hours per day) (Michanek, 1975). Later in the 1960s, it was discovered that blades larger than 2 - 3 cm did not need this period of emersion. From this knowledge the floating raft method was developed, where the netting is fixed horizontally to a floating raft. With this method any suitable deep water site could be utilized. The floating raft method gained popularity as near shore waters became polluted, filled for upland use, or used for other purposes (Tseng, 1981a).

Modern techniques

Conchocelis culture

The modern method of production that is used extensively in the Orient (mainly Japan, Korea, and China) begins with carpospores, which are used to produce a free living conchocelis stock culture. These cultures are maintained in vitro by vegetative growth. A suspension of carpospores is prepared by inducing release from the blades

which have been dried over-night in the shade and immersed in sea water for 4 - 5 hours the next morning, or by filtering the suspension of pulverized blades crushed in a mortar.

From these stock cultures, large scale cultures of conchocelis growing in shells are produced because this makes handling the cultures easier. The germination of carpospores and their boring into the shells is more efficient when the specific gravity is higher than 1.02, daytime brightness is high, and water temperature is between 10 - 15°C (Miura, 1975). In nature, carpospores sink to the bottom and germinate into the conchocelis, which burrows into the shells of various bivalves and molluscs (Michanek, 1975). Inoculation of shells occurs in the spring, after the shells have been cleaned of epiphytes and sessile invertebrates. The shells may be arranged shingle-like in trays or strung together and suspended in tanks. Tanks can be as large a 3.6 x 1.8 x 0.6 m in size, which can hold up to 10 000 pieces of conchocelis-bearing shells. In the hanging shell culture, growth is in accordance with the intensity of light penetrating the water, so highest growth occurs in the shells near the surface. In order to maintain more uniform growth, the hanging lines are turned upside-down, once or twice a month. Sea water in these tanks is maintained at a specific gravity < 1.028, and addition of N, P, and K is usually needed for high growth (Miura, 1975).

During the summer the temperature rises from 15 to 25°C in the conchocelis cultures. Days lengthen, and nutrient conditions are shifted to a higher P:N ratio. This

induces formation of conchosporangia. Photoperiod is artificially reduced to 8 - 10 hours light per day with screens to encourage conchospore maturation and delay release. In order to get co-ordinated mass release of spores, the temperature is dropped to 17 - 18°C, and light levels are raised. After 4 - 6 days at these conditions, the conchospores are released in a co-ordinated manner, in the morning, when exposed to full sunlight (Mumford, 1988).

Net seeding

Seeding of nets with conchospores is usually done in September and October (Miura, 1975). Two methods are used to get the conchospores to attach to the netting. The indoor method involves winding nets onto large reels, and immersing them in deep tanks that contain conchocelis-bearing shells. When conchospores are released, a spore suspension is created and nets are rotated in this suspension until enough spores attach to the netting. The outdoor method is done by placing shells in horizontal rafts, with many layers of nets over these shells. The released spores float and attach to the netting (Mumford, 1988). About 10 - 50 conchospores per cm of net is the ideal number, as higher numbers are associated with the spread of fungal and bacterial disease, as well as self-shading and reduced individual growth rates (Mathieson and North, 1982).

Seeding takes about 1 - 5 days, after which the nets are placed in the field for nursery growth. At this time fertilizer may be applied to the nets (600 mg per m^2) over

two to three days in order to increase survivorship and to prevent discolouration (Michanek, 1975). The nets are placed in the ocean in November and the grow-out season lasts until March. After about 15 - 20 days from the time the nets are seeded, blades become visible to the naked eye as black dots on the twine. When the plants are on the nursery nets, monospores may be produced, increasing the density of plants per net (Michanek, 1975).

Cold storage of nursery nets

After about 25 - 30 days from the time blades become visible, the plants on the nursery nets are 2 - 3 cm long, and some of the nets with attached plants are dried, rolled up, placed into vinyl bags, and frozen at -6 to -30°C. Blades are dried until the water content decreases to 20 - 40%, which usually takes 2 - 3 hours. Drying is necessary because when water around and within cells freezes, they suffer damage and lose their vitality. If freezing is done at temperatures colder than -30°C, crystallization of protoplasm may occur, resulting in cell death (Miura, 1975). When needed, nets can be taken out of cold storage and placed in the ocean for growout. In nature the plants are able to survive periods of freezing as they live in the intertidal during winter. If partially dried and frozen at -20°C, plants will remain viable on the nets for 6 - 12 months. This technique was first used in 1965 in Japan, and it allows the farmer to have many more nets than will fit in his growing area (Oohusa, 1984). This gives insurance against crop failure due to disease or unfavourable conditions.

Grow-out and harvest

Once the plants are over 2 - 3 cm long, they no longer need periodic desiccation, and may be placed in floating rafts. They grow to 15 - 20 cm in a few weeks, and then are harvested. Once a net has been harvested completely, another net is taken out of cold storage and placed in the growing fields. This process will be repeated several times during the growing season (Mumford, 1988). Harvesting was once done by hand and was a labour intensive process. Modern techniques utilize mechanical harvesters that usually consist of a rotating reel with blades. The harvester is usually mounted in a boat that runs under the nets, as the harvesters pull the net sideways over the boat. The mower then cuts the hanging blades from the nets. Another technique uses a cage mounted on a boat with a single operator. The boat is pushed along under the nets, and the harvester cuts off the blades. There are also mechanical harvesters that can be mounted in front of a boat, several feet under the water. Blades are cut from the net under water, and cut material is sucked up into the boat (Mumford, 1988). From a standard net a farmer can expect to yield 35 - 105 kg fresh wt (Chapman, 1980). Modern techniques of artificial net seeding, frozen storage of nets, and mechanized harvesting have resulted in a dramatic increase in production (Oohusa, 1993b). Prior to these techniques, only one net was seeded naturally, and harvested once during a shorter season (Miura, 1975).

Strain selection

In the early 1970s, the practice of strain selection to improve growth and quality became common in Japan. The simple method of selecting plants with specific attributes was done, and currently there are at least 32 cultivars of *P. yezoensis* and *P. tenera*, the two most commonly grown species. Ten of these varieties became accessible to the public in 1980, and since then royalties must be paid to the originators of new strains. Attributes that have been selected include, long narrow shape (gives higher yield on netting), late maturation (prolonged growth), monospore production (dense net seeding), and high phycobilin content (Merrill et al., 1983). Strain selection is one of the main reasons for the increase in production of *Porphyra* recently (Mumford, 1988).

Porphyra linearis also shows evidence that strains exist. Some conchocelis cultures of *P. linearis* fail to sporulate even under favourable conditions. Also, conchocelis grown under identical conditions display variation in growth rate, colour, degree of branching, morphology, and texture of colonies. Rapidly growing vegetative plants are generally ineffectual in conchospore production (Bird et al., 1972). The use of a few genetic strains that grow well may result in the genetic uniformity of farms. This is dangerous as these farms would be subjected to the risk of crop failure through disease outbreaks or unfavourable conditions, affecting all plants equally. This could be prevented by seeding nets with several different strains (Mumford, 1988).

Protoplast isolation

It is now possible to enzymatically break down *Porphyra* blades, isolate individual somatic cells, and regenerate blades from each of the isolated cells. This process shows great potential for by-passing the conchocelis stage during cultivation, and in selecting and propagating clones from plants with desirable traits, such as disease resistance, or high growth rate. There is also potential for combining traits of different plants through somatic hybridization and other genetic engineering techniques (Saga and Sakai, 1984; Mizukami et al., 1992). Individual cells can be induced to develop a callus (i.e., an undifferentiated cell mass) morphology, allowing storage and maintenance of "seed stock", advantageous for cultivation (Polne-Fuller and Gibor, 1987; Xue-wu and Gordon, 1987). Protoplast isolation technology shows great potential for improving modern cultivation techniques, but some fundamental problems exist and must be worked out.

Tissue culture of land plants began in the 1960s (Butler et al., 1990) with carrots, and began to be widely practiced in the 1970s with other commercial crop plants (Zuo-mei, 1984). The procedure for land plants differs in many ways to seaweed procedures, however some similarities exist. In general terms, specific sections of plants are selected and treated with harsh cleaning agents to kill bacteria and other surface dwelling contaminants. Enzymes are used to breakdown cell walls. Individual cells or naked protoplasts are isolated and cultured on a special medium. Cell culture on solidified agar medium usually results in the development of calli, whereas liquid

media results in growth and differentiation into thalli. With land plants, addition of specific hormones such as kinetin and auxin is needed for the proper development of plants from individual cells (Torrey, 1985).

Seaweed tissue culture has developed more slowly because of some unique problems which occur when seaweed cells are isolated (Polne-Fuller and Gibor, 1987). The surfaces of seaweeds are usually heavily infested with various organisms, both macro- and microscopic. Some of these epiphytic organisms grow imbedded in cell walls and between the living cells. This makes non-damaging tissue cleaning very difficult with seaweeds. Algal cell walls are complex due to the presence of macromolecules, and commercially available enzymes capable of breaking down these macromolecules are generally unavailable. Very little is known about the composition of the cell walls of *Porphyra* (Polne-Fuller and Gibor, 1986). Inducing development into blades from single cells can be tricky (culture conditions are usually manipulated, a hit and miss procedure), and little is known about growth and differentiation induction factors in seaweeds. Finally, the culture of seaweeds in a laboratory setting is difficult due to the absence of seed-like structures (i.e., microscopic gametes and spores, as well as alternate life history phases are difficult to manipulate).

Land-based mariculture

Artificial seeding of conchospores, cold storage of nursery nets, and the mechanization of harvesting and processing are all recent developments that have

resulted in dramatic increases in production, but the overall techniques have not changed much for centuries (Oohusa, 1993a). This does not necessarily mean that this is the best and only method of production. There are many problems associated with this method of cultivation.

With increasing industrialization in Japan, many nearshore waters have become polluted and unsuitable for cultivation. To overcome this problem, farmers have begun offshore net setting. Because cultivation is done in the ocean either nearshore or offshore, the farms are subjected to abiotic environmental conditions that can at times become extreme. Some examples of abiotic problems that can arise include: storms that upset the anchoring of nets, temperature fluctuations above or below optimal conditions, sediments entering the water during periods of high runoff which lower the clearness of the water and settle on blades, and suboptimal nutrient levels in the water. Biotic conditions can also become extreme at times. Some examples of extreme biotic conditions include: disease (fungal, bacterial, or viral) outbreaks (Fujita, 1990; Ishio et al., 1971; Kerwin et al., 1991), herbivore invasions, and attachment of epiphytes that compete for light and nutrients (Oohusa, 1993a).

Another problem associated with ocean-based net farming comes from floating debris that can get caught in the net structures. This is a very destructive problem in some areas. The debris consists of floating phaeophycean seaweeds, eelgrass, wood, plastics, and anything else that floats. Debris must be removed before harvesting to

prevent inclusion in the final product, and debris can damage plants and nets. Large floating fences can be constructed around farms, but these are very costly and difficult to clean and maintain (Mumford, 1990).

The traditional method of cultivation requires the largest area of any aquacultural operation, at least 0.20 to 0.25 km^2 for a commercial farm (Egan, 1990). Such a large area is needed because nets must be widely spaced to allow adequate water flow through the entire farm. Usually the area required is six times the actual area of the nets (Miura, 1975). This is the biggest problem with this type of farming in B.C. and the rest of North America. Because of the large size needed there are conflicts with recreational and commercial fisheries, recreational and commercial boaters, waterfront land owners, the Coast Guard (who see large farms as navigational hazards), and many others. As a result, the acquisition of permits and leases has become a very difficult and lengthy process, and is considered to be the single greatest barrier to the expansion of seaweed industries on the west coast of North America (Mumford, 1990).

There is another method of production that can alleviate or reduce most of the problems associated with the traditional method. It is land-based tank culture. In a land-based tank, water can be pumped from a submerged offshore inlet pipe into large tanks. Because the inlet pipe is offshore and ideally very deep, the water should be clean, rich in nutrients, and of constant temperature. Maintainance of ideal growing

conditions is facilitated in tanks, allowing easier environmental manipulation. Therefore, higher growth rates may result from tank culture. For example, *P. tenera* increased its length by 15 - 20% per day under ocean-based culture, but in tank culture its length increased by 24 - 30% per day (Imada et al., 1971). These higher growth rates in tanks would result in higher nori yeilds. If these higher yields off-set the higher costs of production typical of land-based operations, then higher profit margins would result.

Storms and floating debris would not affect a land-based farm and herbivores would not be a problem. There would be no conflicts with ocean-oriented special interest groups, however there may be just as many conflicts with other groups such as waterfront land developers. The cost of water front land and electricity for pumps is expensive (site dependent), but the high prices that *Porphyra* products fetch, and the potentially high yields of tank cultivation may make this type of cultivation economically feasible.

Porphyra poses some technical problems that must be solved before cultivation can be accomplished in tanks. Seeded nets could simply be placed in the tanks for growout, but this would be a waste of space. A better use of the tanks' volume is the production of suspension cultures. Blades in these cultures are not attached to anything, and are suspended freely in the water. Because the tanks represent a three dimensional volume (nets are essentially two dimensional), less area is required for a commercial land-based farm than for an ocean-based farm.

There exists a large volume of knowledge about *Porphyra* as a result of centuries of cultivation. However, there is little information on the optimal conditions for growth in suspension cultures. In fact, there is almost no information on the best techniques for producing suspension cultures of *Porphyra* blades. This thesis reports the results of a series of experiments that represent a step towards the large scale production of *P. yezoensis* in land-based tanks.

Porphrya yezoensis was used in this study since markets already exist for its products and there is a vast amount of information on the culture of this species. Variety U-51 was used because this cultivar has been grown in local waters with good results (J. Merrill, pers. com.). *Porphyra yezoensis* grows well under culture conditions, and a supply of conchocelis inocula was available for use. The Japanese have experimented with tank culture but have taken a high technology, axenic approach using sealed stainless steel culture vessels. This study focuses on techniques that are more commercially viable. Cultures will not be bacteria free. Instead culture conditions will control bacterial numbers by using clean procedures and by changing the media frequently. This will more closely resemble conditions that would be found in large scale, outdoor cultivation. Because this research will take place in a laboratory, the scale is very small and investigations will focus on small, young blades. Older blades may show different responses to factors investigated in this work.

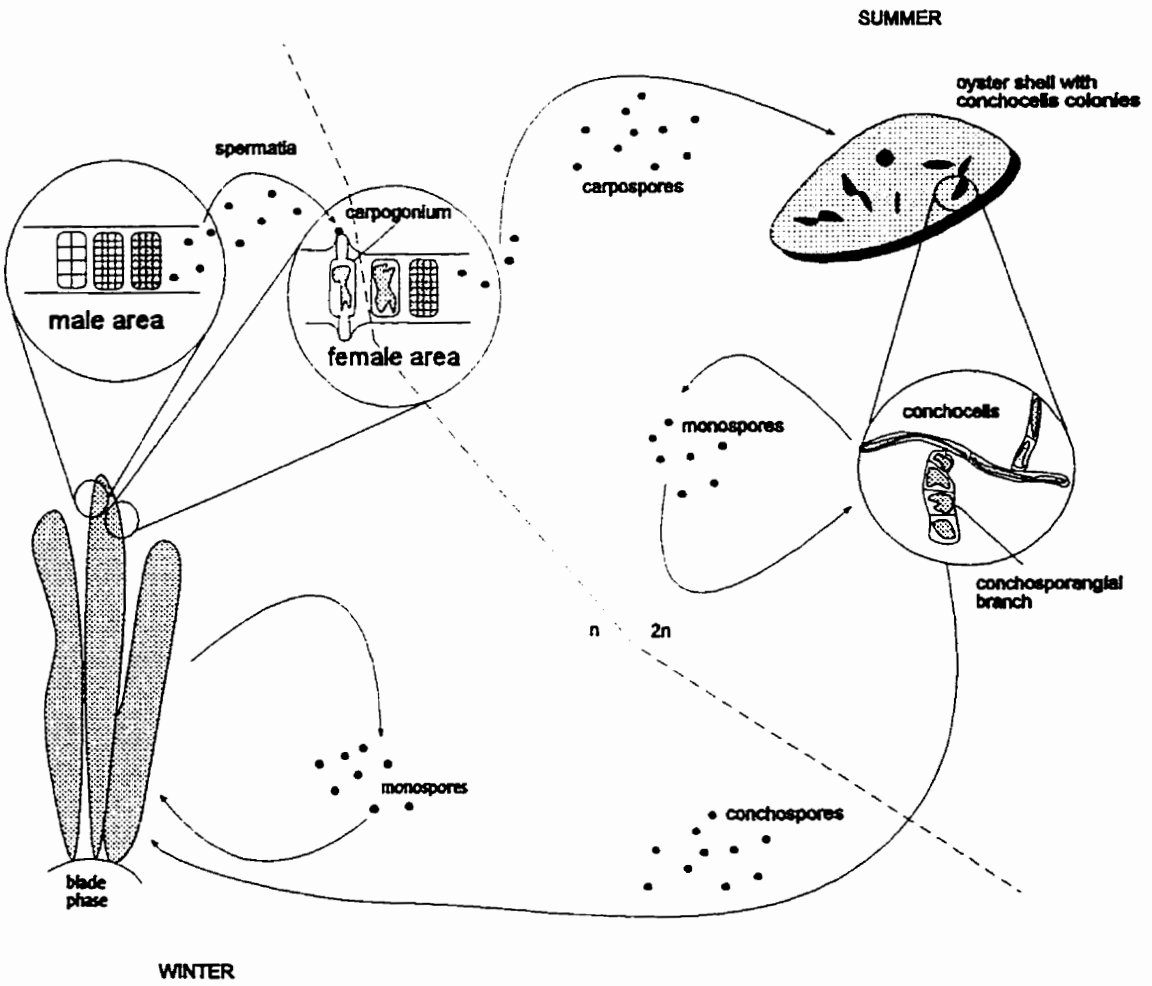
OBJECTIVES

This thesis reports on research addressing two main areas or problems that have never been investigated with *P. yezoensis*. These are:

1. Techniques for the production of blade suspension cultures from conchospores and the vegetative propagation of blades in suspension cultures.
2. The use of tissue analysis for nutrient management, optimizing growth of blades in suspension cultures.

There is great potential for the establishment of a *Porphyra* industry in North America. The market size is large and growing, established techniques are easily adaptable to our waters, there is a vast knowledge about the physiology of this alga, and native species have potential for producing good quality nori. There are areas of research that have not been fully explored, and may lead to improved cultivation techniques. Nutrition, strain production, disease control, vegetative propagation via protoplast isolation, and land-based tank mariculture, are all areas of present research that have potential for improving cultivation, and could result in higher quality products.

FIGURE 1.1. *Porphyra yezoensis* life history.



CHAPTER TWO

PROPAGATION

INTRODUCTION

Modern ocean-based cultivation of *Porphyra* begins with the seeding of conchospores onto nets made of synthetic fibres (Tseng, 1981a; Miura, 1975). Tank cultivation of *Porphyra* could be accomplished by simply placing traditionally seeded nets into tanks for grow-out and harvest (in theory). However this would be a waste of three dimensional space. Better use of the tank's volume can be made by producing suspension cultures of free-floating blades, kept in constant motion by agitation of the medium. This acts to reduce boundary layers, preventing diffusion rates from limiting growth. Tank aeration also acts to expose all blades equally to light and nutrients, resulting in higher yields than are possible in ocean-based net cultivation (Imada et al., 1973).

Commercial tank cultivation of macroalgae is usually done using species which possess suitable characteristics for this type of cultivation (Schramm, 1991). Macrophytes that fragment and continue to grow vegetatively are particularly attractive (e.g., *Gracilaria* (Lapointe et al., 1976), *Chondrus* (Bidwell et al., 1985), and *Gelidium* (Melo et al., 1991)). Vegetative propagation of the crop potentially allows sexual cycles to be by-passed during production, and facilitates the isolation and grow-out (cloning) of individuals with desirable traits (Schramm, 1991). Sexual cycles can be undesirable

because plant growth typically slows down or stops during spore or gamete production, spores and gametes can foul the tank system, and the alternate stage in the life history may not be saleable. Also the alternate stage of a macrophyte may be microscopic or have a morphology that does not lend itself well to tank cultivation. When a heteromorphic alternate life history phase is cultivated, production costs typically rise, because of the specialized equipment and techniques needed for its cultivation.

Unfortunately, *Porphyra yezoensis* does not fragment vegetatively. Each blade is a discrete individual which does not break up under aeration in tanks. Each must begin life as either a monospore or conchospore, therefore large batch cultures of this alga would need to be produced for cultivation to be financially viable. This poses a serious technical problem for tank cultivation and propagation of this alga. Traditional ocean-based cultivation relies on the monospore cycle to produce densely seeded nets, but the conchocelis is still required for the initial net seeding. It is always the source of new blades at the beginning of a growing season because of the separate seasonal occurrence of the gametophyte and sporophyte (Waaland et al., 1990; Sidirelli-Wolff, 1992). Novel techniques for production and propagation of *P. yezoensis* blades in suspension cultures are presented here.

The purpose of the first experiment described below is to determine which general technique results in the most efficient early growth of blade suspension cultures from conchospores. Two general techniques will be tested: fixed substrate and

suspended substrate. Three fixed substrata and four suspended substrata were tested for conchospore settlement and germination. The question posed is which type of technique results in the highest growth rate of young suspended blades?

The second experiment reports on a vegetative method for propagating blades. New blades are produced from existing blades, allowing the conchocelis to be by-passed. The vegetative method involves cutting blades into tissue sections or disks, and inoculating these disks under specific conditions. After 7 - 10 days in culture, cells begin to dissociate from the disks, as monospores do from blades. This is an easy and effective method for vegetatively propagating blades, simplifying the cultivation of *Porphyra yezoensis* in tanks, and lessening the disadvantage that a non-fragmenting alga is subject to in tanks. This technique is investigated here to determine if it is reliable, and has true potential for vegetative blade propagation. Specific points of interest are: optimal tissue section size to use, and optimal blade size to use for tissue section cutting.

MATERIALS AND METHODS

Conchocelis stocks

A free-living supply of *Porphyra yezoensis* (Ueda) variety U-51 conchocelis was made available by the University of Washington, Botany Department (E. Duffield). Conchocelis was cultured in 1 L jars, with aeration lines running through the lids to the bottoms of the jars. All conchocelis stock cultures were housed in one growth chamber.

Culture conditions in this stock chamber were as follows: light source = fluorescent Vita-lights, temperature = 20°C, photon flux density (PFD) = 100 µmol photons m^{-2} s^{-1}, photoperiod = 14L:10D, and medium was kept in constant motion by aeration with aquarium air pumps.

Since this study sought to simulate commercial conditions on a small scale, enriched filtered seawater (f/2) was used, not artificial medium. Seawater was pumped from the bottom of Burrard Inlet by the Vancouver Aquarium. f/2 medium was enriched with nitrate ($NaNO_3$, 880 µM), phosphate (NaH_2PO_4, 36.3 µM), trace metals, and vitamins (McLachlan, 1973) . In blade cultures, nitrate and phosphate concentrations were doubled (1760 and 72.6 µM, respectively) to prevent nutrient limitation during the experiments. The conchocelis medium was changed weekly. Axenic conditions were not attempted because this does not mimic commercial conditions. Prior to enrichment, the seawater was filtered through a 0.22 µm Millipore GS filter.

Experimental culture vessels

Vessels were composed of 400 ml plastic beakers (bottom removed) with funnels sealed to the bottom using silicone sealant. An air line ran into the narrow end of the funnel, and was connected to an aquarium air pump. This type of vessel was ideal for preventing settling of materials in solution, was inexpensive and easily constructed, and was small so many replicates of each treatment could be accomplished in a limited space (one walk-in growth chamber).

Blade growth

Both the length and the width of blades were recorded at weekly intervals. A small sample was taken from each treatment, and the 25 largest blades in the sample were measured, initially with a compound microscope. When the blades became too large to be measured with a compound microscope, a dissecting microscope was used. Specific growth rates (μ, % day^{-1}) were calculated as follows: $\mu = [\ \ln (L_2 / L_1)\ ] / (t_2 - t_1)$, where L_2 and L_1 are mean blade length at times t_2 and t_1, respectively.

Statistical analysis

ANOVA and Tukey analysis, and t-tests were used to determine if significant differences were present among means for each treatment. A two-way ANOVA was used to analyze factorial data from experiment 2. The factors considered in this two-way analysis were tissue section size and blade size used to produce these sections. Tukey analysis was used as a post hoc test to determine pairwise comparison probabilities between treatment means. Tukey results are shown in histograms with letters indicating significant differences in means. In all of these statistical tests, the significance level (α) was set at 0.05.

EXPERIMENT 1. BLADES FROM CONCHOSPORES

Substrate preparation

Oyster and clam shells were collected at Qualicum Beach (Vancouver Island, B.C.) from the high intertidal zone. These shells were extremely sun-bleached, and had

no visible organisms living on them. Supermarket chicken egg shells were collected, and the membranes were removed. The shells were scrubbed, and then separately ground to a fine powder using a mortar and pestle. The powders were then passed through a series of sieves to give three fractions based on particle size: < 74 µm, 74 - 212 µm, and > 212 µm. The 74 - 212 µm fraction was most suitable for conchospore substrate because it stayed suspended under moderate aeration and did not accumulate at the surface. Therefore the 74 - 212 µm fraction was selected and used in these experiments. Monofilament line (0.5 mm diameter) was cut finely by hand with large scissors to give pieces 0.5 to 1.5 mm long.

For the fixed substrate, monofilament line and household (cotton) string were wound around plastic frames that fit within culture vessels. When wound around these frames, the line and string were held near vessel walls.

Conchospore production, maturation, and release

Once conchocelis cultures had grown to a usable size, conchospore production could be initiated. This was accomplished by increasing the temperature to 25°C. After 4 - 6 weeks there were enough conchosporangial branches present to induce maturation of these branches. Maturation was accomplished by reducing the photoperiod to 8L:16D. A mature conchosporangial branch appeared densely pigmented, with very few vacuoles within cells (vacuoles, when present, were never large). Maturation took 2 - 3 weeks to achieve. Release could be delayed until required

by leaving cultures under maturation conditions. When ready, conchospore release was induced by decreasing the temperature to 14°C under a 8L:16D photoperiod. Spore release was confirmed by removing a filament clump from the culture and allowing water to drip off the clump, onto a glass slide which could be examined microscopically. Release was normally achieved 7 - 10 days after decreasing the temperature. PFD must be high to get co-ordinated, mass release of conchospores.

Experimental design

Free-living conchocelis filament balls (3 per treatment) were placed in each of seven funnel vessels (300 ml) when conchospore release was observed. Conchospore-releasing filament balls were exposed to seven different substrata (treatments) for five days to ensure an adequate seeding of substrata with conchospores. After five days, filament balls were removed from the experimental cultures and returned to glass jars in the stock chamber for further conchospore production. The treatments are summarized as follows:

Fixed Substrata:

① Monofilament line (6 m long, 0.5 mm diameter)

② Household string (cotton, 6 m long)

③ Control (vessel surfaces)

Suspended Substrata:

④ Cut monofilament line (1.2 g = 6 m length)

⑤ Clam shell powder (0.15 g)

⑥ Oyster shell powder (0.15 g)

⑦ Egg shell powder (0.15 g)

Five replicates of each treatment were run simultaneously. All treatment vessels were housed in a walk-in Alex Gair growth chamber. Culture conditions were as follows: temperature = 14°C, PFD = 200 µmol photons m^{-2} s^{-1}, photoperiod = 8L : 16D, and medium was kept in constant motion by aeration with aquarium air pumps. Light was provided by fluorescent Vita-lights, with incandescent bulbs present as accessory lights. Culture medium was not changed during the first 28 days of the experiment because this would have resulted in loss of microscopic blades and unattached conchospores. Instead, f/2 nutrients (nitrate and phosphate double conchocelis concentrations) were given in weekly pulses, after the blade length and width were recorded. After day 28, the medium was replaced weekly with fresh f/2 medium.

After the blade length and width were measured on day 21, blades that were attached to fixed substrata were stripped off and became blade suspension cultures after this day. Blades were removed from the line and string by passing the substrate through a small hole punched in a plastic sheet. This was effective in removing almost all blades. Blades attached to vessel surfaces were scraped off these surfaces using a glass microscope slide.

EXPERIMENT 2. VEGETATIVE PROPAGATION

Blades produced from various suspension cultures during the previous experiments were used as raw material for the experiments described below. Glass aquaria were used to grow blades to suitable sizes. All blades used were of the same

age even though they differed significantly in length.

Blades were selected, and tissue sections were cut using variously sized (diameter) glass and plastic tubes. Blades were laid flat on a Plexiglas cutting board and the tubes were used to punch holes in the blades. Blade sections were also ground using a piece of plastic window screen laid flat on the cutting board. The blade sections were laid on top of the screen and were then ground using the end of a glass test tube. Blades selected for tissue sectioning fell into three categories based on length: small = 5 - 7 cm, medium = 10 - 12 cm, and large = 16 - 25 cm. Tissue sections were cut into four sizes (area): ground = variable but always < 8.0 mm^2, small = 8.0 mm^2, medium = 23.8 mm^2, and large = 93.8 mm^2.

Tissue sections were inoculated into 200 ml of f/2 medium in funnel vessels, under 8L:16D photoperiod, 15°C, and 200 µmol photons m^{-2} s^{-1}, with moderate aeration. Nutrients were replenished weekly after measurements were taken. Each funnel vessel contained roughly equal amounts (approximately 380 mm^2) of tissue in terms of total tissue area (i.e., vessels inoculated with small tissue sections received more sections than vessels inoculated with larger sections). Treatments were run in triplicate. Total tissue section area (mm^2) was measured weekly by collecting all intact tissue sections and measuring their diameters. This was not done for ground sections because of the variability of individual section sizes, and the difficultly in measuring the large number of individual tissue sections.

RESULTS

Sample sizes were small in these experiments. As a consequence the assumptions of normality and of equality of variance are important to the interpretation of statistical results. Equality of variance was examined by checking and comparing the sample variances. They differed by less than 25% in all cases, so the assumption of equal variance was considered valid. Normality of data was checked by making histograms for each sample separately. There were no conspicuous departures from normality, so the assumption of normality was considered valid. However, because of the small sample sizes, the results presented in this chapter must be regarded with caution.

EXPERIMENT 1. BLADES FROM CONCHOSPORES

Blade size

Blades attached to suspended substrata grew to a larger size during the 42 day experimental period than those initially attached to fixed substrata (Fig. 2.1). On the final day of the experiment, mean blade length was significantly larger (t-test p value = 0) in suspended cultures compared with fixed cultures (Fig. 2.1).The mean length of fixed blades on day 42 did not include string cultures because no blades remained viable in these cultures by day 42. Suspended blades grew to 1.5 mm in length and 0.65 mm in width, while fixed blades grew to 0.5 mm in length and 0.3 mm in width,

after 42 days in laboratory culture. Day 0 represents the day conchospore-releasing conchocelis filament balls were removed from the culture after 5 days of exposure to substrata.

It appears that the use of $CaCO_3$ suspended substrata (i.e., powdered clam, oyster, and egg shell) allowed the blades to grow to their longest length (Fig. 2.1, ANOVA $p = 0.001$). Cut line gave the shortest blade lengths (Fig. 2.1) of all suspended substrata tested. Cut line cultures gave lengths that were similar to monofilament line and control cultures (fixed cultures).

Growth rate

Suspended blade length grew at a significantly higher rate than fixed blade length, when averaged over the entire 42 day experimental period (Fig. 2.2). Suspended blades grew 10% per day in length, while fixed blades grew 6% per day in length (t-test, $p = 0.014$). When the experimental period was split into two intervals, more detail in terms of growth rates was obtained. The first interval included day 0 to day 28, and the second included day 28 - day 42. During the first interval, suspended and fixed blade growth rates in terms of length did not significantly differ (Fig. 2.2). Blades grew 8% per day in length during this initial interval (t-test, $p = 0.863$). However during the second interval (i.e., after fixed blades had been removed from their substrata), suspended blade growth rates in terms of length were significantly higher than fixed blade growth rates (Fig. 2.2). During the second interval, suspended blades

grew at 13% per day in length, while fixed blades grew at 4% per day in length (t-test, p = 0.002).

Length : width ratio

In general, suspended substrata produced significantly longer, more narrow blades than fixed substrata (Fig. 2.3). Mean L:W ratio of fixed blades was 1.77, but it increased by 10.7% to 1.96 for suspended blades (t-test, p = 0). Suspended blades were generally somewhat lanceolate, while fixed blades were more ovoid. Oyster powder produced the most narrow, lanceolate blades of all materials used (Fig. 2.3). Clam, egg powder, and control blades were slightly less lanceolate (i.e., more ovoid), while monofilament line and cut line blades were the most ovoid of all the blades produced (ANOVA, p = 0).

EXPERIMENT 2. VEGETATIVE PROPAGATION

Tissue disk initial growth

After an initial period of growth for about 10 days, the tissue sections began to disintegrate as they released monospores. This initial period of growth was strongly influenced by the size of the tissue section, but not by the size of blade that the tissue section was cut from, or by the interaction between these two factors (Table 2.1). The initial growth rate of the tissue disks did not significantly differ with blade size used to cut disks (Fig. 2.4A). For all the blade sizes tested, 23.8 mm^2 tissue disks initially grew at a significantly higher rate than 93.8 mm^2 disks (Fig. 2.4B; Tukey test, p = 0.006). The

growth rate of 8.0 mm^2 disks did not significantly differ from either 23.8 mm^2 (Tukey, p = 0.355) or 93.8 mm^2 (Tukey, p = 0.101) tissue disks. In general, the larger the tissue section, the lower the initial tissue section growth rate.

Disintegration rate

Tissue disk disintegration occured mainly along the disk edges. The disintegration rate of tissue disks was also strongly influenced by the size of the tissue section, but not by the size of blade that the tissue section was cut from, or by the interaction between these two factors (Table 2.1). There was no significant pattern of increase or decrease in disintegration rate with increasing blade size (Fig. 2.5A). However, the 8.0 mm^2 tissue sections disintegrated significantly faster than the 93.8 mm^2 sections (Fig. 2.5B; Tukey, p = 0.013). The disintegration rate of the 23.8 mm^2 tissue sections did not significantly differ from either 8.0 mm^2 (Tukey, p = 0.237) or 93.8 mm^2 (Tukey, p = 0.299) tissue sections. The general trend here was decreasing disintegration rate with increasing tissue disk size with all blade sizes tested.

Monosporeling length

Both tissue section size and blade size influenced the mean length of monosporelings after 35 days in culture, while the interaction of these factors did not (Table 2.1). When large blades were used for tissue sectioning, mean monosporeling length was significantly lower than when both small blades (Tukey, p = 0.004) and medium blades (Tukey, p = 0.050) were used (Fig. 2.6A). Mean monosporeling length

did not significantly differ between small and medium sized blades used for tissue sectioning (Tukey, $p = 0.498$). There was a general pattern of decreasing monosporeling length after 35 days with sectioned blade size (i.e., the smaller the blade used to produce tissue disks, the larger the resulting monosporelings after 35 days in culture).

The use of 93.8 mm^2 tissue sections resulted in significantly larger blades than when ground (Tukey, $p = 0.007$) or 23.8 mm^2 (Tukey, $p = 0.050$) sections were used (Fig. 2.6B). Monosporeling length, when 8.0 mm^2 sections were used, did not significantly differ from mean length when ground (Tukey, $p = 0.054$), 23.8 mm^2 (Tukey, $p = 0.297$), or 98.8 mm^2 (Tukey, $p = 0.808$) sections were used. Also monosporeling length from 23.8 mm^2 tissue disks were not significantly different from monosporelings using ground tissue (Tukey, $p = 0.794$). The general pattern here is of increasing monosporeling length with increasing tissue section size (i.e., the larger the tissue section size, the larger the resulting monosporelings after 35 days in culture).

Monosporeling growth rate

The growth rate of monosporelings over the 35 day experimental period was influenced by the blade size used to produce tissue disks, but not by the tissue section size or the interaction between the two factors (Table 2.1). Growth rate of monosporelings using large blades was significantly slower than that of monosporelings from small blades (Fig. 2.7A; Tukey, $p = 0.011$). The growth rate of

monosporelings from medium blades did not significantly differ from either small blades (Tukey, $p = 0.341$), or large blades (Tukey, $p = 0.208$). The general pattern was of decreasing growth rate with increasing blade size used to produce tissue disks. Tissue section size had no significant effect on monosporeling growth rate (Fig. 2.7B).

DISCUSSION

Spore germination (conchospore and monospore) will not occur without initial settlement and attachment. Development of blade polarity will not occur unless the spore first attaches to some substrate. If a spore does not settle and attach, the result is the production of a callus (Polne-Fuller and Gibor, 1990), or no further development and eventually death (Imada et al., 1971). Therefore, techniques for suspension culture production must allow spores to settle and develop polarity. This complicates the production of blade suspension cultures from spores. Some substrate must be seeded with spores (as nets are in traditional methods), then the blades can be removed from this substrate when they attain polarity. *Porphyra* rhizoids are not responsible for the absorption of nutrients and function only to anchor the seaweed (Tseng, 1981a), so detachment should not affect physiology. Alternatively, the substrate could be offered in suspension, so that spores attach and germinate while remaining in suspension. This would reduce the amount of culture handling because blades would never have to be removed from the seeded substrate. The use of suspended substrate for spore

attachment would also reduce tank fouling by spores because settlement on tank surfaces would be reduced.

EXPERIMENT 1. BLADES FROM CONCHOSPORES

Blade size

Cut line cultures produced the smallest blades, in terms of length, of all the suspension substrata tested, and were similar to fixed cultures. This shows that suspension alone does not guarantee higher growth rates of suspended blades. Rough surface relief that is favourable for conchospore attachment seems to be very important in determining the usefulness of a suspended substrate. Imada and Saito (1983) found that powdered material having rough surfaces and high density gave the highest growth rates of *Porphyra yezoensis*. They found that pearl-oyster shell gave the best results with germlings grown on this substrate reaching 5 - 10 mm in length and 2 - 3 mm in width after 30 days of culture. Conchospores can settle and attach more easily to suspended particles when surface relief is rough. Therefore these conchospores germinate sooner and a higher yield of blades results. Oyster shell powder particle surfaces appear the roughest of all suspended particles tested, and this substrate produced the longest blades after 42 days in culture. Rough fixed substrate (household string) did not allow easy removal of blades and resulted in the complete culture failure by day 42.

Mean blade sizes after 42 days of culture were smaller in this study than other investigators have achieved. Imada et al. (1971) was able to obtain blades that were 12 cm long after 40 days in culture and grew at 22% per day, but blades were grown in a green-house, exposed to natural sunlight. Iwasaki and Matsudaira (1958) found that blades grow more rapidly under sunlight than any artificial light tested. This is probably the reason for the low yields found in indoor cultures during this study.

Growth rate

The higher growth rates observed during the second interval with suspended cultures are probably due to two factors. After day 28, the culture medium was changed weekly. Before this day, nutrients were added to the medium but the medium was not changed. This was because of the potential for loss of microscopic blades with medium changes. In suspension cultures, growth rates increased from a pre day 28 mean of 8% per day in length to a post day 28 mean of 13% per day in length. This was probably due to the alleviation of carbon limitation or toxin build-up in the pre day 28 medium when the medium was changed on day 28. In fixed substrate cultures, growth rates decreased from a pre day 28 mean of 8% per day in length to a post day 28 mean of 4% per day in length. This may be due to damage sustained by the blades during removal from their fixed substrata on day 21. However, removed blades did not appear physically damaged. Rhizoids do not participate in nutrient absorption, and serve only to anchor the plant (Norton et al., 1982). Therefore damage to this part of the plant during detachment should not affect growth significantly. Perhaps blades were

morphologically adapted to a fixed blade environment, and when in suspension could not take advantage of the new nutrient rich medium (see Blade Shape, below).

It must be noted that the growth rates given in this study are probably underestimates of the actual values, because the growth rates are in terms of length (i.e., one dimensional). Blades could be growing in terms of volume or mass, and this would not necessarily be manifested in an increase in length. Length and width were easily measured on these small blades. It was not practical to accurately measure wet weight or volume of blades that were < 1 mm in length, so these parameters were not considered.

Blade shape

Suspended substrata produced blades that were significantly more lanceolate than fixed blades were. Oyster powder produced blades that were the most lanceolate of all methods tried. Fixed blades were generally more ovoid. This may be a response to being attached to a smoother substrate. Perhaps in an effort to hold on to a smooth substrate, the rhizoidal area of the blade increases in area, causing the blade to become broader in shape. Kornmann (1986) found that *P. yezoensis* blade shape depended on the substrate used for monospore attachment. Mean L:W of cut line and monofilament line blades (same material used for conchospore attachment) are not significantly different, though one method is suspended and the other is fixed. Surface relief is similar between cut line and monofilament line (cut line is slightly rougher).

Since mean L:W of these two methods are the lowest recorded, and they are amongst the smoothest substrata tested, this supports the arguement that a decreased L:W ratio may be a reaction to being attached to a smoother substrate.

It is more likely that blades were responding to local current conditions when the mean L:W decreased by 10.7% when fixed. Within the culture vessel, water velocity was lowest near the vessel surfaces. The water was essentially still at the surface (Charters et al., 1973). The region of low water velocity can be considered a boundary layer (Neushul, 1971). It was within this layer that conchospores were able to attach (Coon et al., 1972) by chemical bonding (Charters et al., 1972), and germinate into blades. Fixed blades grew within this boundary layer. Suspended blades were in constant motion, and grew in higher water velocities than fixed blades did. Suspended blades were not sheltered by a relatively large boundary layer near vessel surfaces.

Fixed blades were probably grown under conditions of reduced water velocities, even though they were cultivated in the same vessels and under equal aeration rates as suspended blades. Under reduced water flow, nutrients were replenished more slowly (Koehl, 1986). Blades may have grown more ovoid (lower L:W) to intercept more of the water flowing past (Koehl, 1986). This morphological acclimation to low water velocity may have allowed fixed blades to grow as fast as suspended blades (pre day 28 mean). This is an acclimation that is seen in many macroalgae, including *Porphyra abbottae* (Hannach and Waaland, 1989). The ovoid fixed blades, and lanceolate

suspended blades were well acclimated to their particular local current conditions, and initially grew at the same rate (8% per day length).

Once the fixed blades were detached from their substrata, they did not grow as well as suspended blades, nor as well as they previously did while still attached. This was despite the fact that the medium was changed soon after detachment. Suspended blades grew more quickly after day 28 in response to the medium change. Detached blades did not appear damaged. Therefore there was something about the fixed form that caused blades to be at a disadvantage when suspended. I suspect that factors such as relative surface areas and thickness of boundary layers may be involved. This remains to be tested.

It is generally agreed that lanceolate blades are ideal for traditional net cultivation because more blades can fit onto one net, giving a higher yield per net (Mumford, 1988). It remains to be seen whether this is an advantage in tank cultivation. One advantage that lanceolate blades may have over ovoid ones, is that as blades become about 5 cm long, lanceolate blades tend to trap less air than ovoid blades do. This trapped air causes blades to remain at the surface, drying the exposed portion and shading other blades in the same culture. This trapped air may not be a problem in

large commercial tanks because the water current produced in these tanks will be much higher and may not allow blades to remain at the surface, no matter how much air they trap.

EXPERIMENT 2. VEGETATIVE PROPAGATION

Large tissue sections had a lower edge to surface ratio than smaller sections. Disintegration occurred mainly along the edges of the tissue disks (observed in culture), therefore large sections would be expected to disintegrate more slowly than smaller sections. Growth may be occuring near the edges of the sections, however this was not confirmed. Cell division in the blade is diffuse and not concentrated at blade edges. Monospore germination with large tissue sections occured before tissue disintegrated completely. This in situ germination may have given monospores from large sections a head start over those that were released into the medium, settled, attached, and germinated. The smaller sections completely disintegrated, with few monospores germinating while still attached to the tissue section. The higher edge to surface ratio of smaller sections may have caused them to break up more quickly, before the monospores were mature. This edge to surface ratio difference among tissue sections accounts for the observed lower disintegration rate of large sections, and for the longer sizes of the blades from large sections after 35 days in culture.

Both mean monosporeling growth rate and mean length were significantly higher when small blades were used. It is known that smaller blades grow more quickly than larger blades. Monospores derived from small blades may have retained their high growth rate potential, which is a small blade characteristic. Blades typically produce monospores when they are small, and cease this production once they reach several hundred mm in length (Hawkes, 1980). Thus, sections cut from small blades may release monospores more readily, giving the resulting monosporelings a head start over those from sections cut from large blades.

It must be noted that all of the tissue section sizes cut and all of the blade sizes used, successfully produced a suspension culture of monosporelings. Differences were obtained in terms of length and growth rate, but this technique of tissue cutting to stimulate monospore formation and release gave dependably repeatable results. The best tissue disk size to cut appears to be the largest one used in this experiment (larger ones could be tested but were not), while the small blade size is best (5 - 7 cm length). This technique was used with successive blade generations, and was successful in producing a new generation each time during this research.

Ying (1984) found that the growth of monosporelings was faster than that of conchosporelings in the lab as well as in the field. This was also found here. The longest monosporelings after 35 days were obtained using large sections from small blades, and were on average about 17 mm long and grew at about 20% per day. The longest conchosporelings after 42 days in culture were obtained using oyster powder, and were 2.25 mm long and grew at 10% per day. This is another advantage that vegetative propagation has when by-passing the conchocelis.

CONCLUSIONS

This study has shown that the use of $CaCO_3$ suspended substrata for the production of blade suspension cultures from conchospores results in the highest early yields of all methods tested. Ground oyster, clam, or egg shell is ideal for the settlement and germination of conchospores, while remaining in solution. The rough surfaces of these particles promotes attachment of the conchospore. Because these substrata are suspended and their surfaces are ideal for conchospore attachment, denser cultures can be obtained than with fixed cultures. Suspended blades are exposed equally to light and nutrients by the aeration mixing of the medium, so better early growth rates are possible than with fixed methods. It must be noted that *early* growth rates are being discussed here. Further observations of fixed blade cultures,

once detached show that they overcome their disadvantage at detachment, and their growth rates are similar to suspended blades.

Suspended substrata remain in solution while exposed to conchospore-releasing conchocelis filaments, so the cultures never need to be handled once seeded. Also, tank fouling by spores is reduced with the use of suspended substrate. This allows lower production cost on a commercial scale than the fixed substrate methods. With fixed methods, special equipment would have to be manufactured to allow lines or surfaces to be seeded, and then to remove the attached blades from these surfaces. This would be a more labour intensive production method than suspended substrate methods. Cheap sources of $CaCO_3$ could be exploited to produce suitable powders for conchospore attachment. Oyster and clam farms produce an abundance of shells, most of which are considered garbage. Powder production could be automated easily. For all of these reasons, $CaCO_3$ suspended substrata methods are the most suitable for commercial production of blades from conchospores.

A technique that shows promise for vegetatively propagating blades (i.e., bypassing conchocelis) involves the enzymatic breakdown of thallus cell walls, and the axenic isolation of individual naked protoplasts (Polne-Fuller et al., 1984a&b; Butler and Evans, 1990). These protoplasts can be seeded onto nets or other materials where they develop into new blades (Dai et al., 1993). The need for axenic conditions (Gibor

et al., 1981; Chen and McCracken, 1993), and the use of expensive enzyme mixtures (Chen et al., 1994) during protoplast production makes this method difficult on a large scale.

The method described here represents an easy, low-tech method for blade propagation. When needed, desirable blades could be removed from the tanks, chopped up, and the tissue sections inoculated into fresh medium in special vessels in a greenhouse so that culture conditions could be carefully controlled. After two to three weeks, the sections would be completely disintegrated, producing a dense suspended blade culture. Some of these monospore-like cells would settle on the vessel surfaces and would have to be detached after three weeks, but the majority of germinating cells would remain in culture, attached to the remnants of tissue disks. Once the blade cultures had grown to a suitable density, they could be emptied into large outdoor tanks for growout.

This type of vegetative propagation would help to by-pass the conchocelis in the production of large batch cultures of blades, reducing one disadvantage that this non-fragmenting form is subject to in land-based tank cultivation. Since costs typically rise when alternate morphological phases are cultivated, this vegetative propagation

method may also result in higher profits during large scale cultivation. It will also help in selection and amplification of clones with desirable characteristics.

FIGURE 2.1. Mean length of blades on day 42 produced from conchospores using various types of material for attachment and germination (95% conf. limits shown, n = 5). Bars with like-letters indicate no significant difference in mean blade length (upper case used with suspended vs fixed mean). Dotted lines emphasize suspended and fixed means.

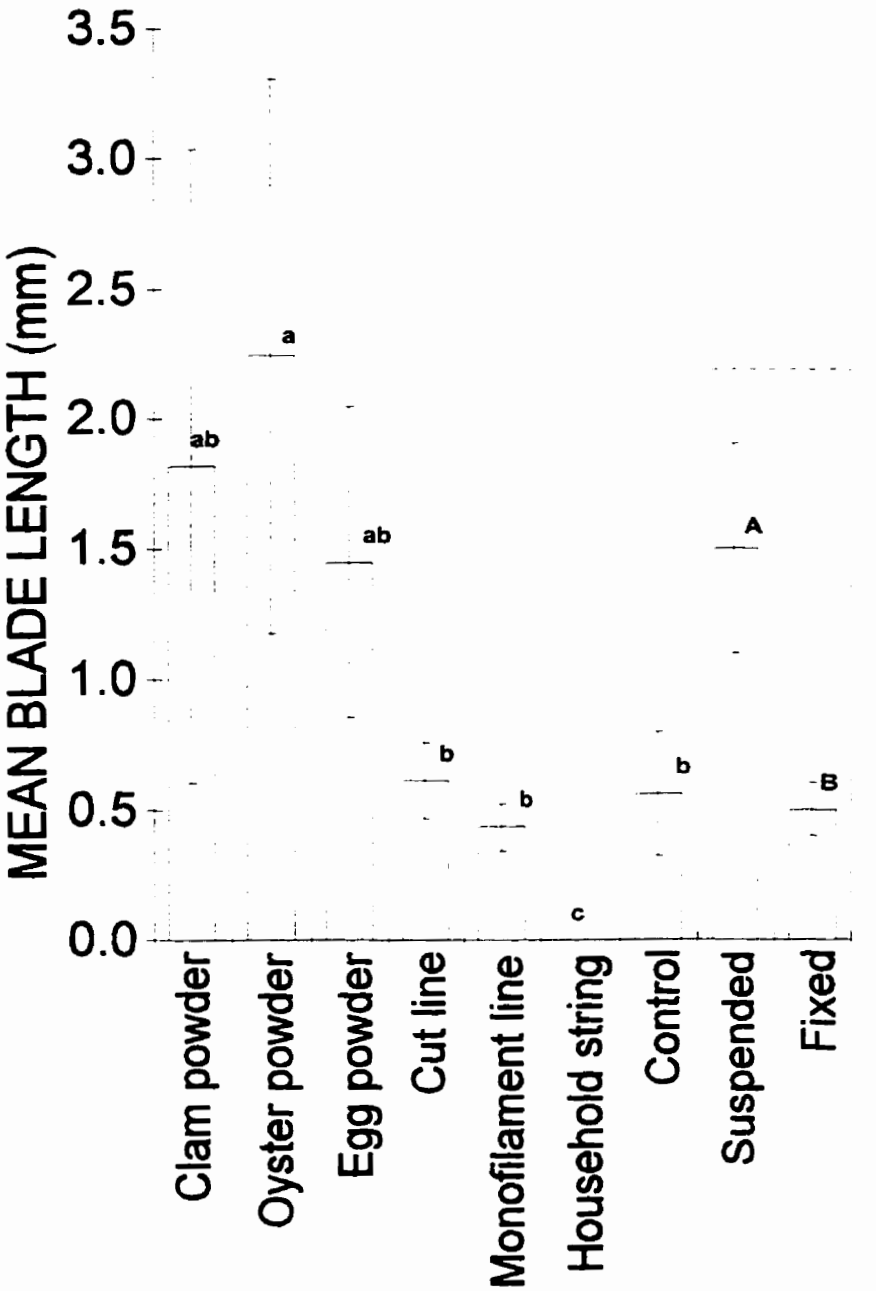


FIGURE 2.2. Mean growth rate (length, % per day) of blades produced from conchospores using suspended and fixed substrata for attachment and germination, over various intervals (95% conf. limits shown, n = 5). Bars with like-letters indicate no significant difference in mean.

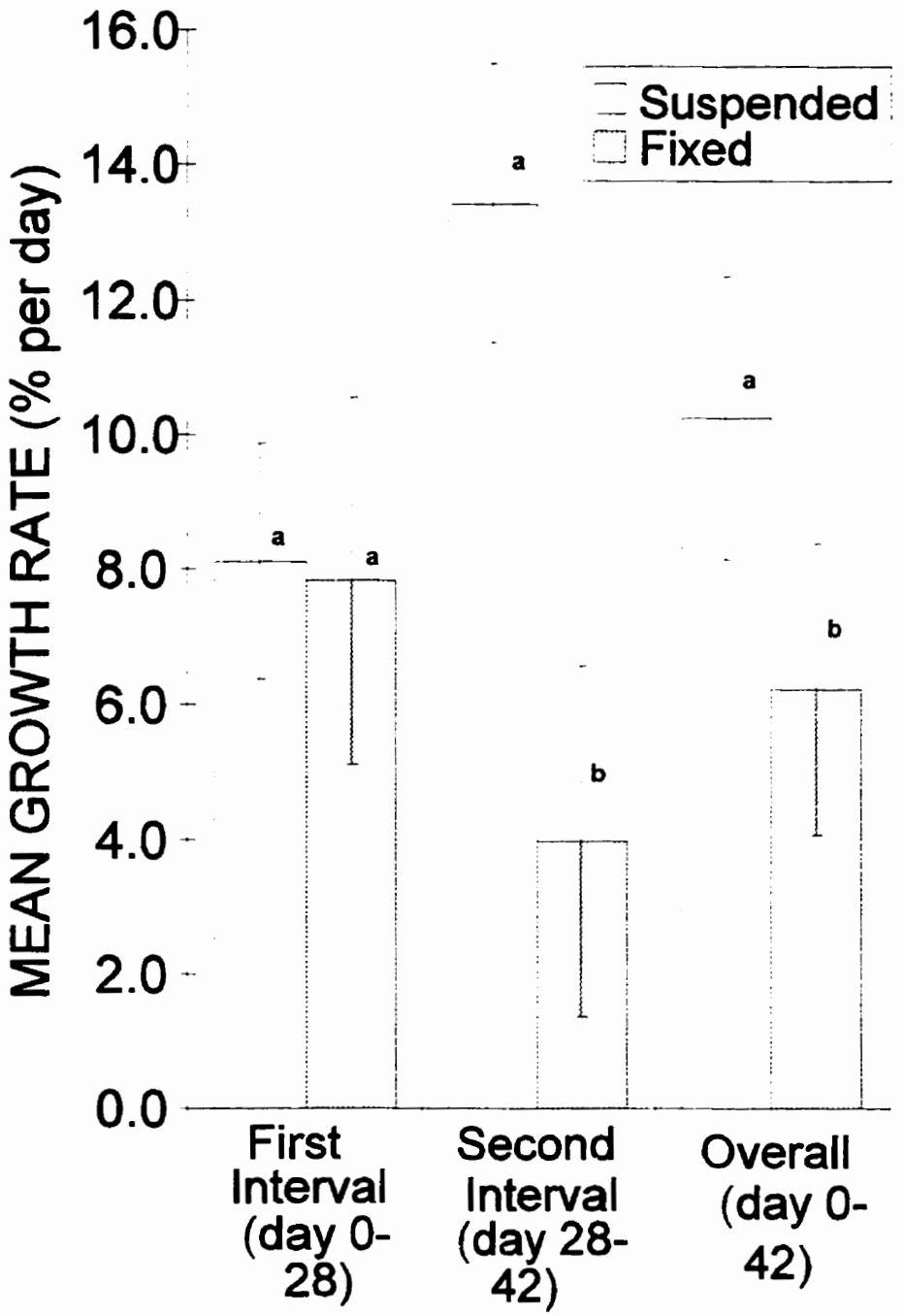


FIGURE 2.3. Mean L:W ratio of blades on day 42 produced from conchospores using various types of material for attachment and germination (95% conf. limits shown, n = 5). Bars with like-letters indicate no significant differences in mean (upper case used with suspended vs fixed mean). Dotted lines emphasize suspended and fixed means.

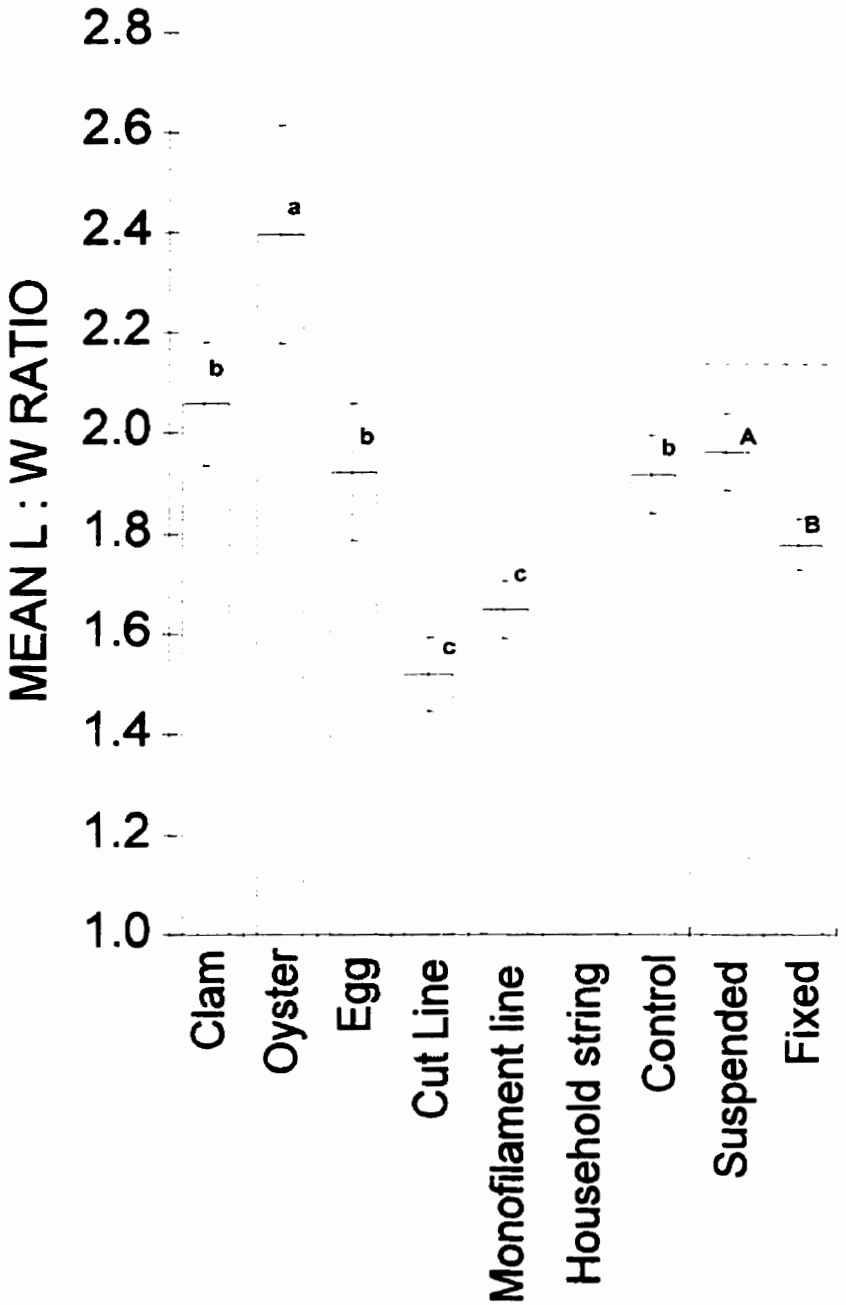


TABLE 2.1. Two-way ANOVA summary for *Porphyra yezoensis* tissue disk growth, disintegration, and monosporeling length and growth (significant results shown in **bold**, $\alpha = 0.050$).

Variable	Factor	F-ratio	p value
Tissue disk initial growth	Section size	6.579	**0.007**
	Blade size	3.233	0.063
	Section size X Blade size	0.437	0.780
Tissue disk disintegration	Section size	4.524	**0.017**
	Blade size	0.119	0.888
	Section size X Blade size	0.144	0.965
Monosporeling length	Section size	5.441	**0.005**
	Blade size	6.814	**0.005**
	Section size X Blade size	1.428	0.245
Monosporeling growth rate	Section size	1.436	0.257
	Blade size	5.074	**0.015**
	Section size X Blade size	1.096	0.393

FIGURE 2.4. Initial growth rate (% per day) of tissue disks as a function of blade size and tissue disk size (95% conf. limits shown, n = 3).

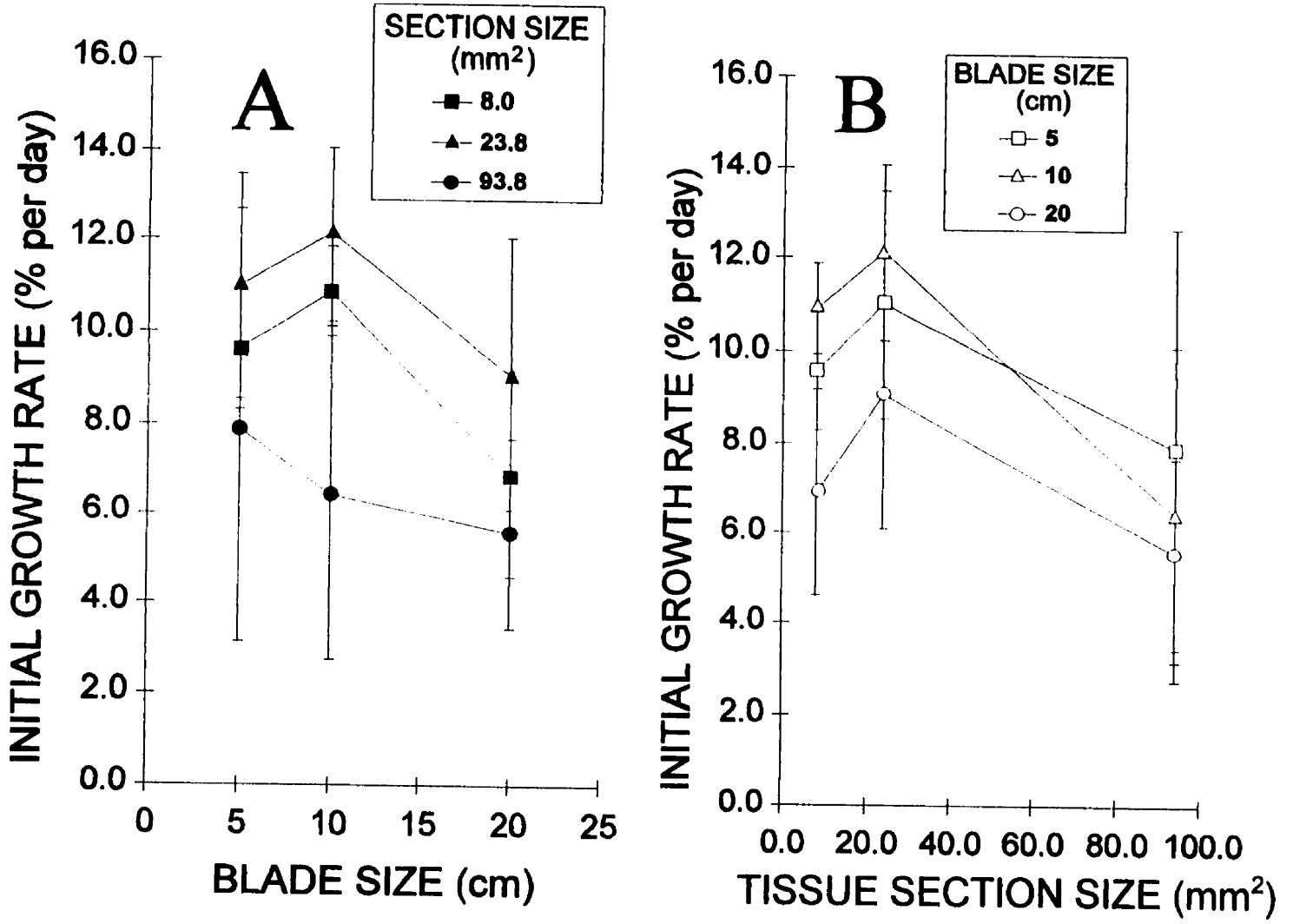


FIGURE 2.5. Disintegration rate (% per day) of tissue disks as a function of blade size and tissue disk size (95% conf. limits shown, n = 3).

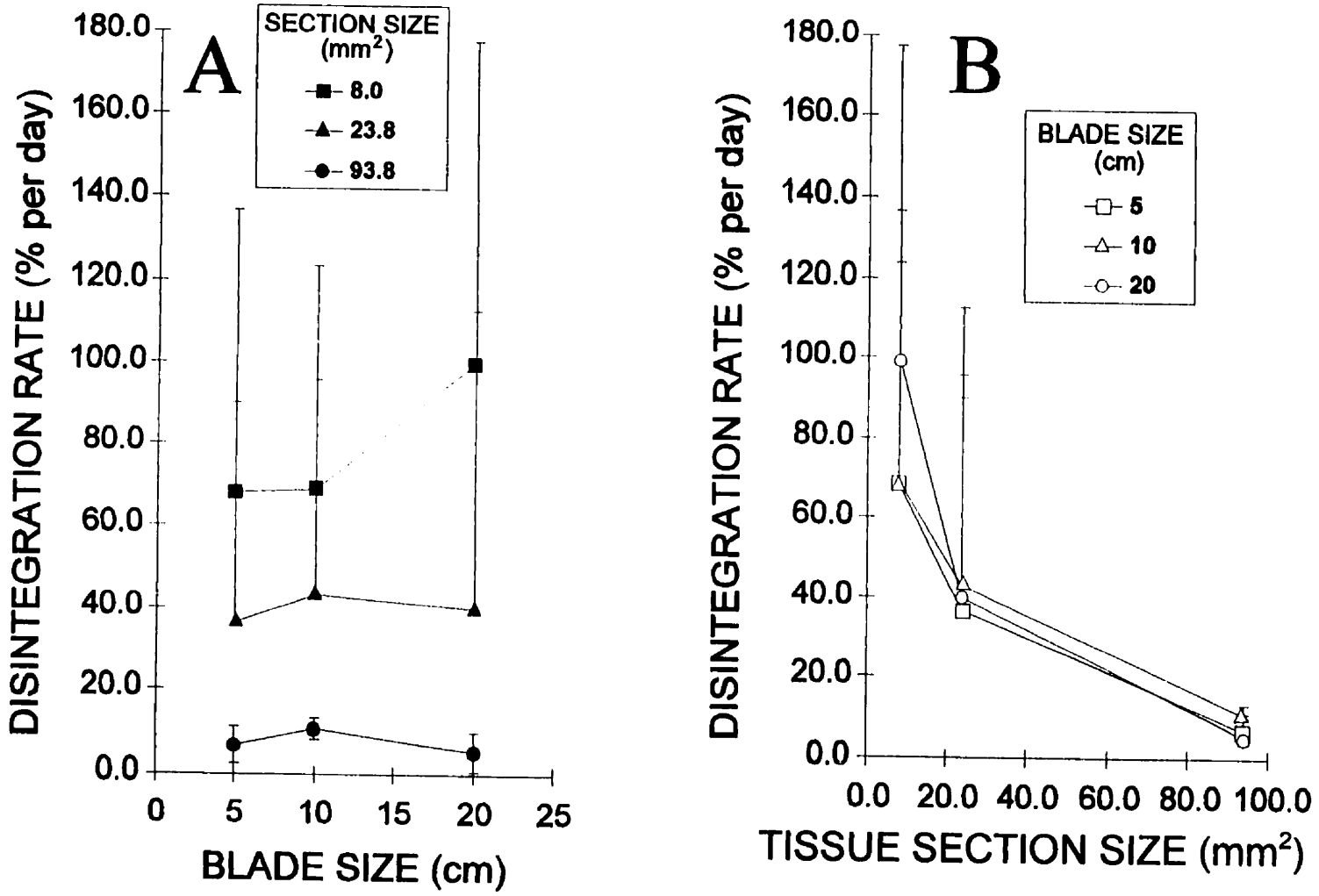


FIGURE 2.6. Length of monosporelings on day 35 as a function of blade size and tissue disk size (95% conf. limits shown, n = 3).

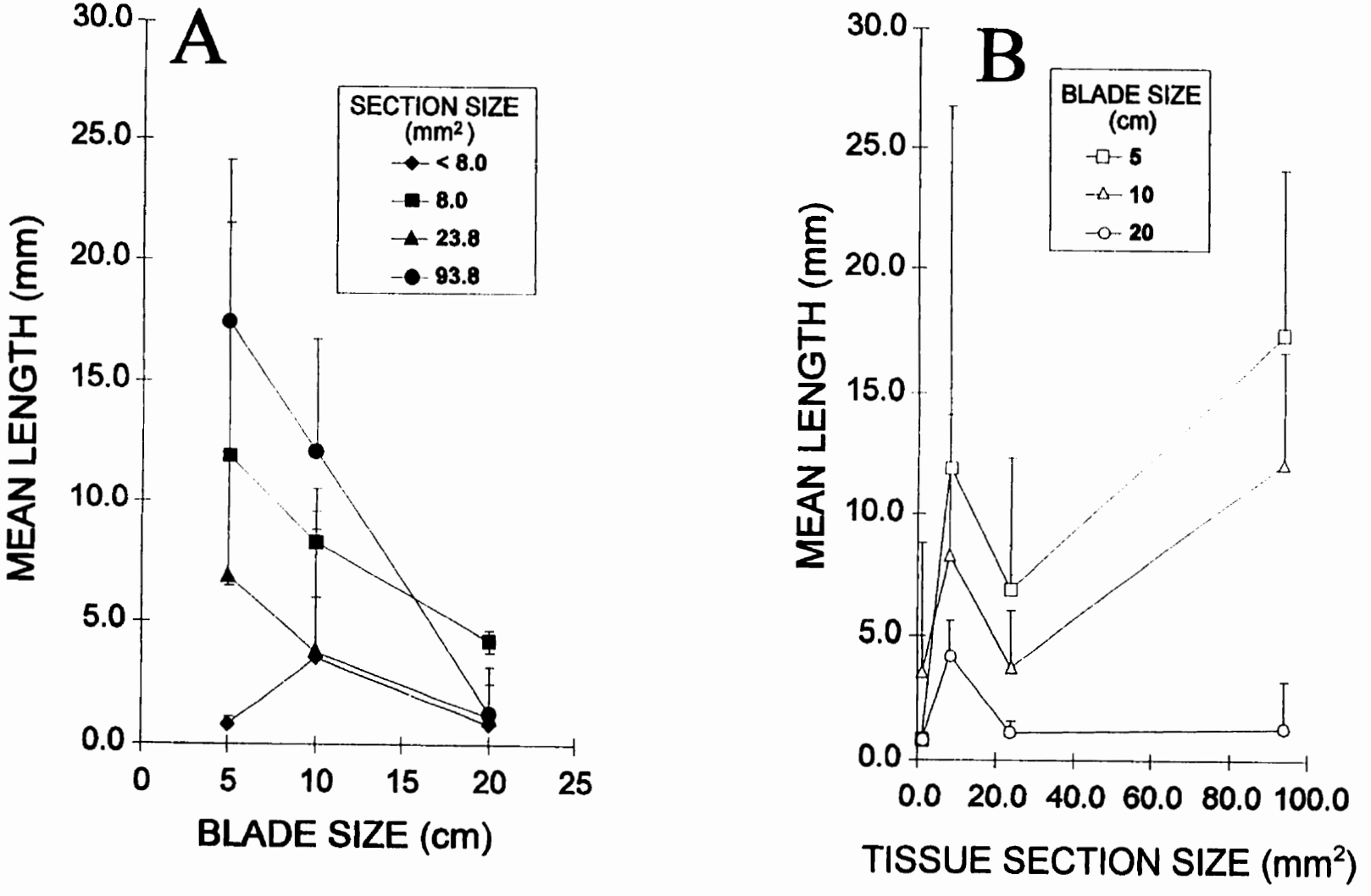
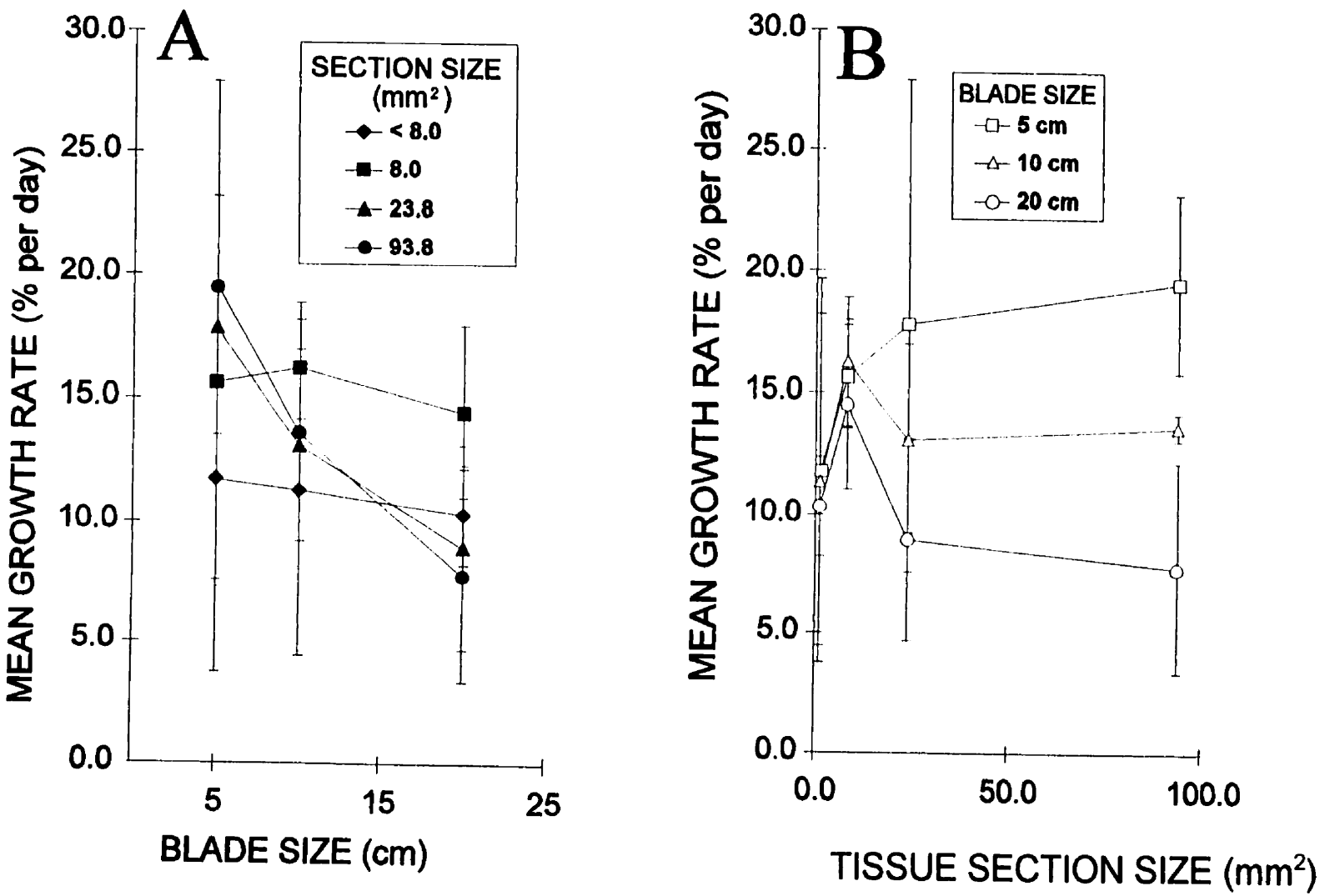


FIGURE 2.7. Growth rate (length, % per day) of monsporelings as a function of blade size and tissue disk size (95% conf. limits shown, n = 3).



CHAPTER THREE

CULTURE CONDITIONS

INTRODUCTION

Before nutrient experiments with *Porphyra yezoensis* blades could begin (Chapter Four), appropriate culture conditions had to be experimentally determined. Conditions of interest include photoperiod, temperature, and stocking density. The experiments in this chapter were designed to define the experimental growing conditions necessary to obtain maximum growth rate in the absence of light and nutrient limitation.

Short day conditions are optimal for the growth of the blade phase (Sidirelli-Wolff, 1992; Waaland et al., 1990), while long days (>13 h of light per day) trigger reproduction and the subsequent disintegration of the thallus (Dixon and Richardson, 1969; Iwasaki, 1961). Because the following experiments are to be performed under artificial conditions with a predetermined photoperiod (i.e., not outdoors), this photoperiod must be selected carefully. The literature is vague, with different authors using different photoperiods, or not reporting photoperiod at all (usually outdoor experiments).

Temperature is another parameter that must be considered. The literature is vague in terms of optimal temperature for growth during experiments. High

temperatures (25-30°C) are known to be lethal (Bird, 1973), and to facilitate pathogen infection (Molina et al., 1988). Also, *Porphyra* growth is inhibited by temperatures that are higher in the dark than in the light (Oohusa, 1993a).

The nutrient experiments in Chapter Four are on nitrogen and phosphorus relationships with growth. Carbon must not become limiting during these experiments. The easiest way to ensure that carbon does not become limiting is to monitor pH (Bidwell et al., 1985). Photosynthesis during the day drives pH up, and respiration at night decreases pH (Gao et al., 1991). If pH does not return to natural levels (i.e., pH≈ 8.0) at night, this indicates that carbon is becoming depleted in the seawater (DeBusk and Ryther, 1984). By choosing a stocking density that results in high growth, without overly depleting dissolved carbon in seawater for the duration of the experiment, it is possible to prevent carbon limitation from occuring.

This chapter describes experiments performed to determine optimal photoperiod, temperature, and stocking density for the nutrient experiments described in Chapter Four, with blades in suspension cultures.

MATERIALS AND METHODS

Culture vessels

Porphyra yezoensis blades were obtained by the monospore method explained in the Chapter Two. The blades were allowed to grow to at least 0.5 cm in length before

use. Trough (half-pipe) culture vessels were constructed, consisting of PVC pipes (20 cm diameter, 86 cm length) cut in half longitudinally with an air tube (3 cm diameter PVC pipe) glued and sealed to the outside bottom of each trough. Small holes were drilled through the bottom of the trough into the air tube. This air tube was connected to a large air pump to provide aeration, keeping blades in constant motion, and reducing boundary layers (Neushul et al., 1992). Aeration was also used to maintain temperature and nutrient distribution in each compartment (Bidwell et al., 1985).

Each trough was sub-divided into 4 compartments by Plexiglas dividers sealed with silicone aquarium sealant. Each compartment held 3 L of culture medium (Fig. 3.1). As long as the air pump was operating, no mixing of medium among compartments occurred (there were no pump failures throughout these experiments). Each compartment was covered with a Plexiglas lid to prevent excessive medium losses due to evaporation.

Culture conditions

Fluorescent Vita-lights were used to give a photon flux density (PFD) of 160 μmol photons m^{-2} s^{-1} (the maximum PFD possible with this experimental set up). Seawater was filtered through 0.22 μm Millipore GS filters before use. The medium was enriched using the f/2 recipe of McLachlan (1973). These nutrients were given twice per week to prevent nutrient limitation. All experimental treatments were done in triplicate in a walk-in Alex Gair growth chamber.

Photoperiod and temperature

A 3 X 3 factorial experiment was done with suspended blades given all combinations of 3 temperatures (10, 15, and 20°C) and 3 photoperiods (8, 10, and 12 hours of light per day). Blades were inoculated in vessel compartments at low densities (1 g fresh wt) to prevent C limitation (pH was monitored in the morning, 0.5 h before the lights came on). During a 3 week experiment, blades were removed 3 days per week (Monday, Wednesday, and Friday) from their compartments, blotted dry, weighed, and harvested back to the original 1 g fresh wt by removing excess blades. This was done for 3 weeks. Blades were given 7 days to acclimatize to culture conditions before growth rates were recorded. A two-way ANOVA ($\alpha = 0.050$) was used to determine the significance of temperature and photoperiod effects on growth, as well as any interactions between the two parameters.

Stocking density

To determine optimum stocking density, blades were cultured under 8L:16D, 15°C, 160 µmol photons m^{-2} s^{-1}, using f/2 medium with replenishment and filtering as in the previous experiment. Stocking densities from 1 - 8 g fresh wt per 3L medium were used in triplicate, and growth rate was monitored as before during this 3 week experiment. pH was monitored throughout the day and in the morning, just before the lights came on. A control treatment was used as a base line for pH measurements. The control (containing no seaweed) was under the same conditions as the treatments with

seaweed. ANOVA (α = 0.050) was used to determine the significance of stocking density effects on growth and production.

RESULTS AND DISCUSSION

Photoperiod and temperature

Growth of *P. yezoensis* blades in suspension cultures was significantly affected by temperature, but not by photoperiods with 8 - 12 h of light per day (Fig. 3.2). Blades grown under each photoperiod tested grew quickest at 15°C, followed by 10 and 20°C (two-way ANOVA p value = 0.019). There was no significant relationship between photoperiod and growth (p value = 0.190), and there was no interactive effect between photoperiod and temperature on growth (p value = 0.922).

Coastal seawater temperatures in winter are typically lower than 15°C in the NE Pacific (Bergdahl, 1990). However for the purposes of this thesis, 15°C resulted in the highest growth rates, so this temperature was used in the experiments to follow in Chapter Four. Yamamoto et al. (1991) found that the optimum temperature for *P. yezoensis* growth in outdoor raceway tanks fell within the range 10 - 17°C, similar to the optimum found here. *Porphyra columbina* (Avila et al., 1985) and *P. umbilicalis* (Hernandez et al., 1993) also grew best at 15°C.

Growth rate (Fig. 3.2) was unaffected by the photoperiods tested (at least for three weeks). Therefore any photoperiod with 8 - 12 h of light per day could be used.

Commercial cultivation of this seaweed occurs in the winter in temperate regions, and because this study is intended to be of use to commercial cultivators, the nutrient experiments to follow were conducted under an 8L:16D photoperiod. Iwasaki and Matsudaira (1958) found that maximal growth of *P. tenera* was obtained using 8 - 9 h of light per day.

Stocking density

The higher the stocking density, the slower the growth rate (Fig. 3.3, ANOVA p value = 0). However, production peaks at the higher stocking densities used in this study (Fig. 3.3, ANOVA p value = 0). The highest stocking density tested was 8.0 g per 3L. Production begins to level off at this high stocking density (Fig. 3.3) and, judging from the decrease in growth rates shown in Figure 3.3, at higher densities production would probably drop fairly rapidly beyond 8.0 g per 3L.

The use of high densities (where growth rates are lower, but production is higher) may be more efficient commercially (Neish and Knutson, 1977). Epiphyte growth would be inhibited (Lignel et al., 1987), and production would be higher with higher densities. The actual numbers reported here (i.e., 1 - 8 g per 3L) are of little use to industry, because they will certainly differ in large scale tanks. However, the general pattern of growth and production with stocking density will be the same in large tanks. It is difficult to compare these results with those of other authors, as the vessels used here were small and shallow, with a high surface area to volume ratio. A similar

relationship with production and stocking density was found by Lapointe and Ryther (1978) using *Gracilaria tikvahiae*.

Figure 3.4 shows typical pH dynamics throughout the 21 day experimental period. After 1 day in culture, dark respiration is sufficient to return pH back to control levels (pH = 7.8). This baseline is lower than the natural pH of the seawater (pH = 8.0), and may be due to aeration increasing dissolved carbon. At the end of the experiment, after 21 days in culture, only the 1.0 g per 3L culture was able to return pH to 8.0 via respiration. The maximum pH of 8.4 during the day indicates that at this culture density, dissolved carbon is still at a high enough concentration such that it is probably not limiting (Braud and Amat, 1996). In general, the lower the stocking density, the higher the growth rate and the lower the carbon depletion. Therefore, the nutrient experiments to follow in Chapter Four are all done with 1.0 g fresh wt per 3L stocking density.

Even though growth rates were lower in high density cultures, the blades appeared healthy. Very high density cultures can be held in small tanks, as stock cultures, and the blades remained healthy for months, with little growth or production occurring. When needed, these blades can be taken from the high density stock cultures, and used in experiments. They begin growing immediately once inoculated at a lower density. Bidwell et al. (1985) found similar effects with *Chondrus crispus* under high densities.

CONCLUSIONS

During this experiment it was found that, of the conditions examined, maximal blade growth occured at: temperature = 15°C, stocking density = 1.0 g fresh wt per 3 L, photoperiod (8 - 12 h light) = no effect on growth. Typically commercial cultivation occurs during winter in temperate regions, as a result 8 h of light per day was used in the nutrient experiments in Chapter Four (15°C, 1.0 g fresh wt per 3L).

FIGURE 3.1. Culture trough design.

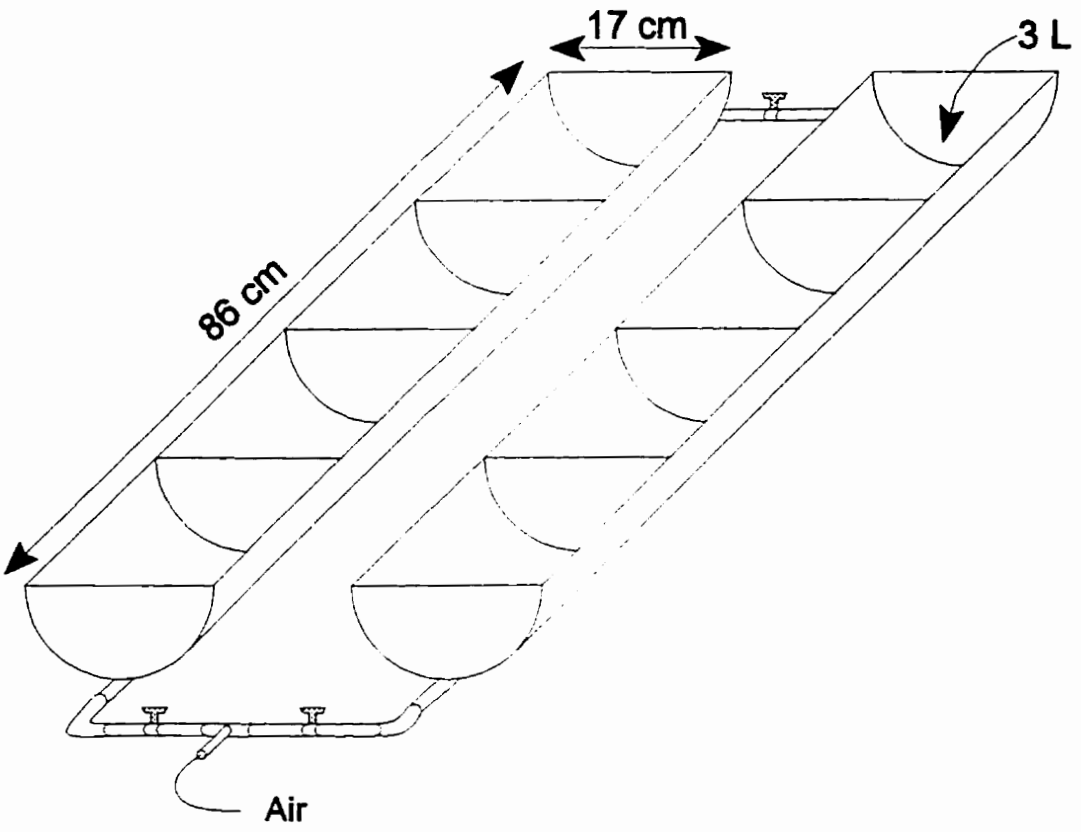


FIGURE 3.2. The effect of photoperiod and temperature on the growth rate of blades (95% confidence limits shown, n = 3).

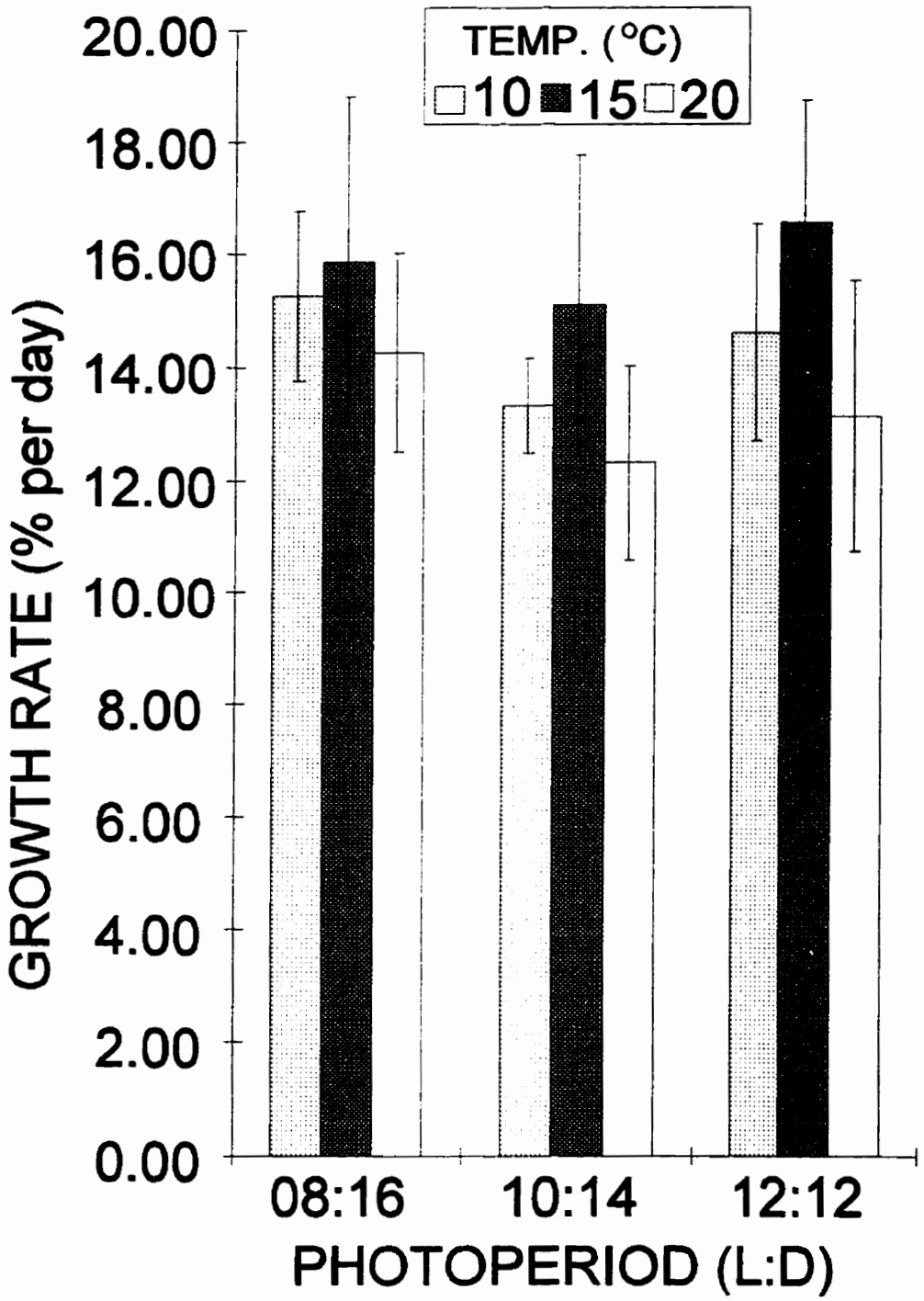


FIGURE 3.3. Growth rate and production of blades grown at varying stocking densities (95% confidence limits shown, n = 3).

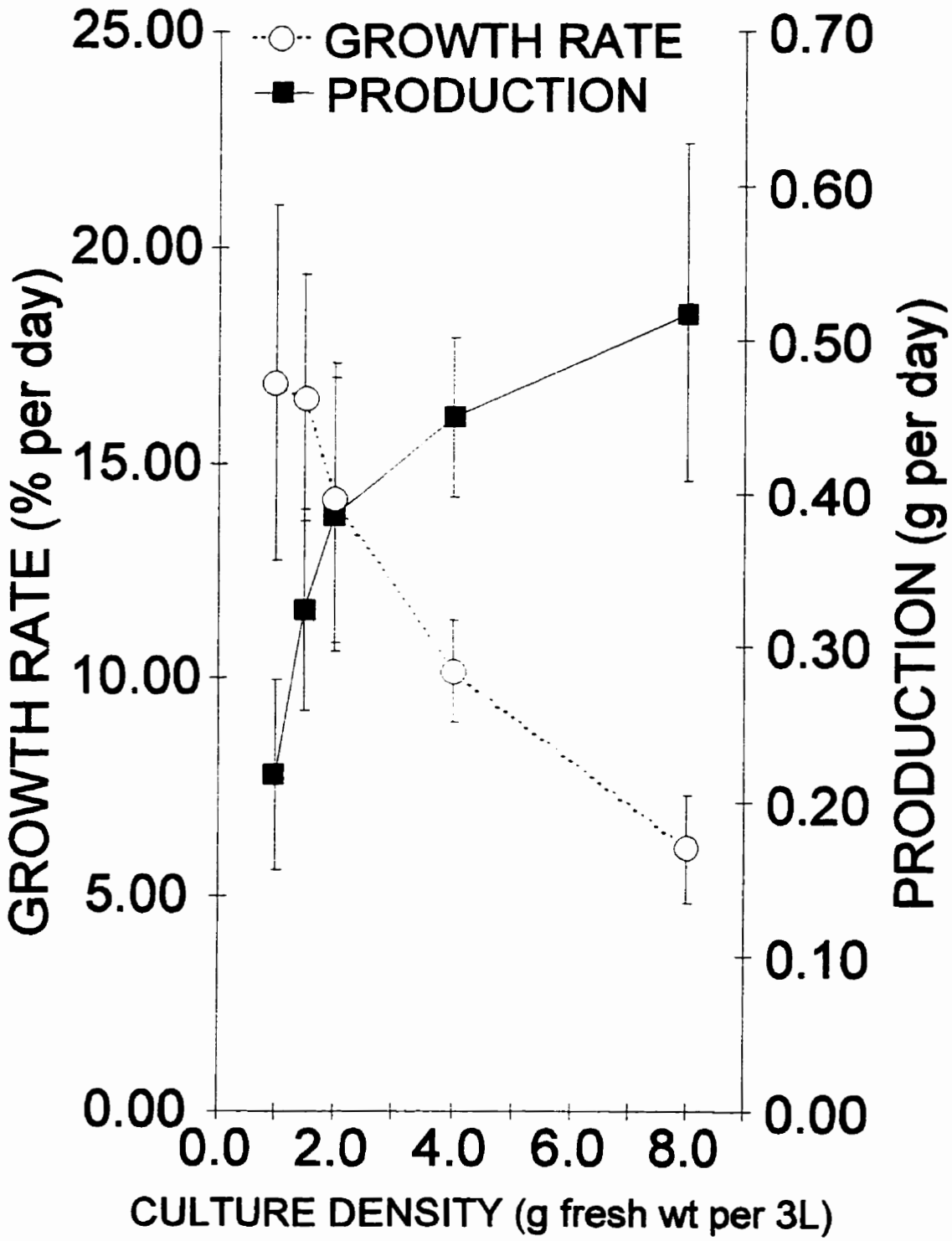
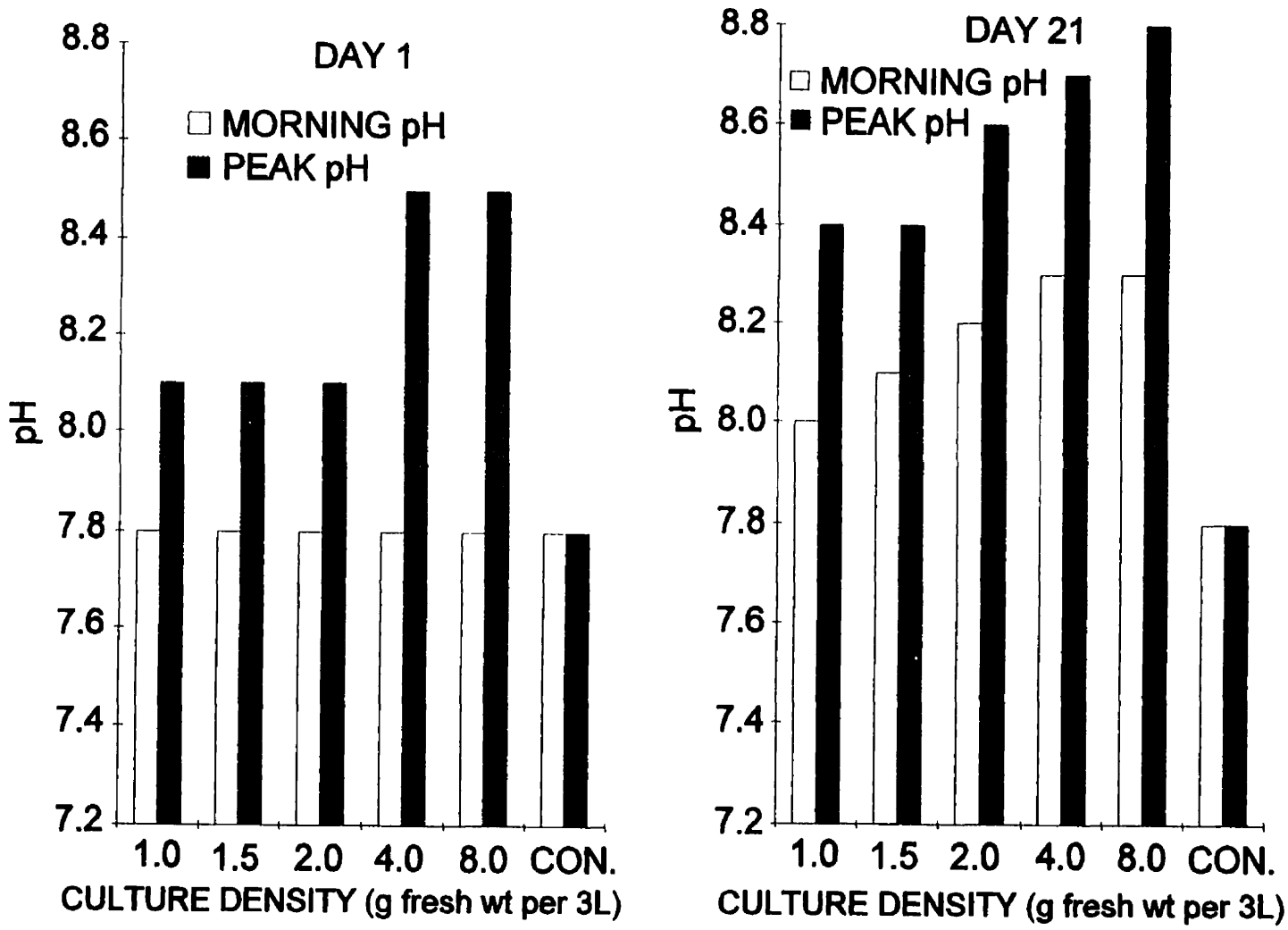


FIGURE 3.4. Typical pH dynamics of blade culture medium at 1.0 g fresh wt per 3L, 08:16 L:D, and 15°C throughout the 21 day experimental period.



CHAPTER FOUR

TISSUE NITROGEN AND PHOSPHORUS

INTRODUCTION

Tank cultivation has its greatest advantage over traditional ocean-based cultivation in that one can control the growing environment of the plants with greater efficiency (Lignell et al., 1987). Though light is the most important variable limiting growth during cultivation, It would be uneconomical to try and control the light environment of large-scale tanks. The next most important variable limiting growth is carbon, followed by nitrogen and phosphorus. Carbon limitation can be easily prevented with the use of a pH sensing device, attached to a CO_2 tank. When pH increases during the day during photosynthesis, the pH sensor triggers the release of CO_2 into the aeration system, bringing pH back down to acceptable levels. This technology is effective in eliminating C limitation and has become standard in the industry; therefore carbon was not investigated in this study.

Nutrients such as N and P are not as quickly diluted and washed away in a tank (especially if in-flowing water is shut off during fertilization) as they are in the ocean. The optimal growing conditions occur when all nutrients added are assimilated into algal crop biomass, leaving nothing behind for epiphytes and other algae. Ideally, the crop is growing at its maximal rate in a "desert" of low nutrients. To accomplish this, detailed studies into algal responses to nutrients are needed. This allows the

manipulation of the nutrient environment to optimize yields.

Very little work has gone into the N and P nutrition of *Porphyra yezoensis*, even though it is farmed extensively in Asia. In traditional ocean-based farms, it is very difficult to monitor the nutritional status of blades. Also, this status varies widely throughout the farm depending on such factors as the local flow conditions. In temperate coastal waters, nutrient levels are typically high in the winter when *Porphyra* cultivation takes place (Kain, 1991). For these reasons, farmers use simple guidelines when considering algal nutrition on their farms. If the $NH_4^+ + NO_3^-$ concentration < 3 μM (50 mg m^{-3}), then the seawater is considered infertile, and fertilizer must be applied. A spraying technique is usually used to apply the fertilizer. If the $NH_4^+ + NO_3^-$ concentration is about 7 μM (100 mg m^{-3}) it is regarded as semi-fertile. At this nitrogen concentration, growth is supported without fertilizer, but the product is of medium grade. An $NH_4^+ + NO_3^-$ concentration of about 15 μM (200 mg m^{-3}) and above is considered fertile enough to support growth and high quality without fertilizer application (Lobban and Harrison, 1994; Tseng, 1981a&b). Phosphorus concentrations are not usually considered by farmers, and tissue analysis is rarely done. Tanks allow greater control over nutrient conditions, and provide uniform growing conditions throughout the farm. In order for this advantage to be exploited, knowledge of the relationships between growth and tissue N and P is needed.

It would seem at first that supplying nutrients at saturating amounts for uptake rate would be desirable, however this means that nutrient levels are always high, leaving nutrients for epiphytes and weed species (Schramm, 1991). There is a better way of managing nutrients for optimal growth. This involves tissue analysis for total nitrogen and phosphorus. Tissue analysis is a direct measurement, and can tell the aquaculturalist whether the algae need nitrogen or phosphorus. Most macroalgae tend to store excess nitrogen in their tissues for growth during periods of low nitrogen concentration (Thomas and Harrison, 1985).

This study will determine if N and P are stored in the tissues of *Porphyra yezoensis*. If storage occurs, then the critical levels (i.e., the concentration at which growth is saturated) and the subsistence levels (i.e., the minimum concentration at which growth occurs) of this nutrient will be determined under both high and low light conditions. Nitrogen will be given as NO_3^- or NH_4^+ because it is possible that critical nitrogen and phosphorus levels will differ depending on the nitrogen source (Wheeler and Björnsäter, 1992).

MATERIALS AND METHODS

Culture conditions

Porphyra yezoensis blades were obtained by the monospore method explained in Chapter Two. The blades were allowed to grow to at least 1 cm in length before use. The blades were cultured in trough vessels (described in Chapter 3) under a 8L:16D

photoperiod, 15°C temperature, using fluorescent Vita-lights to give a photon flux density (PFD) of 160 µmol photons m^{-2} s^{-1} in high light cultures, and 50 µmol photons m^{-2} s^{-1} in low light cultures. PFD levels were adjusted by raising or lowering the trough vessels relative to the light source. Low light levels were achieved by using window screen to shade these compartments. Each compartment was covered with a Plexiglas lid, to prevent excessive loss of medium due to evaporation. All experiments were done in a walk-in growth chamber. Due to limited space, each treatment was performed initially in duplicate, then each experiment was repeated. Therefore each treatment was performed 4 times.

Seawater was filtered through 0.22 µm Millipore GS filters before use. The seawater was then enriched with trace metals and vitamins following the f/2 recipe of McLachlan (1973). Nitrate ($NaNO_3$), ammonium (NH_4Cl), and phosphate (NaH_2PO_4) loads (concentration * volume given / frequency of addition) were varied according to which nutrient of interest was under investigation. When tissue N was being studied, phosphate loads were kept high to prevent phosphorus limitation. When tissue P was being considered, nitrate or ammonium loads were kept high to prevent nitrogen limitation (Table 4.1). Note that N and P loading is given in Table 4.1, not concentration. The supply rate is more important in determining nutrient limitation, so this parameter was used instead of concentration (Lobban and Harrison, 1994). These nutrient loads compare well with ecological ranges in N and P supply. Culture medium was not changed during the experiment. Nitrogen and phosphate were added directly

to the compartments from stock solutions. Culture pH was monitored and never raised above 8.4 in any culture throughout the experiment, therefore carbon was assumed to not be limiting.

Experimental design

Each compartment was inoculated with 1 g fresh wt of blades. Experiments were always initiated on Friday, and nitrogen and phosphate enrichments were done on Mondays, Wednesdays, and Fridays during the experiment (frequency of addition = 0.43 per day). On these same days, blades were removed from the compartments using an aquarium fish net, excess surface water was blotted from the blades using paper towels, and the blades were weighed. Before returning blades to their compartments, they were harvested back to their original 1 g fresh wt. A sample of harvested blades was saved from each compartment for tissue nutrient analysis. Blades were allowed to acclimatize to culture conditions for 7 days in high light cultures, and for 14 days in low light cultures before data were recorded. The extra 7 days acclimation time in low light cultures was necessary because growth processes were slowed under these conditions. Growth rate and tissue nutrient levels stabilized after these acclimation periods (semi-steady state conditions). The experiments ran for 21 days in total.

Starvation experiment

A nutrient starvation experiment was done at the end of 21 days in culture using nitrogen and phosphorus sufficient blades (given nitrate) under high light conditions. This was done to determine how quickly blades use their stores of nitrogen and phosphorus, and to confirm critical and subsistence levels of N and P. After day 21, blades were given either NO_3^- or PO_4^- at the highest levels shown in Table 4.1. Total P depletion was measured using blades that were given high levels of NO_3^-, and total N depletion was measure using blades that were given high levels of PO_4^-. Blades were treated the same as in the previous experiment, with fresh wt measurements, harvests, nutrient additions, and tissue analysis done the same way. Growth (or death) was monitored for an additional 12 days. After this day blades ceased growth and began to die.

Tissue nutrient analysis

Total tissue N and P was determined by alkaline persulfate digestion (Björnsäter and Wheeler, 1990; D'Elia et al. 1977).This technique has the advantage of allowing the measurement of total N and P from a single extraction. Some modifications were made to the procedures used by the aforementioned investigators. An algal sample was taken and weighed (0.0100 - 0.0200 g fresh wt) on an analytical balance, and placed into 50 ml Pyrex culture tubes with screw caps. The culture tubes contained 5 mL deionized water (DIW) and 30 mL of oxidizing reagent (3.0 g NaOH and 6.7 g low N (<0.001%) potassium persulphate dissolved in 1 L DIW). The tubes were capped tightly

and autoclaved for 1 h at 110°C and 15 psi. Tissue was not dried so that analysis could be done immediately after taking samples, therefore tissue nutrient levels are reported as % fresh wt. This gave faster results than if blades had been dried, and this short-cut is useful in an commercial aquaculture setting (Ulrich, 1952). All surface water was removed before weighing, and samples were immediately submerged in oxidizing reagent, reducing any errors resulting from the use of fresh material.

After the tubes cooled enough to be handled safely, 3 mL of a 0.3 M HCl solution was added to each tube to acidify the solution. Then 4.0 mL of borate buffer were added (30.9 g boric acid, and 100 mL of 1 M NaOH, made up to 1 L with DIW). The volume of each tube was brought up to 51 mL with DIW. Under alkaline conditions all nitrogenous compounds were converted to NO_3^- and all phosphorus containing compounds were converted to PO_4^-. Total N and P were then measured colorimetrically.

Phosphate was measured using the standard method of Strickland and Parsons (1972). Nitrate was measured using a spongy cadmium method (Jones, 1984). This method converts all NO_3^- to NO_2^- for analysis. This method is accurate and allows many samples to be processed in a short time.

Statistics

Mean growth rate and tissue N or P for each treatment was based on days 10 to 21 for high light conditions and on days 17 to 21 for low light conditions. These means were then pooled for each treatment replicate. Therefore, each mean and confidence interval shown is based on a sample size of 4. All statistical analyses were done using Systat for Windows (version 5.0). Critical tissue levels of a nutrient were defined as the tissue level at which an increase of 0.01% fresh wt of tissue N or P results in less than a 0.10% increase in growth rate.

RESULTS

In all figures that follow, results from high light cultures are represented by solid diamonds, while results from low light cultures are shown by hollow circles. All error bars shown are 95% confidence intervals (n = 4). In figures with two graphs, the first graph (A) shows data using NO_3^- as the N source, while the second (B) shows data using NH_4^+ as the N source (except Fig. 4.6).

Tissue N

Figure 4.1 shows that the relationship between growth rate and internal total N has the form of a rectangular hyperbola, regardless of the N source. The data fit very well into the Droop equation (Droop, 1983) with a slight modification (i.e., raised to the 2^{nd} power): $\mu = \mu_{max} [1 - (Q_0 / Q)^2]$, where μ is the growth rate (% per day), Q is the tissue concentration of the nutrient within the algal cells (% fresh wt), Q_0 is the lowest

level of Q at which the alga can grow (the subsistence level, % fresh wt), and μ_{max} is the maximal growth rate (% per day) at infinite Q (South and Whittick, 1987). Specific parameters for the modified Droop equation are given in Table 4.2.

Figure 4.1 and Table 4.2 show that regardless of the light level or N source, Q_o (i.e., the subsistence N level) and the critical N level differ very little. Because of this a mean Q_o (0.15% fresh wt) and critical N (0.40% fresh wt) were calculated. R^2 values are all very high (0.83 - 0.93) showing that the data fit the modified Droop equation (given above) very well. High light cultures grew more quickly (μ_{max} mean = 12.6% per day) than low light cultures (μ_{max} mean = 8.4% per day). The maximal growth rate differed between the high light cultures. NO_3^- cultures grew at a higher rate (14.7% per day) than NH_4^+ cultures (10.4% per day) under the high light conditions. Blades that had a tissue N level > 0.40 appeared very dark purple to black in colour. As the tissue level dropped < 0.40, the blades became more green in colour. Near the Q_o level, blades were bright green, with no trace of purple or reddish colouration.

Tissue P

Figure 4.2 shows that the relationship between internal tissue P and growth rate is linear, and not a rectangular hyperbola. The linear relationship parameters are shown in Table 4.3. The data fit well (R^2 values ranged from 0.97 - 0.99) into the standard linear equation: $\mu = m(Q) + b$, where μ is the growth rate (% per day), m is

the slope of the line, Q is the tissue concentration of the nutrient within the algal cells (% fresh wt), and b is the y intercept.

Figure 4.2 and Table 4.3 show that the subsistence level for P was much higher when NH_4^+ was the N source (mean Q_O = 0.09% fresh wt) than when NO_3^- was the N source (mean Q_O = 0.02% fresh wt). Again, high light cultures grew more quickly than low light cultures, but Q_O did not differ between high and low light blades given the same N source. The slope of the relationship between growth and tissue P was greater when NO_3^- was the N source (Table 4.3). P-limited blades did not differ much in colour from P-sufficient blades, however the blade texture and strength changed with P limitation. P-limited blades fell apart very easily and felt slippery. Aeration was sufficient to break P-limited blades apart, contributing to their ultimate death.

Tissue N : P

Growth rate was greatest at a molar N:P of 10 - 17 when NO_3^- was given, and at a molar N:P of 10 - 15 when NH_4^+ was given (Fig. 4.3). Molar N:P less than these ranges occurred in blades given low N loads (Table 4.1), while N:P greater than these ranges occurred in blades given low P loads. The ranges were the same for high and low light cultures given the same N source. Figure 4.3 also shows that growth rate drops off much more severely in low P load blades (N:P > 15) given NH_4^+, than blades given NO_3^-. Low P load NH_4^+ blades began to die (i.e., growth rate < 0% per day) at N:P > 17 - 20, while death in low P load NO_3^- blades didn't begin until N:P = 35.

Tissue N was greatest at a molar N:P of 13 - 17, regardless of N source or PFD (Fig. 4.4). Molar N:P < 13 occurred in low N load blades, and N:P >17 occurred in low P load blades. Figure 4.4B shows that both high and low light blades given NH_4^+ at low P loads (N:P > 17) had tissue N levels as high as blades within the 13 - 17 N:P range. Also tissue N levels were higher within the optimal 13 - 17 N:P range in low light blades given NH_4^+, than in low light blades given NO_3^- (Fig. 4.4).

Tissue P was greatest at a molar N:P of 10 - 17, regardless of N source or PFD (Fig. 4.5). Low light cultures given NH_4^+ had a higher tissue P content within the N:P range of 10 - 17 (Fig. 4.5B) than low light cultures within this same N:P range given NO_3^- (Fig. 4.5A). Molar N:P < 10 occurred in low N load blades, and N:P > 17 occurred in low P load blades.

Starvation experiment

Figure 4.6A shows that when only PO_4^- was given to high tissue N blades (0.67% fresh wt) under high light conditions, growth rate remained high for 5 days, until tissue N fell below about 0.36% fresh wt. Once tissue N was < 0.36% fresh wt, growth rate decreased quickly until the cultures began to die on day 12. The blades began to die once tissue N fell below about 0.15% fresh wt.

A similar pattern is seen in figure 4.6B, when only NO_3^- was given to high tissue P blades (0.09% fresh wt) under high light conditions. Growth rate remained high for 5

days, until tissue P fell below about 0.05% fresh wt. Once tissue P fell below this level, growth rate decreased just as quickly at is did in figure 4.6A until blades began to die on day 12. The blades began to die once tissue P fell below about 0.03% fresh wt.

Figure 4.7 used the same data as figure 4.5. This time N:P is shown along with growth rate. Growth rates (dashed lines) followed a similar pattern, whether the blades were given no N, or no P. Growth rates began to drop in low N cultures once N:P < 10. Growth rate also dropped in low P cultures once N:P > 23.

DISCUSSION

Tissue N and P

Porphyra yezoensis has the ability to store N in excess of its needs for growth. The relationship between growth rate and internal total N has the shape of a rectangular hyperbola, indicating luxury storage of N (Droop, 1974). The critical and subsistence levels for N were unchanged when either NO_3^- or NH_4^+ was given, or when light levels were 50 or 160 μmol photons m^{-2} s^{-1}. Under high light, NO_3^- is a better N source in terms of growth rate than NH_4^+. This was somewhat unexpected since NH_4^+ is directly assimilated into amino acids, and NO_3^- must be reduced before assimilation (Lobban and Harrison, 1994). Under low light there is no advantage in using either of the N sources in terms of growth rate.

A critical internal tissue N level of 0.40% fresh wt and a subsistence level of 0.15% fresh wt seem to be maintained by the blades, regardless of N source or light level. Chao-yuan et al. (1983) found that the maximal growth rate of *P. yezoensis* (11.6% per day) was attained with a N content of 4.7% dry wt. Assuming a fresh wt to dry wt ratio of 10, this compares well to the 0.40% fresh wt (i.e., 4.0% dry wt) critical level found in this study. The critical tissue N level is 2% dry wt for *G. tikvahiae* (Hanisak, 1990), and 1.9% dry wt for *Codium fragile* (Hanisak, 1979). This suggests that *Porphyra yezoensis* has a greater N requirement than *G. tikvahiae* or *C. fragile* (assuming a fresh : dry wt ratio of 10).

The fact that the light environment did not alter the critical level of N was unexpected. Light was expected to alter cell constituents such as pigment amounts, as well as the amount of N needed for maximal growth rate (Lobban and Harrison, 1994). This photoadaptation did not occur in *P. yezoensis*. This alga is always found in the high intertidal zone, where light levels are always relatively high (compared to the subtidal environment). Perhaps *P. yezoensis* does not have the ability to increase or decrease pigment levels in response to light, so light has no effect on critical tissue N levels (Herbert and Waaland, 1988). This differs with the interactive effect of light and N seen with *Gracilaria tikvahiae* (Lapointe and Duke, 1984), and with *Macrocystis pyrifera* (Shivji, 1985). The relationship between N and growth rate varies with the light intensity in these algae. The critical levels of N can be expected to differ depending on the light environment of these algae (Lapointe and Duke, 1984).

The two PFDs used in this experiment (50 and 160 µmol photons m^{-2} s^{-1}) were very low compared to natural values that would be obtained in a commercial grow-out facility. On a photosynthesis vs. irradiance curve, 160 µmol photons m^{-2} s^{-1} is probably not near the saturation point for photosynthesis (Lapointe and Duke, 1984). If severe light limitation was occuring in both the high light and low light cultures, this would prevent a meaningful discussion of light effects on critical N levels. However there is no evidence of light limitation in the high light cultures. A growth rate of 14% per day in high light cultures given NO_3^- is evidence that light limitation is not occuring. Imada et al. (1971) obtained a maximal growth rate of 15 - 20% per day during outdoor cultivation, where PFDs were much higher than those used in this study.

Though PFDs will certainly be much higher during commercial scale tank cultivation, 100% of the incident irradiance will be absorbed by the dense seaweed cultures that are necessary for profitable production. Therefore, the bottom of the tanks will be in complete darkness. An individual blade, circulating in a tank will alternate between short bursts of high irradiance, followed by complete darkness. Blades in the small trough vessels used in this study are exposed to either 50 or 160 µmol photons m^{-2} s^{-1} constantly for 8 hours per day. There is no dark period in the experimental 1 g per 3L cultures in the small trough vessels used here. This may be the reason why growth rates were high compared to the PFDs used, and why high light cultures were not severely light limited though PFDs used were very low compared to natural

irradiance levels. Therefore, the applicability of the results to the high light environments in outdoor conditions is not limited by the low PFDs used.

Porphyra yezoensis does not have the ability for luxury storage of P (over the range of P and N:P supply given here), as indicated by a linear relationship between growth rate and tissue P. P storage may have occured with lower N:P supply ratios, but these were not tested here. A higher subsistence level for P when NH_4^+ was given, than when NO_3^- was given, suggests an increased requirement for P, or a decreased ability to utilize tissue P for growth when the N source is NH_4^+. The slope of the relationship between growth rate and tissue P was greater when NO_3^- was given. This suggests that *P. yezoensis* blades are more efficient at using tissue P for growth when NO_3^- is the N source, than when NH_4^+ is the N source.

Perhaps the greater efficiency of tissue P use when NO_3^- is the N source is the reason for NO_3^- being a better N source (in terms of growth rate) under high light conditions than NH_4^+. Under low light, the pressure to utilize tissue P for growth is not as great as under higher PFD, and therefore no advantage is seen between N sources. Under high light, growth rates are higher and an efficient use of tissue P is necessary to support the higher demands for tissue P utilization. Because blades given NO_3^- are more efficient at using tissue P for growth, these blades have an advantage when light levels are high. It has traditionally been assumed that N is the most important limiting nutrient in temperate oceans and during mariculture (Flores-Moya et al., 1997),

however this study shows that P levels also play a very important role in determining growth rate of *P. yezoensis*.

Perhaps *P. yezoensis* has a lower vacuole storage capacity for NH_4^+ than for NO_3^-. This would mean that NH_4^+ would have to be assimilated into amino acids in order for it to be stored in tissue. This assimilation is driven by ATP, which requires P to function. This would account for the higher requirement for tissue P found when NH_4^+ was the N-source. However, without a measurement of the inorganic NO_3^- and NH_4^+ pool, this is just speculation.

Chao-yuan et al. (1983) concluded that NH_4^+ is a better N source than NO_3^- for *P. yezoensis*. However, uptake rate was the only parameter considered. Even if NH_4^+ is taken up at a faster rate than NO_3^-, this does not mean that growth rate is enhanced with the use of NH_4^+. The blades may be storing NH_4^+ as tissue N, and not using it for growth. This study goes one step further by relating internal tissue N and P to growth. Based on these parameters, it seems that NO_3^- is the better N source in terms of growth. This agrees with Iwasaki (1967), who found that NO_3^- is the better N source for *Porphyra tenera*.

For *Gracilaria tikvahiae*, growth rate is identical whether NH_4^+ or NO_3^- is given (Hanisak, 1990). Both *Gracilaria foliifera* and *Neoagardhiella baileyi* have higher growth rates with the use of NH_4^+ than with NO_3^- (DeBoer et al., 1978). This preference is very

dependent on the macrophyte being considered. No general trends can be determined in the advantage that one N source has over another in terms of growth rate.

The change in colour from dark purple-black to bright green in N depleted blades suggests a loss of the pigment phycoerythrin, or the proteins associated with this pigment (Ryther et al., 1981/82). Other algae such as *Chondrus crispus* (Neish et al., 1977), *Pterocladia capillacea, Gracilaria foliifera, Agardhiella subulata,* and *Ceramium rubrum* (DeBoer, 1981) show a similar loss of colour in N-depleted seawater. The loss of colour in low N seawater suggests that a relationship between tissue N and phycoerythrin exists (Darley, 1982). It is possible that this reddish proteinaceous pigment is acting as a N sink as it does in *Porphyra abbottae* (Hannach, 1989), however this study did not measure pigment levels so only the suggestion of a link can be made here. The fragmentation of *P. yezoensis* tissue given low P is also seen in *C. crispus* (Neish et al., 1977). P is found in phospholipids, essential components of cell membranes (DeBoer, 1981). This suggests that P has a role in maintaining the structural integrity of *P. yezoensis* blades.

This study was performed on blades in the artificial and controlled environment of a growth chamber. It remains to be seen if blades will have a critical N level of 0.40% fresh wt under natural sunlight, and whether the critical N level varies seasonally. This study's findings are a good starting point for further, larger-scale outdoor trials.

Tissue N : P

Optimal N:P for growth, tissue N, and tissue P were all similar regardless of N source or light level. These factors were all optimal within the N : P range of 13 - 15. When N:P is within this range, growth rate, tissue N, and tissue P are all at their highest. This suggests that N:P is a better indicator of *P. yezoensis* nutritional status than either tissue N or tissue P alone (Wheeler and Björnsäter, 1992). The fact that the relationship is constant under low and high light, and regardless of N source, makes N:P a particularly useful measurement commercially, with N:P < 13 - 15 indicating N limitation, and N:P > 13 - 15 indicating P limitation. The critical N level (0.40% fresh wt) is also constant no matter what the N source or light level is. As long as tissue N > 0.40% fresh wt (the higher the N content, the higher the quality of the resulting processed nori; Johnston, 1971) and N:P = 13 - 15, blades are neither N- nor P-limited, and should be growing at their highest rate possible (as long as other factors such as carbon, and micronutrients are not limiting).

Marine phytoplankton have an average N:P ratio of 16:1 (the Redfield ratio). Marine macrophytes show a greater range of Redfield ratios, with a median of 30:1 (Lewis and Hanisak, 1996). This suggests lower P requirements (or higher N requirements) in macrophytes. Since the optimal N:P ratio found in this study ranges from 13 - 15, *P. yezoensis* has P requirements that are closer to those of marine phytoplankton than other marine macrophytes. This may be due to its simple

monostromatic structure (fewer structural proteins needed means lower N:P). This also illustrates the importance of P nutrition to *P. yezoensis*.

Wheeler and Björnsäter (1992) found that for *Porphyra* sp. and *Codium fragile*, N:P < 12 suggests N limitation and N:P > 17 suggests P limitation. Though this range is slightly wider than that found here, these results agree well with the findings of this study. Atkinson and Smith (1983) list a C:N:P ratio of 137:23:1 for *P. yezoensis*. Hernandez et al. (1993) give a C:N:P ratio of 258:20:1 in the winter, and 495:38:1 in the spring for *Porphyra umbilicalis*. The ratios found in these wild collected blades suggests P limitation. Experiments by Flores-Moya et al. (1997) confirm that *Porphyra leucosticta* is often P-limited in the spring.

Under P limitation, growth rate is affected much more severely when NH_4^+ was given. This fits with the suggestion that blades given NH_4^+ cannot use tissue P for growth as efficiently as blades given NO_3^-. Under P limitation (N:P > 13 - 15), blades given NH_4^+ are able to store tissue N at levels as high as N and P sufficient blades (N:P = 13 - 15). Perhaps blades are physiologically geared towards utilizing this usually ephemeral N source, so that they take advantage of NH_4^+ when it is available, even if blades cannot use the N for growth because of P limitation.

Under low light, N and P sufficient blades given NH_4^+ are able to store more tissue N than blades given NO_3^-. One explaination is that blades are storing N, taking

advantage of a usually patchy N source (NH_4^+) while they can, even if they cannot use the N for growth because of light limitation. Also, tissue P levels were higher in N and P sufficient blades given NH_4^+ under low light than blades given NO_3^- under low light. This increased tissue P may be in response to the increased storage of NH_4^+, as an attempt to regulate N:P, keeping it within the range 13 - 15, optimal for growth. The potential for N accumulation was greater with NH_4^+ than with NO_3^- in *Neoagardhiella baileyi*, *Gracilaria foliifera* (D'Elia and DeBoer, 1978), and *Ulva lactuca* (DeBusk et al., 1986) for the same reasons mentioned above.

Starvation experiment

The results of the starvation experiment serve to confirm both the critical and subsistence levels of N and P, and the optimal N:P ratio. Growth rate did not decrease at all until tissue N fell to 0.36% fresh wt (N:P < 10) and tissue P fell to 0.05% fresh wt (N:P > 23). This agrees well with previous findings in this study for critical N (0.40% fresh wt), and optimal N:P (13 - 15). Death occurred when tissue N fell to 0.15% fresh wt and tissue P fell to 0.03% fresh wt. This also agrees well with this study's previous findings for subsistence N (0.15% fresh wt), and subsistence P with NO_3^- as the N source (0.02% fresh wt). Growth rates were equally affected by P and N limitation, illustrating the importance of both N and P in regulating growth.

N and P reserves were used up relatively quickly. Growth rates stayed high for only 5 days before N and P reserves fell to limiting levels. *Gracilaria tikvahiae* is able to

grow on its nutrient reserves for 2 weeks before limitation occurs (Hanisak ,1990). Low nutrient-storage capability is characteristic of opportunistic species (Lobban and Harrison, 1994), and may pose a problem in managing nutrients so that other weed species are left with little nutrient for uptake and growth. It appears that *P. yezoensis* may have a nutrient storage capacity that is as low as other opportunistic weed species (like *Enteromorpha* and *Ulva*). However there are alternative weed control methods that may be effective (like air exposure).

CONCLUSIONS

The two forms of nitrogen given to *P. yezoensis* in this study (NO_3^- and NH_4^+) differ in their abilities to promote growth. In general, growth is higher with NO_3^- than with NH_4^+. This may be due to the increased efficiency (i.e., lower subsistance quota, and increased slope of the relationship between tissue P and growth rate) of tissue P utilization by blades when NO_3^- is the N source. However, the potential for N accumulation is higher when NH_4^+ is the N source. Blades can store more N during light and phosphorus limitation when NH_4^+ is given, but this increased N storage does not translate into increased growth rate. These experiments were done with small blades and it remains to be seen whether the results are similar using larger blades. However, because *Porphyra* has a simple monostromatic structure (i.e., fewer structural components than other seaweeds such as kelp for example), it is expected that results will probably be similar for large blades.

Tissue analysis should be used in the cultivation of *P. yezoensis*. It is a valuable tool that is the most direct way of determining the nutritional status of blades. It is used extensively with agricultural plants, and has contributed to the success of this industry (Hanisak, 1979; DeBoer, 1981). The critical nutrient concentration is the keystone for assessing the nutrient status of a crop (Ulrich, 1952). This type of analysis should become standard in the cultivation of macrophytes. Maintaining a N:P of 13 - 15, and a tissue N of ≥ 0.40% fresh wt should result in growth that is neither N- nor P-limited. Because final product quality has been linked to tissue N content, it is probably desirable to keep tissue N closer to the 0.70% fresh wt range (Mencher et al., 1983). It remains to be seen if these figures remain unchanged in an outdoor commercial setting.

TABLE 4.1. Range of N and P loads given to blades during experiment.

Tissue N experiments (load = µmol per day)				
NO_3^- load	2.14 - 257.14		NH_4^+ load	2.14 - 171.43
PO_4^- load	21.43		PO_4^- load	21.43
N:P supply ratio	0.1 - 12.0		N:P supply ratio	0.10 - 8.0
Tissue P experiments (load = µmol per day)				
NO_3^- load	128.57		NH_4^+ load	128.57
PO_4^- load	0.26 - 16.29		PO_4^- load	0.43 - 21.43
N:P supply ratio	7.9 - 494.5		N:P supply ratio	6.0 - 299.0

FIGURE 4.1. Relationship between tissue N and growth rate (95% confidence limits shown, n = 4; high light = solid diamonds, low light = hollow circles; A = NO_3^- - N source, B = NH_4^+ - N source).

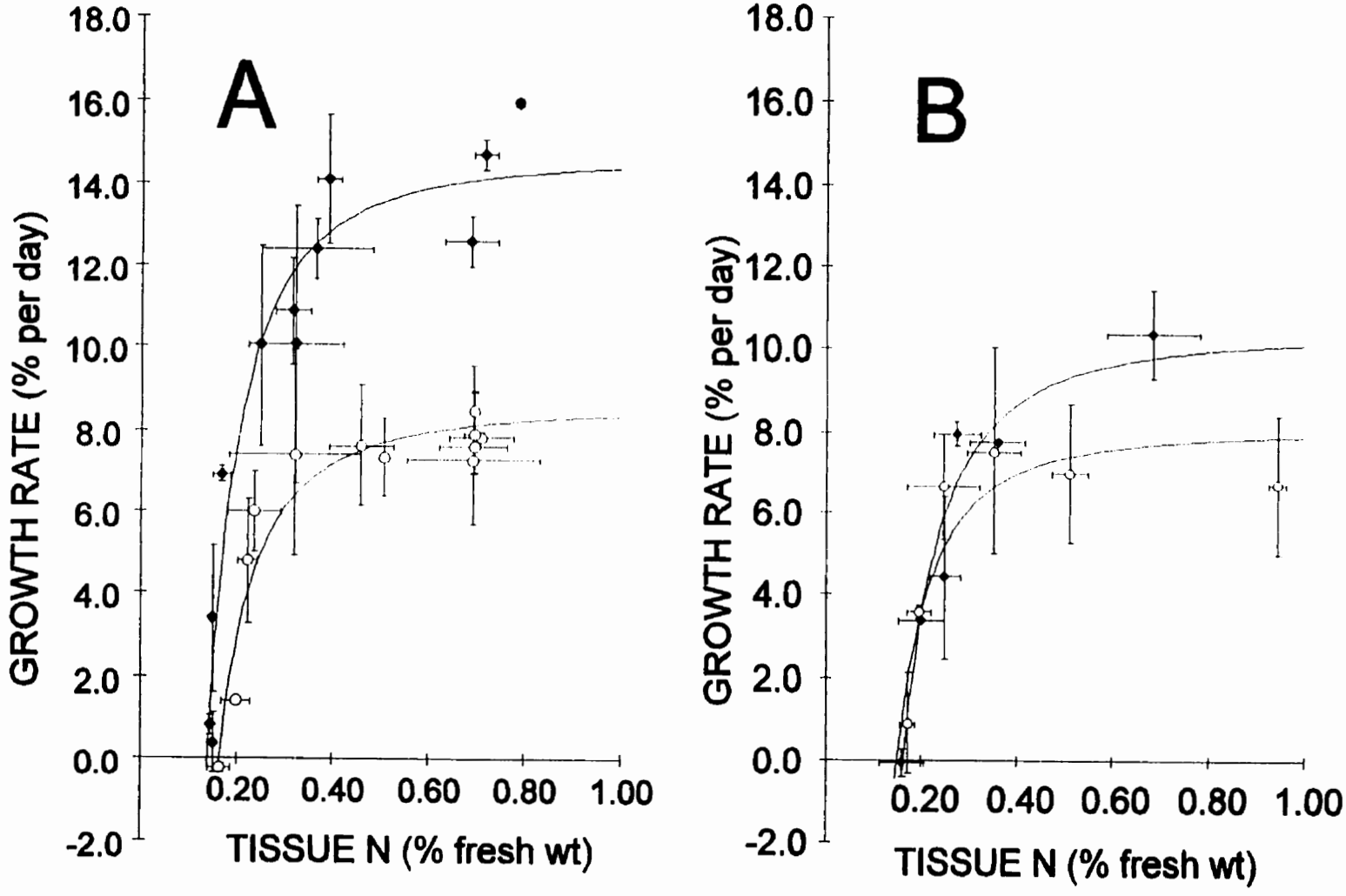


TABLE 4.2. Modified Droop equation parameters and regression statistics for the relationship between tissue N and growth rate shown in Figure 4.1. Modified Droop equation: $\mu = \mu_{max} [1 - (Q_0 / Q)^2]$.

LIGHT	N source	μ_{max} (% per day)	Q_0 (% fresh wt)	Crit. N (% fresh wt)	R^2
HIGH	NO_3^-	14.7	0.14	0.37	0.93
HIGH	NH_4^+	10.4	0.16	0.41	0.93
LOW	NO_3^-	8.6	0.16	0.41	0.92
LOW	NH_4^+	8.1	0.15	0.39	0.83
		MEAN	0.15	0.40	

FIGURE 4.2. Relationship between tissue P and growth rate (95% confidence limits shown, n = 4; high light = solid diamonds, low light = hollow circles; A = NO_3^- - N source, B = NH_4^+ - N source).

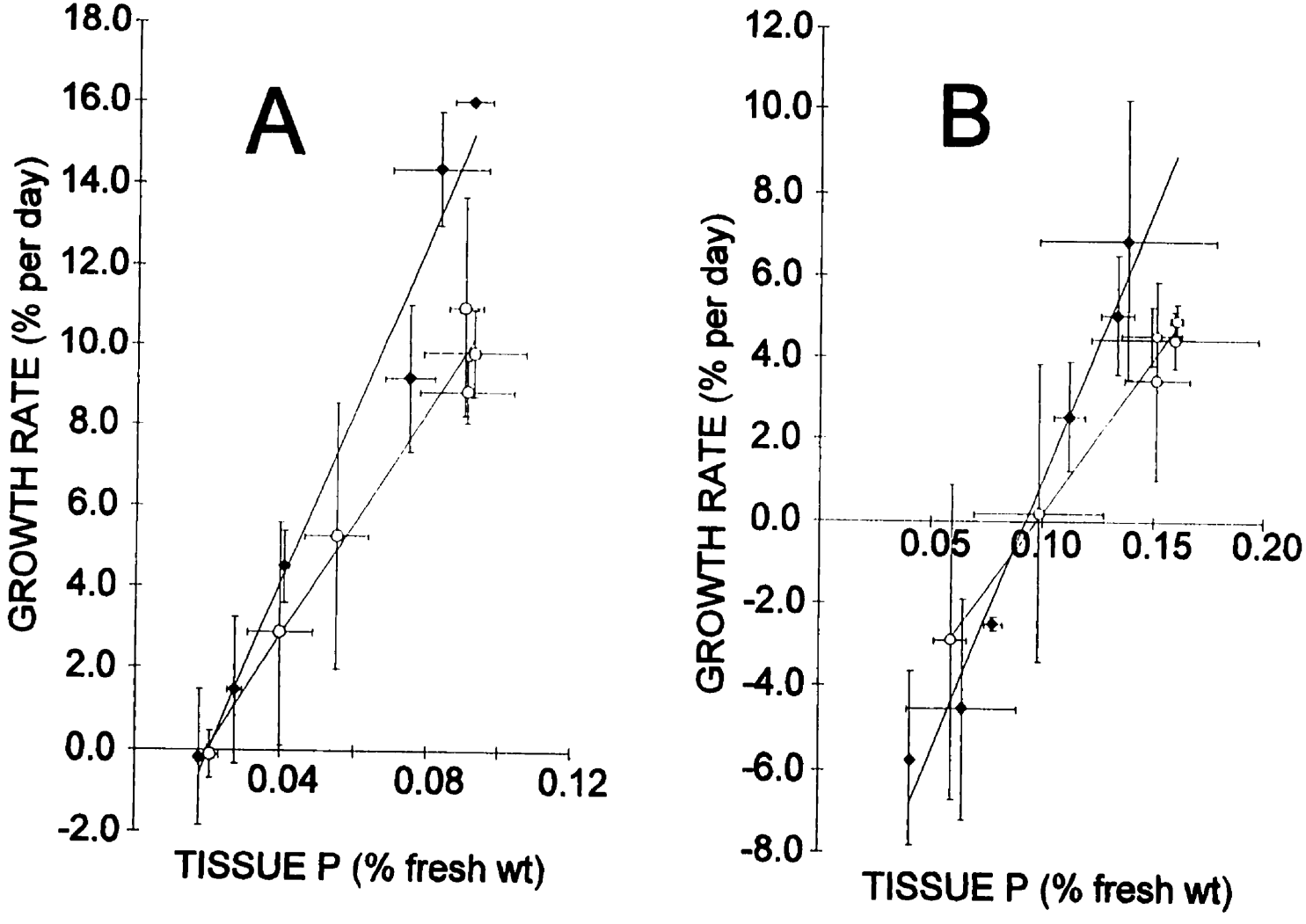


TABLE 4.3. Parameters and regression statistics for the linear relationship between tissue P and growth rate shown in Figure 4.2. Linear equation: $\mu = m(Q) + b$.

LIGHT	N source	m	b	Q_0 (% fresh wt)	R^2
HIGH	NO_3^-	210.84	-4.30	0.02	0.99
HIGH	NH_4^+	132.03	-12.25	0.09	0.98
LOW	NO_3^-	137.70	-2.67	0.02	0.97
LOW	NH_4^+	74.93	-7.30	0.10	0.98

FIGURE 4.3. Relationship between molar N:P and growth rate (high light = solid diamonds, low light = hollow circles; A = NO_3^- - N source, B = NH_4^+ - N source).

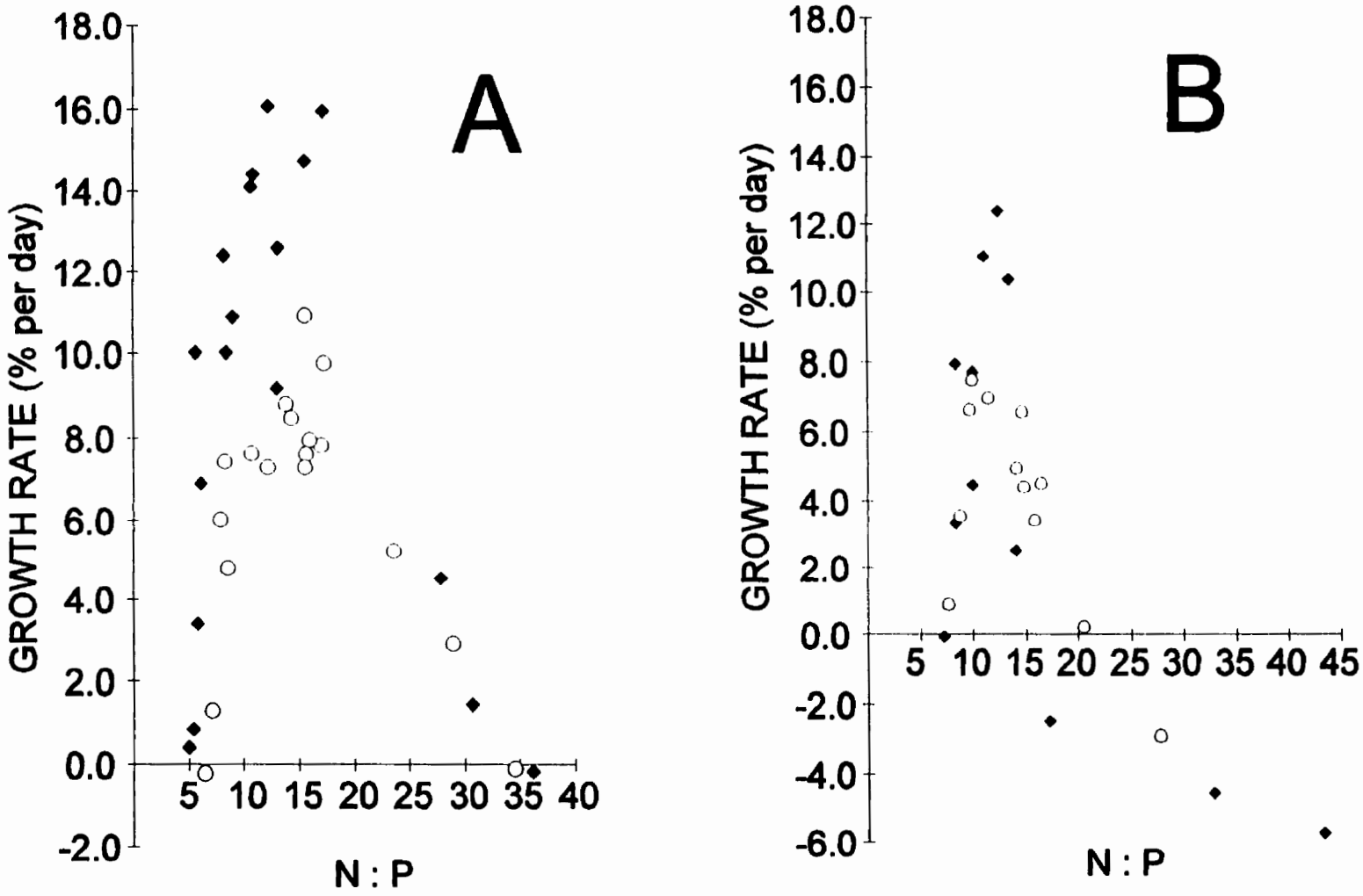


FIGURE 4.4. Relationship between molar N:P and tissue N (high light = solid diamonds, low light = hollow circles; A = NO_3^- - N source, B = NH_4^+ - N source).

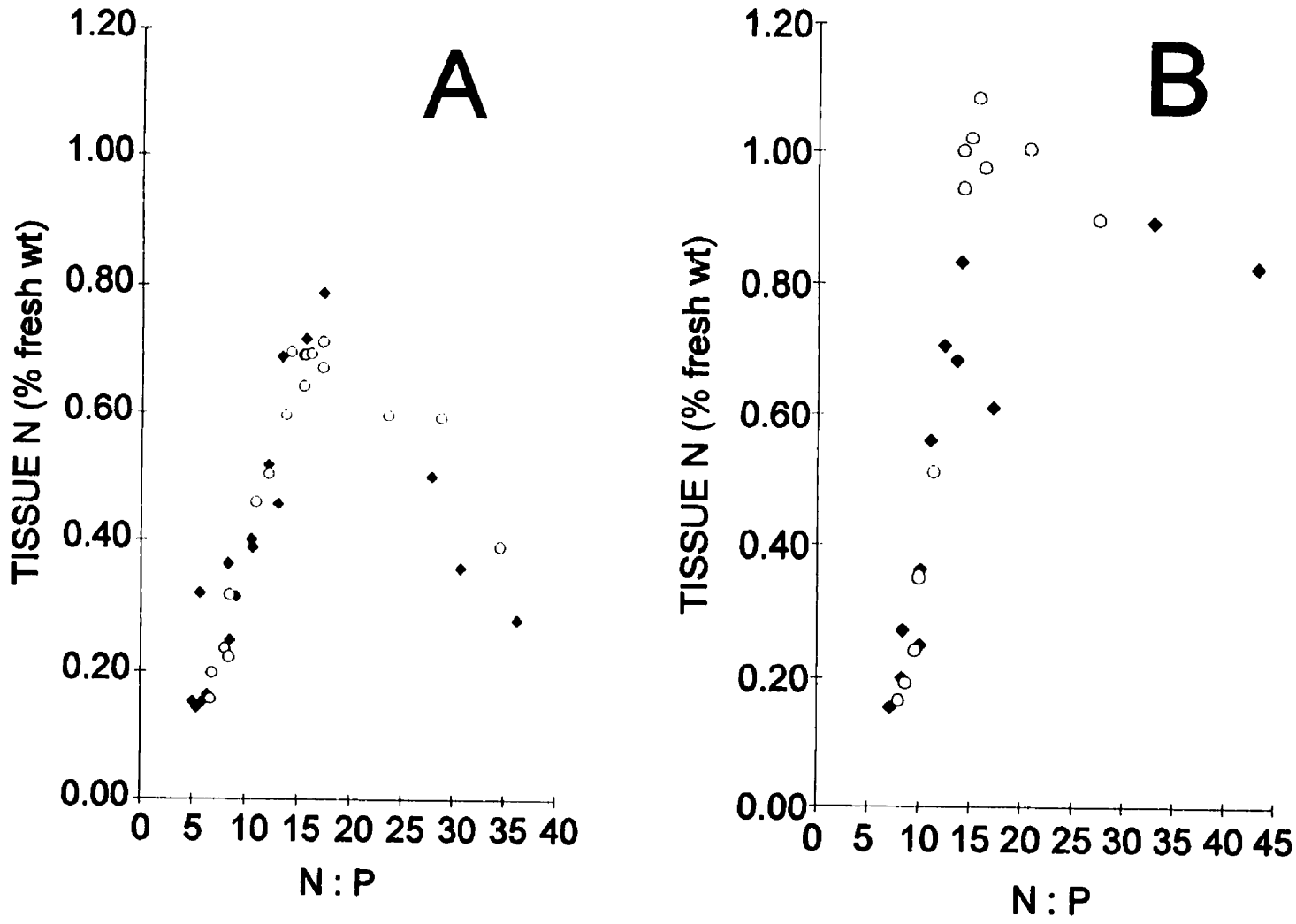


FIGURE 4.5. Relationship between molar N:P and tissue P (high light = solid diamonds, low light = hollow circles; A = NO_3^- - N source, B = NH_4^+ - N source).

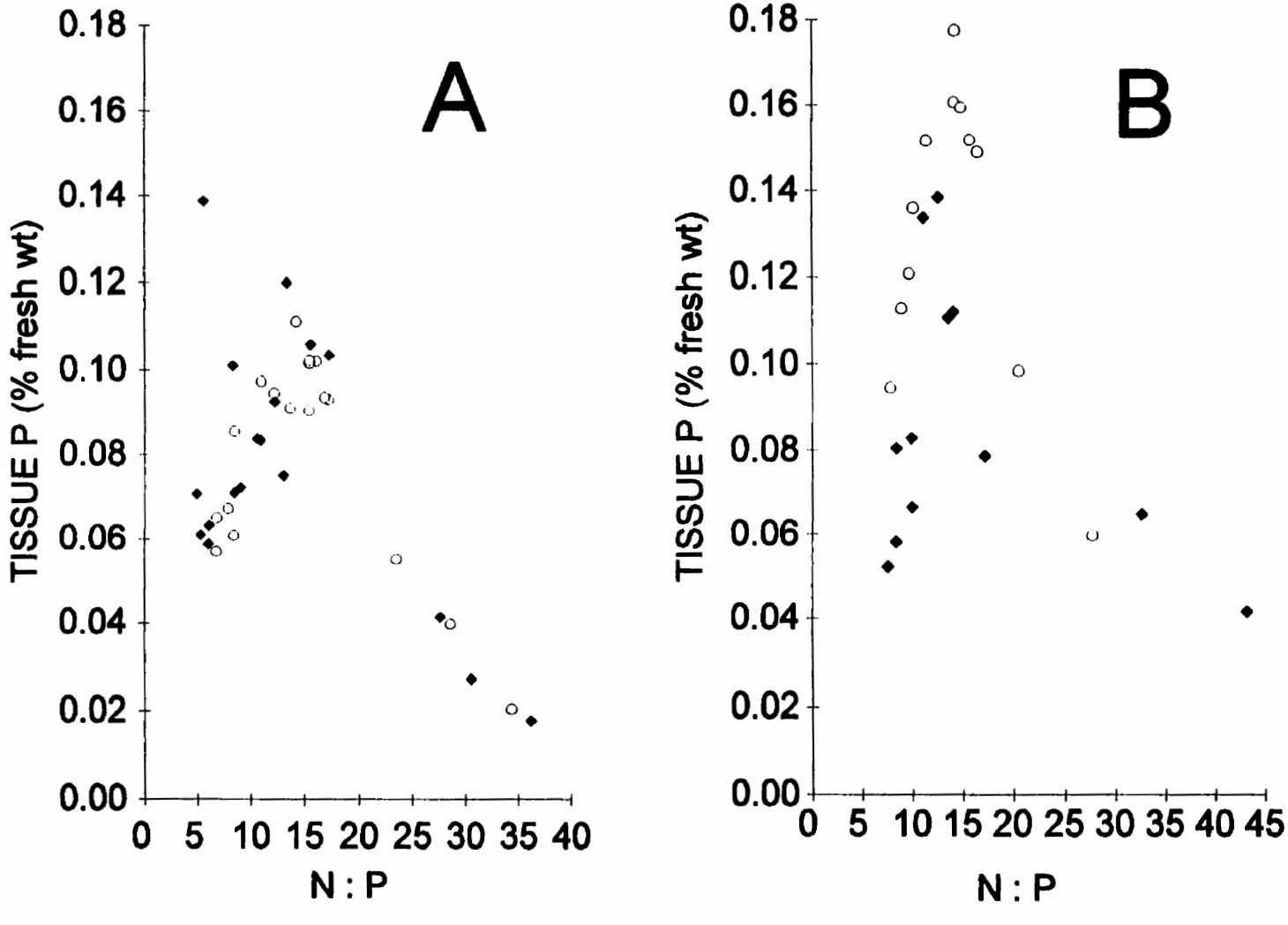


FIGURE 4.6. Results of starvation experiment. A) Growth and tissue N of N- and P-sufficient blades, starved of N by giving them only PO_4^- . B) Growth and tissue P of N- and P-sufficient blades, starved of P by giving them only NO_3^- (95% conf. limits shown, n = 4).

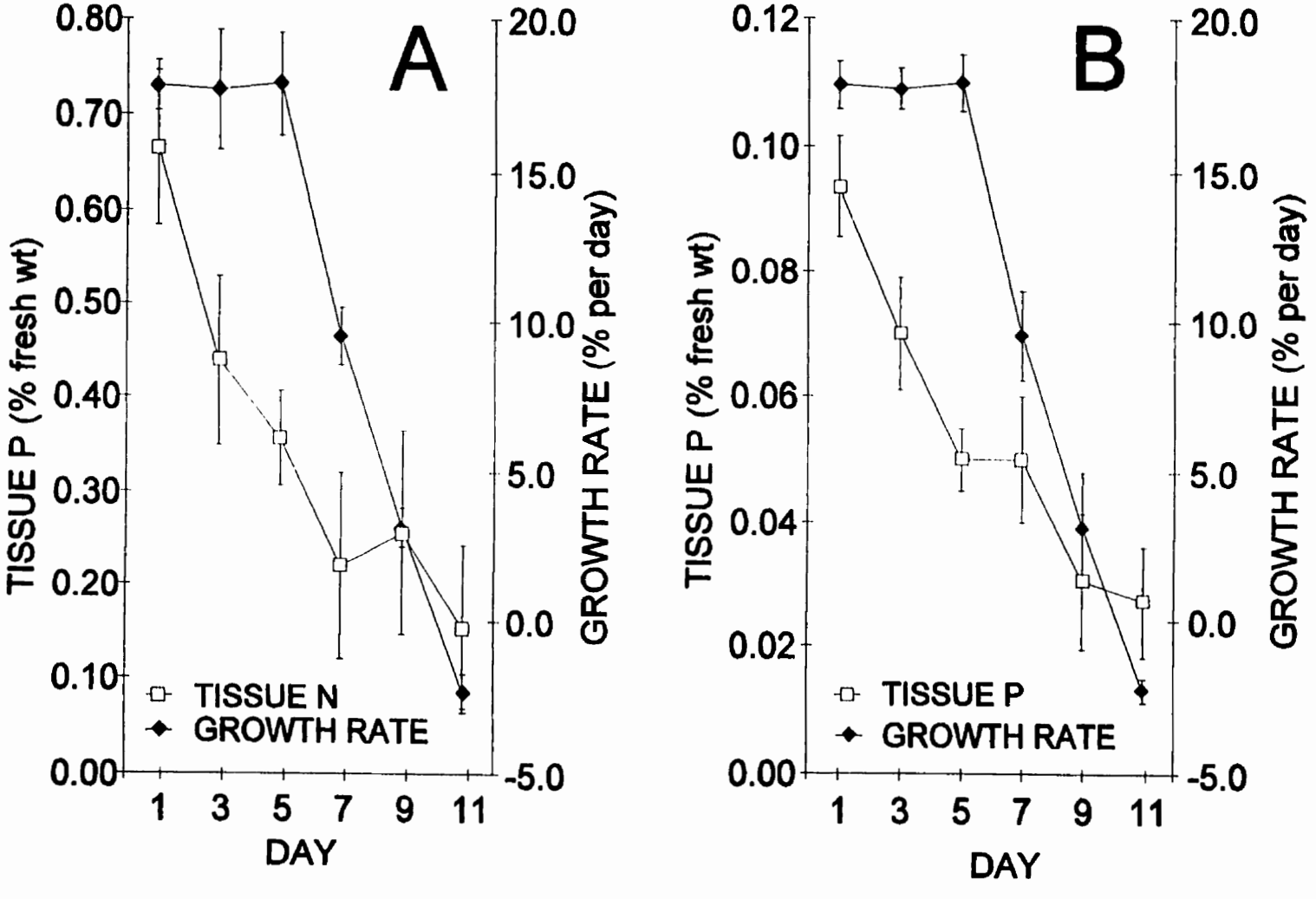
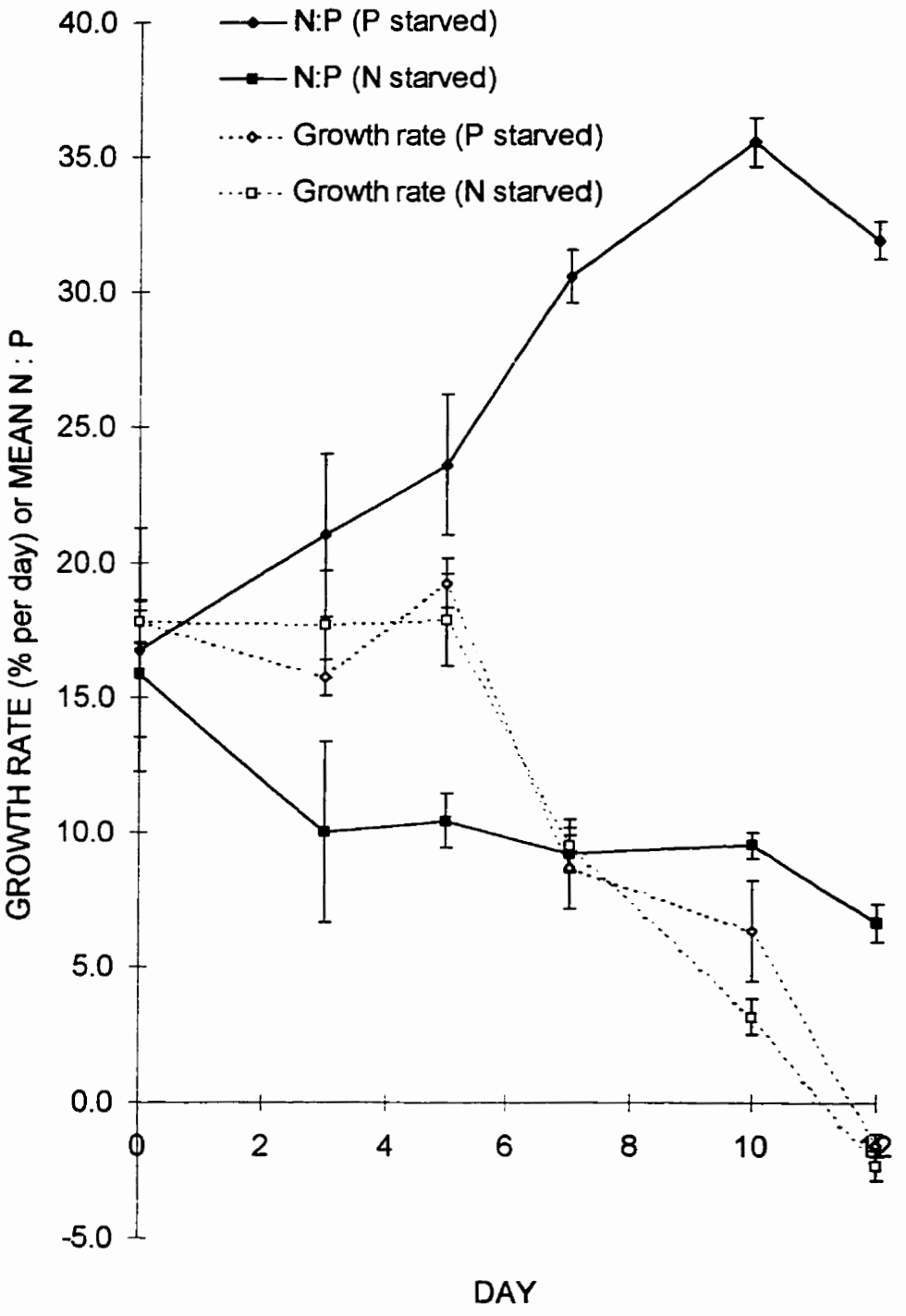


FIGURE 4.7. Results of starvation experiment, showing N:P and growth rate changes as blades were starved of N or P (95% conf.limits shown, n = 4).



CHAPTER FIVE

GENERAL CONCLUSIONS

One of the main purposes of this thesis was to investigate the feasibility of cultivating suspension cultures of *Porphyra yezoensis* in land-based tanks. Usually a fragmenting, filamentous seaweed such as *Chondrus, Gelidium,* or *Gracilaria* is chosen as a candidate for tank culture because tank aeration is sufficient to break plants up, thereby easily propagating these algae. When a non-fragmenting seaweed such as *P. yezoensis* is grown in tanks, it is at a disadvantage in this respect and propagation is more difficult. Unexpected spore or gamete release normally results in tank fouling, so conditions must be controlled such that reproduction occurs only when needed.

The propagation experiments in Chapter Two demostrate that suspension cultures of *P. yezoensis* can be produced quite easily via conchospores or vegetatively via monospores. Both suspended and fixed substrata were seeded with conchospores, producing dense cultures of suspended blades. Though early growth rates were higher using suspended substrata, the use of fixed substrata also worked well.

Porphyra yezoensis may be at a disadvantage during tank cultivation compared to filamentous, fragmenting seaweeds, but the vegetative method of propagation described in Chapter Two may reduce this disadvantage. Again, early growth rates were highest using large tissue sections from small blades, but all sections from all

blades worked well, and dense vegetatively propagated suspended blade cultures were the result. It is not difficult to produce blade suspension cultures and to propagate them vegetatively. Therefore, the potential for successful large-scale tank cultivation of *P. yezoensis* is high.

Chapter Four describes the effect of tissue N and P on the growth of *P. yezoensis* blades in suspension. The blades have the ability to store tissue N but not tissue P (over the range of P supply given). Subsistence quotas for tissue N and P were given, as well as critical tissue N. The effect of varying N source (NO_3^- and NH_4^+), and light level was investigated, and the tissue N:P ratio was shown to be a useful measure for future commercial production. Also an important role for tissue P was investigated; it was shown that tissue analysis for N and P is a useful technique for determining whether blades are N- or P-limited. Tissue analysis is more a more direct indicator of nutritional status than ambient N and P.

Tank cultivation has its greatest advantage over ocean-based cultivation in that culture conditions can be managed more effectively. The nutrient environment can be manipulated such that it is optimal for growth or nutrient storage. However this advantage cannot be exploited unless specific nutrient effects on growth are known. In Chapter Four it was shown that by keeping N:P = 13 - 15, and tissue N $\geq$ 0.40% fresh wt blades will grow at their maximal rate, unlimited by N or P, regardless of light level or N source. Blades have a greater potential for N storage when NH_4^+ is given, but have a

greater potential for growth when NO_3^- is given. When given NO_3^-, blades can make more efficient use of tissue P, allowing blades to grow at a higher rate than when given NH_4^+. The information in Chapter Four allows the exploitation of one of the great advantage tanks have over ocean-based operations for *P. yezoensis*. Tank conditions can now be manipulated such that tissue N and N : P are at their optimal levels.

The next step is to test some of the propagation techniques on a larger scale. Does the use of suspended substrata for spore attachment result in efficient large scale production? Is the quality of processed nori as high when grown in suspension cultures? Also there is much work yet to be done with nutrition. For example, it would be useful to examine the inorganic internal pools of NO_3^- and NH_4^+. Is NH_4^+ stored, unassimilated in vacuoles at the same level as NO_3^-. Also, what are the effects of micronutrients such as zinc and iron?

Overall this thesis has shown that the production and propagation of *P. yezoensis* blades in suspension cultures can be accomplished readily, therefore the potential for successful tank cultivation is high. Also, now that the effect of tissue N and P on the growth and tissue composition of blades in suspension is known, the tank nutrient environment can be managed such that growth is maximal. This thesis represents a step toward land-based mariculture of this commercially important species.

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IMAGE EVALUATION
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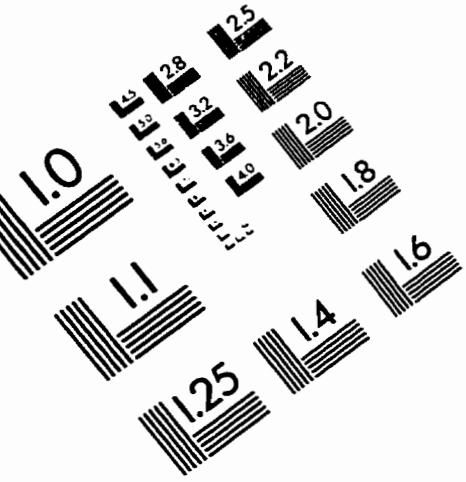

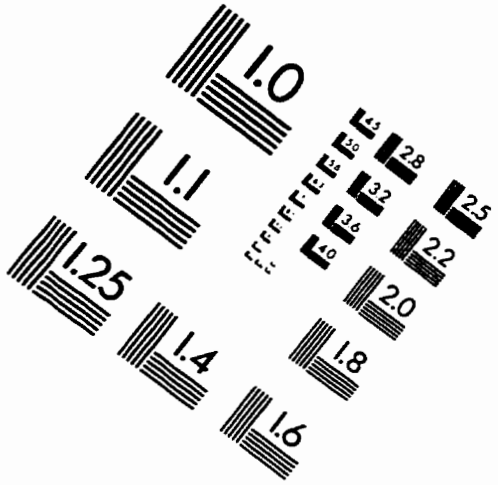

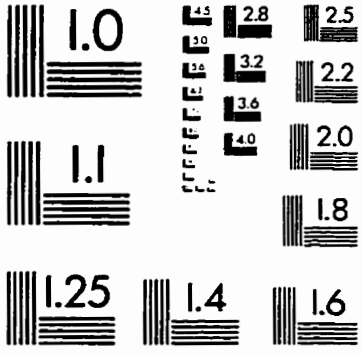

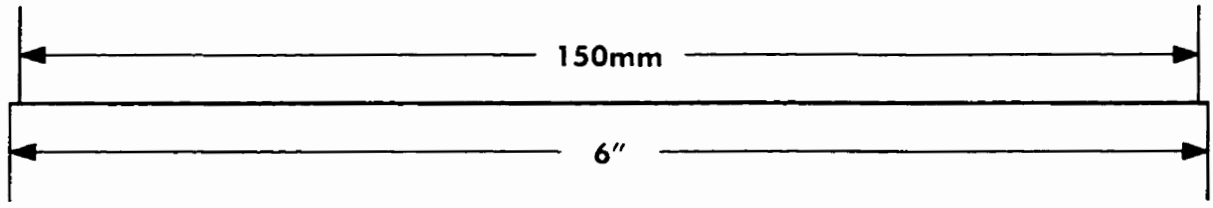

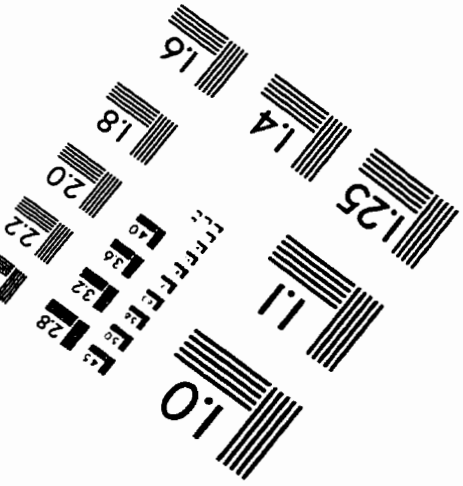

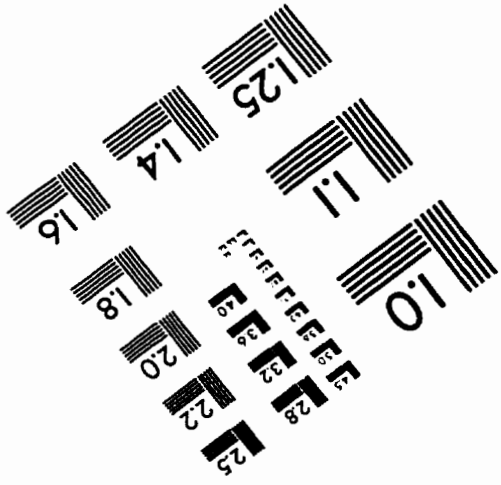

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