AN ABSTRACT OF THE DISSERTATION OF

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Title: Enhanced Production of Pacific Dulse (*Palmaria mollis*) for Co-culture with Abalone in a Land-based System.

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Abstract approved: _____
Christopher J. Langdon

Palmaria mollis (Pacific dulse) is increasingly being used in land-based marine aquaculture. This work elucidates nutrient, inorganic carbon, light, salinity, and temperature requirements of *P. mollis* as food for abalone species.

Nutrient additions every 5 to 7 days resulted in no significant difference in *P. mollis* growth compared with daily nutrient additions. The addition of *f* medium trace metals (Guillard and Ryther, 1962) significantly increased growth compared with additions of PO_4^- and NO_3^- alone. The use of $NaNO_3$ as an N source was found to be superior compared with NH_4NO_3. *Palmaria mollis* growth was most sensitive to phosphorus additions, with a declines in growth above concentrations of 83.3 μM - P d^{-1}.

Palmaria mollis growth was positively correlated with concentrations of dissolved inorganic carbon. A pH of 8.9 produced growth rates that were not significantly different than at pH 8.3. A seawater flushing regime that increased flushing during the light cycle but lowered flushing during the dark cycle resulted in growth rates equal to those obtained by maintaining pH at 8.5 with CO_2.

At a specific light density (SLD) of 0.0048 to 0.036 mol photons $g^{-1}[fw]d^{-1}$, growth was highly correlated with light. Growth was best at a salinity of 30±1‰ and a temperature of 12° C for low light and 14° to 18° C at high light. Growth under higher temperatures was correlated with increased light. Lowering culture temperature at night from 15° C to 11° C significantly increased growth compared with a constant daily temperature of 15° C.

There was no significant difference in growth of *Haliotis discus hannai* cultured in seawater compared to seawater supplemented with PO_4^-, NO_3^-, and 0.75*f* + Zn (d^{-1}). The Japanese abalone *H. discus hannai* showed the highest growth when fed on *P. mollis* supplemented with nitrate loads ranging from 1176 to 2353 $\mu M\ d^{-1}\ NO_3^-$ - N and the red abalone *H. rufescens* when fed on *P. mollis* supplemented with nitrate loads of 2353 to 2942 $\mu M\ d^{-1}\ NO_3^-$ - N. Both abalone species grew better on *P. mollis* supplied with 0.75*f* + Zn (d^{-1}) trace metal solution (Guillard and Ryther, 1962).

Enhanced Production of Pacific Dulse (*Palmaria mollis*) for Co-culture with Abalone in a Land-based System.

by
Carl L. Demetropoulos

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Carl L. Demetropoulos, Author

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Studies on *Palmaria mollis* were initially started as part of my dissertation at the University of Washington. I am indebted to both the university, Dr. Bruce Miller and the Friday Harbor Labs for the fine instruction, mentoring and research skills I received there.

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: NITROGEN, PHOSPHORUS, AND TRACE METAL NUTRITION	5
ABSTRACT	5
INTRODUCTION	7
MATERIALS AND METHODS	9
RESULTS	18
DISCUSSION	41
REFERENCES	52
ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF SEAWATER EXCHANGE, PH, AND INORGANIC CARBON CONCENTRATION ON GROWTH	58
ABSTRACT	58
INTRODUCTION	59
MATERIALS AND METHODS	62
RESULTS	68
DISCUSSION	80
REFERENCES	85
ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF STOCKING DENSITY, LIGHT, SALINITY, AND TEMPERATURE ON ALGAL GROWTH RATES	88
ABSTRACT	88
INTRODUCTION	90

TABLE OF CONTENTS (Continued)

MATERIALS AND METHODS 92

RESULTS 101

DISCUSSION 113

REFERENCES 120

ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF *PALMARIA MOLLIS* NUTRIENT ENRICHMENT AND BIOCHEMICAL COMPOSITION ON ABALONE GROWTH AND CONDITION 124

ABSTRACT 124

INTRODUCTION 126

MATERIALS AND METHODS 128

RESULTS 137

DISCUSSION 155

REFERENCES 164

CONCLUSIONS 169

BIBLIOGRAPHY 172

APPENDICES 184

LIST OF FIGURES

Figure		Page
1.1	Specific growth rate of *Palmaria mollis* (± SE) as a function of nutrient application rate (with equal nutrient loads among all treatments) under artificial low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or artificial high light (PFD = 42.34 mol photons m^{-2} d^{-1}	21
1.2	Specific growth rate of *Palmaria mollis* (± SE) as a function of additional zinc (total Zn = 1.82 μM d^{-1}) under artificial low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or artificial high light (PFD = 42.34 mol photons m^{-2} d^{-1}	23
1.3	Specific growth rate of *Palmaria mollis* (± SE) as a function of different *f* metal concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}) under a moderate PFD of 37.44 mol photons m^{-2} d^{-1}	24
1.4	Specific growth rate of *Palmaria mollis* (± SE) as a function of pulse fertilization with either $NaNO_3$ (linear) or NH_4NO_3 (cubic spline) as the N source	25
1.5	Specific growth rate of *Palmaria mollis* (± SE) as a function of continuous fertilization with either $NaNO_3$ or NH_4NO_3 as the N source	26
1.6	Specific growth rate of *Palmaria mollis* (± SE) as a function of nitrate additions (as $NaNO_3$) under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) and high light (PFD = 51.64 mol photons m^{-2} d^{-1})...	30
1.7	Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue iron concentration (μg g^{-1}[dw] ± SE) for cultured *Palmaria mollis*	31
1.8	Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue zinc concentration (μg g^{-1}[dw] ± SE) for cultured *Palmaria mollis*	32

LIST OF FIGURES (Continued)

1.9 Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue manganese concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis* 33

1.10 Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue copper concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis* 34

1.11 Tissue phosphorus concentration of *Palmaria mollis* as a function of specific growth rate under low light (PFD = 23.68 mol photons $m^{-2}\ d^{-1}$) or high light (PFD = 51.64 mol photons $m^{-2}\ d^{-1}$) 38

1.12 N:P ratio of *Palmaria mollis* as a function of specific growth rate under low light (PFD = 23.68 mol photons $m^{-2}\ d^{-1}$) or high light (PFD = 51.64 mol photons $m^{-2}\ d^{-1}$) 39

1.13 Specific growth rate of *Palmaria mollis* as a function of tissue C:N ratio under a photon flux density ranging from 22.5 to 51.64 mol photons $m^{-2}\ d^{-1}$ 40

2.1 Specific growth rate of *Palmaria mollis* (± SE) taken weekly as a function of seven dissolved inorganic carbon (DIC) additions and a seawater control under photon flux densities ranging from of 26.4 to 36.2 mol photons $m^{-2}\ d^{-1}$ 71

2.2 Specific growth rate of *Palmaria mollis* (± SE) as a function of seven dissolved inorganic carbon (DIC) treatments and a seawater control under photon flux densities ranging from 26.4 to 36.2 mol photons $m^{-2}\ d^{-1}$ during a 28-day experiment 72

2.3 Specific growth rate of *Palmaria mollis* (± SE) and pH (± SE) as a function of exchange rate under natural light (mean PFD = 43 mol photons $m^{-2}\ d^{-1}$) in translucent tanks 73

2.4 Specific growth rate of *Palmaria mollis* (± SE) as a function of either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 sparaging to control pH at constant seawater exchange (7.5 d^{-1}) 78

2.5 Summary of specific growth rate of *Palmaria mollis* as a function of average daytime pH under natural Hawaiian light (mean PFD ≈ 48 mol photons m^{-2} d^{-1}) from the last two weeks of the two seawater exchange rate experiments 79

3.1 Specific growth rate and yield of *Palmaria mollis* (± SE) measured weekly over a 35-day period as a function of six aerial densities (0.6, 2, 3, 4, 5, and 6 kg m^{-2}) under natural light (PFD = 39 to 52 mol photons m^{-2} d^{-1}) 103

3.2 Specific growth rate of *Palmaria mollis* as a function of specific light density (SLD) 104

3.3 Specific growth rate of *Palmaria mollis* (± SE) as a function of specific light density (SLD) and photon flux density (PFD) for two salinity treatments (30±1‰ and 35‰) under natural light over a 35-day period 106

3.4 Specific growth rate of *Palmaria mollis* (± SE) as a function of specific light density (SLD) and aerial photon flux density (PFD) for two salinity treatments (30±1‰ and 35‰) under natural light over a 28-day period 107

3.5 Specific growth rate of *Palmaria mollis* (± SE) as a function of temperature at an average natural PFD of 55 mol photons m^{-2} d^{-1} (0.016 mol photons g^{-1} [fresh wt.] d^{-1}) and an artificial PFD of 13 or 29 mol photons m^{-2} d^{-1} (0.010 and 0.021 mol photons g^{-1} [fresh wt.] d^{-1}, respectively), from the temperature and temperature/light interaction experiments 111

LIST OF FIGURES (Continued)

3.6 Specific growth rate of *Palmaria mollis* (± SE) as a function of lowering temperature from 15°±1C during the day to 11°±1C during the night both with and without CO_2 sparging 112

4.1 Shell length increase of *Haliotis discus hannai* (μm SL d^{-1} ± SE) feed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 141

4.2 Specific growth rate of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 142

4.3 Daily food consumption of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 143

4.4 Food conversion efficiency of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals ... 147

4.5 Shell length increase of *Haliotis rufescens* (μm SL d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 148

4.6 Specific growth rate of *Haliotis rufescens* (%d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 149

LIST OF FIGURES (Continued)

4.7 Daily food consumption of *Haliotis rufescens* (%d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 150

4.8 Food conversion efficiency of *Haliotis rufescens* (%d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals 153

LIST OF TABLES

Table		Page
1.1	Specific growth rates of *Palmaria mollis* (%d^{-1}[dw] ± SE), nitrogen (%dw ± SE) and protein contents (%dw ± SE), as a function of nutrient application rate under low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or high light (PFD = 42.34 mol photons m^{-2} d^{-1}) after 28 days	22
1.2	Specific growth rates of *Palmaria mollis* (%d^{-1}[dw] ± SE), tissue phosphorus (%dw), and protein content as a function of phosphate load d^{-1} under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}) after 49 days	28
1.3	Specific growth rates of *Palmaria mollis* (%d^{-1}[dw] ± SE) and tissue composition for carbon (C), nitrogen (N), phosphorus (P), molar ratios, and protein as a function of nitrate load d^{-1} (as $NaNO_3$) under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}) after 49 days	37
1.4	Predicted optimal tissue [dw] N, P, N:P, C:N and trace metal concentrations (μg g^{-1}[dw]) for maximum growth of *Palmaria mollis* as a function of low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1})	46
2.1	Mean pH's (± SE) and SGR's (± SE) of *Palmaria mollis* cultured under different seawater exchange rates over a 28-day period	74
2.2	Mean pH's (± SE) during the light phase, and SGR's of *Palmaria mollis* (± SE) cultures receiving either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 to control pH at constant seawater exchange (7.5 d^{-1})	77

LIST OF TABLES (Continued)

4.1 Summary of the seven average daily-equivalent nutrient loads applied to each bioassay treatment with *H. discus hannai* 132

4.2 Survival, linear growth rate (μm SL d^{-1} ± SE), specific growth rate (%[dw] d^{-1} ± SE), of *Haliotis discus hannai* (SL ≈ 24mm to 34mm) after 61 days of exposure to various nutrient regimens differing in nitrate load and the addition of 0.75 *f* + Zn (d^{-1}) trace metals 140

4.3 Dry weight protein (± SE), carbohydrate (± SE), and total lipid (± SE) of *Palmaria mollis* as a function of nitrate load d^{-1} under a moderately high light (PFD = 51.64 mol photons m^{-2} d^{-1}) 154

APPENDICES

Appendix		Page
1	Composition of the *f* medium (Guillard and Ryther, 1962) used as the basis for nutrient additions in *Palmaria mollis* culture	185
2	Summary of two ANOVA tests for specific growth rate of *Palmaria mollis* as a function of nitrate additions (as $NaNO_3$) under artificial low light (PFD = 23.68 mol photons m^{-2} d^{-1}) and high light (PFD = 51.64 mol photons m^{-2} d^{-1})	186
3	ANOVA test for specific growth rate of *Palmaria mollis* as a function of either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 sparaging to control pH at constant seawater exchange (7.5 d^{-1})	187
4	ANOVA tests for specific growth rate of *Palmaria mollis* as a function of temperature and light	188
5	ANCOVA test for specific growth rate (%[dw] d^{-1} ± SE) of *Haliotis discus hannai* (SL ≈ 24mm to 34mm) after 61 days of exposure to various nutrient regimens differing in nitrate load and the addition of 0.75 *f* + Zn (d^{-1}) trace metals	189

DEDICATION

This work is dedicated to the marine naturalist, Dr. Lee F. Braithwaite, who first showed me how strange and wonderful the marine environment could be and then taught me how to reveal its mysteries through disciplined use of the scientific method. For this and many other things, I will always be indebted to him.

INTRODUCTION

The major limiting factor in expansion of the abalone industry continues to be the availability of suitable and inexpensive algal foods. Traditional commercial abalone culture systems in the U.S. are based on supplying abalone with harvested kelp. Unfortunately, the nutritional quality of kelp varies with season and kelp can degrade after a few days in abalone culture systems, leading to deterioration in water quality and accumulation of ammonia unless high water exchange rates are maintained. The result is that farm-reared abalone have not been produced in sufficient quantity and quality to fill the void in world market demand.

Our goal at the Hatfield Marine Science Center (HMSC) has been to produce a high yield, commercial method for the co-culture of abalone and *Palmaria mollis* (Pacific dulse) using low flow-through or recirculated seawater. In this way we hoped to help create a more profitable abalone culture industry over a larger geographic range, including areas with good water quality remote from natural kelp beds. The research has shown that understanding and manipulating both the biology of *P. mollis* and the culture environment can solve most of the economic problems associated with scaling-up to mass culture.

Presently, one of the most important problems associated with establishing land-based marine aquaculture farms revolves around mitigating the effects of both disease introductions and nutrient-laden effluent on the surrounding environment. For example, the California

abalone industry has recently been severely impacted by "withering foot syndrome", a disease caused by a rickettsia bacteria. The bacteria are present in coastal waters of Southern California and abalone cultured in open systems are liable to be infected. Closed, recirculation systems would avoid potential infections of cultured abalone, ensuring "high health" abalone seed for stock restoration and grow-out to market size. Furthermore, low flow-through or recirculation systems reduce impacts of effluents on the marine environment. Finally, reducing water exchanges also lowers pumping costs that currently represent up to 30% of the operational costs for land-based aquaculture farms (Huguenin, 1976).

While phytoplankton- and bacteria-dominated recirculating marine systems have been developed, problems associated with maintaining such systems have led researchers to look at alternatives such as macroalgae biofilters (Shpigel and Neori, 1996). The interest in development of living feeds capable of both sustained vegetative growth and acting as in situ biofilters for land-based tank systems has produced studies that have demonstrated the key biological and economic role of seaweeds in land-based systems throughout the world. Since commercially produced finfish and shellfish typically assimilate only 20-35% of the nitrogen (as protein) in feed formulations, with the rest being flushed away mainly as dissolved ammonia, farmers have a substantial economic and environmental incentive to maximize feed conversion of protein into valuable biomass rather than pollution.

Macroalgae biofilters have several significant advantages over bacterial biofilters. Firstly, macroalgae

remove all the nitrogenous excretory products from cultured organisms, while nitrifying bacteria filters do not remove nitrate. Secondly, macroalgae are more easily maintained and, therefore, are more reliable as biofilters and not subject to unpredictable "crashes" due to changes in microbial communities or treatment of the target species for disease. Lastly, excess macroalgae from the biofilter can be used as food for cultured organisms, such as abalone.

Ryther et al. (1975) were among the first to show how biological treatment of nutrient-rich effluents could efficiently and inexpensively be used to culture phytoplankton, bivalves, and seaweeds in a polyculture system. Recent advances in macroalgae culture have set the stage for the aquaculture industry in the U.S. to make the transition to marine recirculating polyculture systems with macroalgae maintaining water quality and providing food for cultured marine grazers.

At the HMSC we have studied the use of *Palmaria mollis* (Pacific dulse) to reduce nitrogenous wastes from the effluents of flow-through, intensive, land-based salmon and abalone cultures. We have found that the rate of ammonia uptake by Pacific dulse is high and sufficient to offset waste loads from high commercial stocking densities of abalone (Evans and Langdon, 2000). Furthermore, preliminary experiments at HMSC have shown that abalone growth rates as high as 3.8 mm/mo (increase in shell length) and mortality rates less than 3% can be achieved over a six month period in a semi-recirculating system with 15% renewal of seawater d^{-1} (1 vol wk^{-1}) with *P. mollis* used as both as a food and biofilter.

The present study shows that an understanding of specific nutrient requirements for *P. mollis* can result in a significant improvement in both growth rates of *P. mollis* and of abalone, such as *Haliotis discus hannai* and *H. rufescens* for, which it provides the primary feed.

ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: NITROGEN, PHOSPHORUS, AND TRACE METAL NUTRITION

ABSTRACT

Pacific dulse (*Palmaria mollis*) is a valuable algal feed for both red abalone (*Haliotis rufescens*) and Japanese abalone (*Haliotis discus hannai*). Land-based, tumble-culture techniques capable of producing commercial quantities of *P. mollis* are still in development. An understanding of nutrient requirements and management strategies for *P. mollis* production has resulted in significant increases in yields. This work reports on experiments that further elucidated these areas of research.

All experiments were conducted under moderate to high artificial light of 24 to 52 mol photons m^{-2} d^{-1} and low seawater exchange (1 vol d^{-1}), in order to minimize seawater costs. Application of nutrients every 5 to 7 days resulted in no significant difference in *P. mollis* growth compared with daily applications. Nutrient additions during the dark cycle compared with the light cycle were found to be effective in controlling epiphytes. The addition of *f* medium trace metals (Guillard and Ryther, 1962) significantly increased growth rates compared with that of cultures supplied with just nitrate and phosphate alone. The concentration of Zn was increased to match that of Mn (1.37 μM d^{-1}), with resulting improvement in yields. $NaNO_3$ as a source of nitrogen was found to be superior for long-term growth (9 weeks) compared with NH_4NO_3, although NH_4NO_3

was superior on a short-term basis (first 2 to 5 weeks). While *P. mollis* growth was not significantly different between additions of 1176 to 2942 μM d^{-1} NO_3^- - N (as $NaNO_3$), an increasing trend in growth was observed with increasing nitrate concentration up to 2942 μM d^{-1} NO_3^- - N (as $NaNO_3$). *Palmaria mollis* was most sensitive to phosphorus, which produced a sharp decline in growth above concentrations of 83.3 μM d^{-1} (as NaH_2PO_4), in spite of 'luxury consumption' of phosphorus. Tissue nutrient concentrations and ratios (N, P, trace metals, N:P and C:N) in the context of improved growth rates and nutrient management are discussed.

INTRODUCTION

Over the past two decades advances in mass culture of seaweeds (Hanisak and Ryther, 1984; Bidwell et al., 1985; Neori et al., 1996) have set the stage for the aquaculture industry to make the transition from open to closed or semi-closed polyculture systems with macroalgae acting as the primary biofilter. Interest in development of these living feeds, capable of both sustained vegetative growth and functioning as in situ biofilters, has resulted in a number of valuable studies. Several good examples that clearly demonstrate the key role of seaweeds in commercial, land-based marine polyculture systems were conducted at the National Center for Mariculture in Elat, Israel, (Cohen and Neori, 1991; Shpigel and Neori, 1996; Neori et al., 1996; Neori et al., 2000).

Palmaria mollis (= *Rhodymenia palmata* var. *mollis*, Setchell and Gardner 1903) (Rhodophyceae, Bangiales) is found in the low intertidal and subtidal rocky shore from Southeastern Alaska to San Luis Obispo County, California. At the Hatfield Marine Science Center (HMSC), Oregon State University, research has shown the effectiveness of *P. mollis* or Pacific dulse in removing NH_4^+, NO_3^-, and PO_4^- from aquaculture effluents generated by land-based salmon and abalone culture (Levin, 1991; Evans and Langdon, 2000).

The purpose of this study was to assess the ability of cultured *P. mollis* to not only act as a biofilter but also to provide high yields. In order to accomplish this, a nutrient management strategy of feed for abalone was needed for enhancing yields of *P. mollis* over a range of naturally occurring photon flux densities (PFD's) under low seawater

exchange conditions. The proper application rate and concentration of specific nutrients needed to be determined for mass culture so that adequate nutrition was provied without the adverse affects of over supply leading to either depressed growth or the proliferation of epiphytes and weed species (Schramm, 1991). Some important culture data was already known. Pulse fertilizing had been shown to produce good growth rates and be effective in controlling epiphytes in other cultured Rhodophyte species (Neish and Fox, 1971, Lapointe, 1985; Pickering et al., 1993). Macrophytes were known to require a greater range of inorganic nutrients than higher plants (Dring, 1982), which can quickly become limiting in land-based, low exchange culture systems. Indeed, it was known that as many as 25 elements might be required for proper seaweed nutrition (Harrison and Druehl, 1982). Nutrients that have been considered essential supplements under low flow conditions likely include: N, P, Fe, Zn, Mn, and Cu (Sunda, *pers. comm.*).

Since pumping seawater represents one of the most expensive operating costs of land-based aquaculture systems (Huguenin, 1976) it was also important to construct a system that utilized a low seawater exchange rate. Previous experiments with *P. mollis* under low exchange rates achieved a specific growth rate (SGR) of 6.1% d^{-1} at uptake saturating levels of N and P (Evans and Langdon, 2000). This was in contrast to an SGR of 7.9% d^{-1}, when Guillard and Ryther's (1962) *f* medium was used (Demetropoulos, *unpublished data*). These results prompted the need for a more in depth study of the nutritional requirements of *P. mollis*.

MATERIALS AND METHODS

General methods

All experiments used healthy, whole *P. mollis* rosettes provided by HMSC (OSU, C-3 strain; Demetropoulos and Langdon, 2001). These had been previously cultured under an exchange rate of 6 vol d^{-1} and a moderate PFD of 30 mol photons m^{-2} d^{-1} under a 12:12 h photoperiod (Evans and Langdon, 2000). Triplicate 3.8 l opaque-sided vessels were placed in a seawater bath and aerated vigorously at 1.8 l min^{-1}. Vessels were stocked with *P. mollis* at a density of 0.5 kg m^{-2} (damp wt) or 2.6 g l^{-1} (damp wt) and supplied with sand-filtered seawater at a flow rate of 1 vol d^{-1}, maintained at 16±1° C and salinity of 31±1‰. A hexagonal "box" covered with reflective mylar was placed around the vessels to block out ambient light and evenly distribute artificial light. Either 1000 or 1500 W metal halide lamps were used to illuminate the treatments. These were adjusted to produce a PFD of 300 to 1400 mol photons m^{-2} s^{-1} (PAR) or 22.5 and 51.64 mol photons m^{-2} d^{-1} at the surface of the culture vessels. One advantage to these lamps was moderately high UV output (UV spectral power = 0.03 W nm^{-1} 1000 $lumens^{-1}$; Osram Sylvania *pers. comm.*) that better simulated outdoor conditions than other available lamps. Cultures were acclimated for one week prior to measurements.

Cultures were not maintained using sterile techniques in order to approach the "commercial reality" of air/water born contamination. All enrichments were added during the dark cycle so as to offset chlorophyte and epiphyte growth

(Hanisak, 1987; J. McLachlan, *pers comm.*). Typically the contents of the culture vessels were damp-weighed weekly using either the spin cycle of a washing machine or a salad spinner and thinned to their original stocking density at the beginning of each week. These methods of measuring growth were found to be highly repeatable (SD±0.02). Specific growth rate was calculated according to the equation: SGR = 100 $\ln(W_t/W_0)/t$ (DeBoer, et al., 1978), where SGR is expressed as %[dw]d^{-1}, W_f and W_i are the final and initial algal dry weights, and t is time (days), The equation assumes-steady state exponential growth (DeBoer et al., 1978) based on final dry weights.

At the end of each experiment, whole rosettes were dipped in 0.5 M ammonium formate to remove external salts, rinsed with deionied water to remove ammonium, and either freeze-dried at −80° C or oven dried (60° C, 48 hr) and kept at −80° C for future analysis.

Nutrient application rate

This experiment determined how *P. mollis* growth was affected by nutrient addition rate under constant nutrient load. It was designed as a 6 X 2 factorial experiment, with six application rates (1xd^{-1}, 1x3 d^{-1}, 1x5 d^{-1}, 1x7 d^{-1}, 1x9 d^{-1}, and 1x11 d^{-1}) at two light levels (22.5 or 42.34 mol photons m^{-2} d^{-1}). Since load = [conc. of added nutrients in culture] X culture volume/frequency of addition (Lobban and Harrison, 1997), the nutrient load for each treatment was held constant by varying concentrations over the range of different application rate treatments.

Thus, treatment 1x7 d^{-1} received 7 times the concentration on day 7 vs. treatment 1xd^{-1}, which received nutrients every day. $NaNO_3$, NaH_2PO_4, and trace metals were supplied at 1*f* molar concentrations d^{-1} (Guillard and Ryther, 1962).

Sand-filtered seawater for the six enrichments was pretreated by passage through a *P. mollis* nutrient-filter consisting of a 120 l tank with 1000 g of *P. mollis* in tumble-culture followed by filtration through 120 μm and 30 μm cartridge filters. Illumination was provided by 1000 W metal halide lamps on a 16:8 h photoperiod. Window screen was placed over experimental treatments to obtain the lowest light level. The experiment ran for 28 days.

Trace metal additions

Two experiments were conducted to determine if modifications to the *f* medium trace metal solution resulted in increased growth of *P. mollis*. Previous work had shown that addition of the *f* medium trace metals could produce better growth in *P. mollis* than additions of N and P alone (Demetropoulos, *unpublished data*). Both experiments had the same design. $NaNO_3$ and NaH_2PO_4 were supplied at the equivalent of 1*f* molar concentrations d^{-1} (Guillard and Ryther, 1962). N and P were added to the culture vessels every 3 days with the total nutrient load (3*f*) being equal to what the vessels would receive if they were fertilized daily (cf., Nutrient application rate exp.).

1) Zinc additions: Zinc was the only individual trace metal in the *f* medium that was evaluated separately from other trace metals in this study for several reasons.

Literature values indicated that typical molar concentrations of Zn in wild *P. palmata* could match or exceed those of Mn (Morgan et al., 1980a; Sirota and Uthe, 1979). Further, Munda (1978) found Zn exceeded that of Mn by 50% in the tissues of *P. palmata* and Sunda (*pers. comm.*) suggested that the concentration of Zn may need to be as high as Mn in *Palmaria spp.*. Finally, cultures of *P. mollis* under high natural light, removed Zn (as supplied by the 1*f* nutrient medium) to below detectable concentrations more quickly than other trace nutrients (Demetropoulos, *unpublished data*).

Zinc (as $ZnSO_4$) was added to 1*f* medium at a concentration equal to that of Mn (1.82 μM d^{-1}). The chelator, ethylenedinitrilo tetra-acetic acid (EDTA), already present in the *f* medium, was assumed to be sufficient for chelating additional $ZnSO_4$ (Sunda, *pers. comm.*). The standard 1*f* nutrient medium (without extra Zn) was used as a control. NaH_2PO_4 was used as the source of P (average concentration of 83.3 μM d^{-1} provided as 10 mg l^{-1} d^{-1} NaH_2PO_4) and $NaNO_3$ was used as the source of N (mean conc. of 1.765 μM d^{-1} provided as 150 mg l^{-1} d^{-1} $NaNO_3$). Nutrients were added every 3 days as described in the nutrient application rate experiment. Illumination was provided by 1000 W metal halide lamps on a 16:8 h photoperiod producing a PFD of 22.5 or 42.34 mol photons m^{-2} d^{-1} for the low and high light levels respectively. SGR's were measured after 35 days.

2) Concentration of Zn-modified *f* medium trace metals: This experiment was conducted to determine the best concentration of Zn modified *f* trace metals to apply to cultures. Based on the results of the Zn experiment (see

results), Zn was added in a concentration equal to that of Mn (1.37 μM d^{-1}). In combination with 1*f* (N and P), six modified *f* medium trace metal concentrations were tested: N&P only (control), 0.25, 0.5, 0.75, 1, and 1.25*f*. The control was 1*f* (N and P) alone. Nutrients were added every 3 days (cf., Nutrient application rate exp.). The experiment ran for 42 days with SGR's being measured and compared in the last 14 days. Space constraints would not allow the experiment to be run at high and low light intensities so an intermediate PFD of 37.44 mol photons m^{-2} d^{-1} (@650 μmol photons m^{-2} s^{-1}) was provided through use of 1000 W metal halide lamps on a 16:8 h photoperiod.

Ammonium nitrate vs. sodium nitrate

These two experiments were conducted to test the effects of NO_3^- vs. NH_4^+ on yields of *P. mollis*. Illumination was provided by 1000 W metal halide lamps, supplying approximately a PFD of 37.44 mol photons m^{-2} d^{-1} on a 16:8 h photoperiod.

1) Pulse fertilization with ammonium: Two nitrogen treatments, each providing 2353 μM – N d^{-1} as either $NaNO_3$ or NH_4NO_3 were evaluated. NaH_2PO_4 was used to provide 83.3 μM – P d^{-1}. Trace metals were added as 1*f* Zn-modified trace metal mix. Nutrients were added every 3 days. The experiment ran for 28 days.

2) Continuous fertilization with ammonium: Since pulse fertilizing resulted in cultures receiving a relatively high NH_4^+ concentration (7059 μM – N every 3 days), the effects of daily or continuous additions of NH_4^+ (supplied

as NH_4NO_3) at a lower concentration with the same N load were determined. Three different ratios of NH_4^+:NO_3^- were tested, each ratio providing 2353 μM – N d^{-1}: A) NH_4^+:NO_3^- [0:1 molar]; B) NH_4^+:NO_3^- [1:1 molar]; C) NH_4^+:NO_3^- [2:1 molar]. $NaNO_3$ and $(NH_4)_2SO_4$ were used as sources of N. NaH_2PO_4 was used to provide 83.3 μM – P d^{-1} and 1*f* provided trace metals. In contrast to the above experiment, all enrichments were pumped into the culture vessels once a day over a period of 12 hours via peristaltic pumps. The experiment ran for 56 days.

Phosphorus and nitrogen additions

In the following two experiments, SGR's of *P. mollis* were assessed as a function ambient N and P concentrations at two light levels. First N was held at a fixed concentration and P was varied. Next, P was held at a fixed concentration and N was varied. Illumination was provided by 1500 W metal halide lamps on a 16:8 h photoperiod producing a PFD of either 23.68 or 51.64 mol photons m^{-2} d^{-1} for the low and high light levels. N, P and trace metals were added to the cultures every 3 days (cf. nutrient application rate exp.). Modified *f* medium (0.75*f* + Zn) provided trace metals (cf., Trace metal additions experiment).

1) Phosphate additions: This experiment was designed as a 7 X 2 factorial, eight PO_4^- concentrations (seawater control, 5, 10, 15, 20, 30, 45, and 60 mg l^{-1} d^{-1} as NaH_2PO_4) at two light levels (23.68 or 51.64 mol photons m^{-2} d^{-1}). $NaNO_3$ was used as the source of N (mean conc. of 2942 μM d^{-1}

provided as 250 mg l^{-1} d^{-1} $NaNO_3$). Inorganic N and P concentrations provided by seawater at a single exchange d^{-1} ranged between 1.52 – 5.34 μM - N and 0.36 – 0.70 μM - P. SGR's were measured after 35 days.

2) Nitrate additions: This experiment was designed as a 7 X 2 factorial, seven NO_3^- concentrations (seawater control, 50, 100, 150, 200, 250, and 300 mg l^{-1} d^{-1}) at two light levels (23.68 or 51.64 mol photons m^{-2} d^{-1}). These NO_3^- values were chosen because they represented an upper range considered potentially applicable to commercial production (Evans and Langdon, 2000; Waaland *pers. comm.*). NaH_2PO_4 was used as the source of P (mean conc. of 83.3 μM d^{-1} provided as 10 mg l^{-1} d^{-1} NaH_2PO_4). Inorganic N and P concentrations provided by seawater at a single exchange d^{-1} ranged between 1.52 to 5.34 μM - N and 0.36 to 0.70 μM - P. SGR's were measured after 49 days.

Biochemical analysis

All tissue nutrient work was performed by Fruit Growers Lab, Santa Paula, CA. Frozen samples were ground to a fine powder. Tissue C and N were measured using a LECO, CNS 2000. Tissue P and trace metals were determined by the dry ash method (thermal Jarrall Ash) followed by measurement with an inductively coupled argon-plasma spectrophotometer. Nitrate was determined spectrophotometrically by the Griess-Ilasvay (cadmium reduction) method.

Crude protein was determined by multiplying tissue N by 6.25. Since high NO_3^- additions and subsequent storage in intracellular NO_3^- pools were likely to interfere with this

determination, plants were starved for 3 days prior to analysis. This reduced intracellular NO_3^- by approximately 96% in *P. mollis* (Demetropoulos, *unpublished data*; cf., Lobban and Harrison, 1997).

Statistics

Unless otherwise stated, SGR's (% d^{-1} [dw]) under the various nutrient regimes were measured during the last week of culture. The effects of nutrient application, trace metals, ammonium, and N:P enrichment on SGR and biochemical assay were assessed by two factor ANOVA and differences among individual treatments were compared by the Student-Newman-Keuls (SNK) multiple comparison test ($\alpha = 0.05$). Simple linear regression was used to test for significant relationships among experimental variables ($\alpha = 0.05$).

RESULTS

General

Initially a light brown precipitate was noticed the day after nutrient applications in many of the treatments. This was thought to be due to a phosphate and trace metal interaction. Subsequently, (N & P) and (metals) were added separately and the precipitate was eliminated. Additionally, pH values for the faster growing treatments were approximately 8.6 to 8.75 during the last two or three days of each week and thus some DIC limitation was likely (Demetropoulos and Langdon, 2002b).

Nutrient application experiment

This experiment showed no difference in growth for cultures provided nutrients daily vs. those provided the same daily average nutrient load at intervals of up to 7 days. However, there was a significant decline ($P < 0.0001$) in growth when nutrient loads were provided at intervals of 9 and 11 days (Fig. 1.1). Mean SGR for nutrient applications of once d^{-1} (1x1) through once every 7 d^{-1} (1x7) under the low light level was equal to 9.04±0.27 and under the high light level was equal to 11.4±0.34 (Table 1.1). Daily addition of nutrients caused epiphytes (primarily *Ectocarpus siliculosis*, and *Enteromorpha intestinalis*) to become a substantial problem in the last 2 weeks of the trial, accounting for as much as 15% of dry

weight yields. In contrast, cultures with nutrient additions every 5 and 7 days were very healthy.

Protein content tended to decline with decreasing application rate (Table 1.1). Tissue NO_3^- - N was statistically not different from 0 for all treatments after 3 days without nutrient additions, indicating NO_3^- pools did not significantly affect tissue N content and subsequent protein measurements.

Trace metal experiments

Increasing the molar concentration of Zn added daily to cultures to equal that of Mn (1.37 μM) resulted in significant (ANOVA, SNK; $P = 0.0026$) increases in SGR's at the high light level but not at the low light level (ANOVA, SNK; $P = 0.449$) (Fig 1.2). No significant differences (ANOVA, SNK; $P = 0.416$) were found between SGR's of *P. mollis* fertilized with either 0.5*f*, 0.75*f*, 1*f*, and 1.25*f* which were all significantly higher ($P < 0.0001$) than growth of *P. mollis* with additions of either 0.25*f* or N and P alone (control) (Fig 1.3).

Ammonium nitrate vs. sodium nitrate as a nitrogen source: pulse fertilization with ammonia

During the first 14 days of the experiment, cultures supplied with 2353 μM – N d^{-1} as NH_4NO_3 had significantly higher SGR's than those supplied with $NaNO_3$ (Fig. 1.4). However, by the fourth week SGR's of NH_4NO_3-supplied

cultures had declined to approximately 2% d^{-1} while $NaNO_3$-supplied cultures maintained a consistent SGR of approximately 12% d^{-1} (Fig. 1.4). Evidence for toxicity of NH_4^+ could be seen in the pale coloration and deterioration of apical cells of the thalli.

Ammonium nitrate vs. sodium nitrate as a nitrogen source: continuous fertilization with ammonia

Like the first ammonium experiment, cultures supplied with 2353 μM – N d^{-1} as NH_4NO_3 initially had significantly higher SGR's than those supplied with $NaNO_3$ (Fig. 1.5). However, the high SGR's lasted for 42 days under the NH_4NO_3 (1:1 molar) treatment and 28 days under the NH_4NO_3 (2:1 molar) treatment after which time growth of cultures supplied with NH_4^+ declined while SGR's of $NaNO_3$-fertilized cultures remained relatively high at approximately 13.5 to 14.5 % d^{-1} (Fig 1.5).

Figure 1.1: Specific growth rate of *Palmaria mollis* (± SE) as a function of nutrient application rate (with equal nutrient loads among all treatments) under artificial low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or artificial high light (PFD = 42.34 mol photons m^{-2} d^{-1}. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at the 1*f* molar concentrations (Guillard and Ryther, 1962). Letters a, b, c, x, y and z indicate significant differences (ANOVA, SNK; $P < 0.0001$). The experiment ran for 35 days.

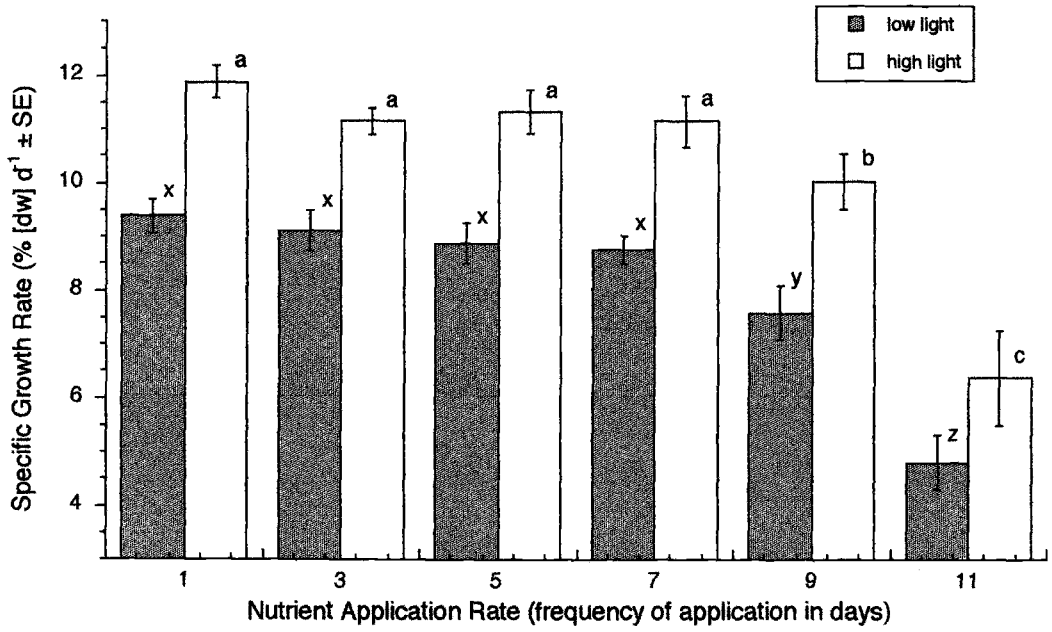


Table 1.1: Specific growth rates of *Palmaria mollis* (%d^{-1} [dw] ± SE), nitrogen (%dw ± SE) and protein contents (%dw ± SE), as a function of nutrient application rate under low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or high light (PFD = 42.34 mol photons m^{-2} d^{-1}) after 28 days.

Application rate (in days)	Light	SGR (%d^{-1}±SE)	N (%dw±SE)	Protein (%dw±SE)
1 (continuous)	low	9.38±0.32	5.83±0.52	36.4±3.3
3	low	9.12±0.39	5.38±0.43	33.6±2.7
5	low	8.89±0.38	4.99±0.45	31.2±2.8
7	low	8.77±0.27	4.42±0.53	27.6±3.3
9	low	7.59±0.51	4.39±0.44	27.4±2.8
11	low	4.81±0.51	4.62±0.61	28.9±3.8
1 (continuous)	high	11.88±0.30	4.69±0.33	29.3±2.1
3	high	11.17±0.25	4.52±0.50	28.3±3.1
5	high	11.36±0.40	3.44±0.65	21.5±4.1
7	high	11.17±0.48	3.81±0.46	23.8±2.9
9	high	10.10±0.52	3.40±0.71	21.3±4.4
11	high	6.39±0.88	3.80±0.42	23.8±2.6

Figure 1.2: Specific growth rate of *Palmaria mollis* (± SE) as a function of additional zinc (total Zn = 1.82 μM d^{-1}) under under artificial low light (PFD = 22.5 mol photons m^{-2} d^{-1}) or artificial high light (PFD = 42.34 mol photons m^{-2} d^{-1}. Cultures were supplied with $NaNO_3$ and NaH_2PO_4 at 1*f* molar concentrations (Guillard and Ryther, 1962). Letters a, b, and c indicate significant differences (ANOVA, SNK; *P* = 0.0026). The experiment ran for 35 days.

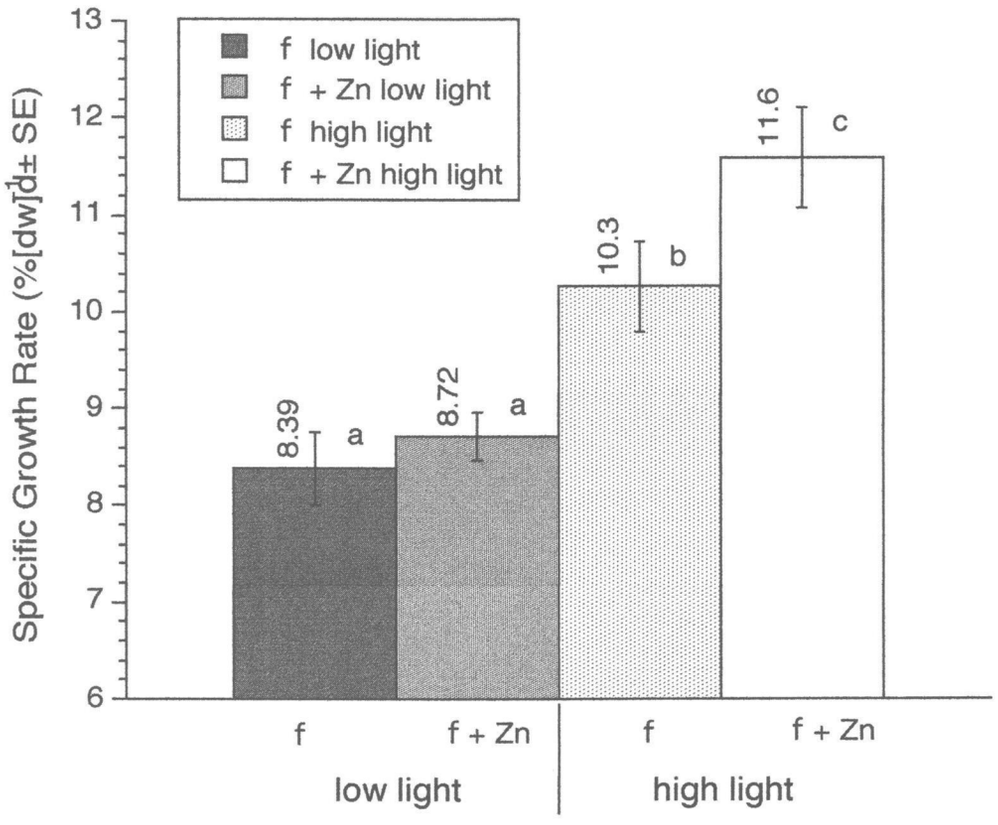


Figure 1.3: Specific growth rate of *Palmaria mollis* (± SE) as a function of different *f* metal concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}) under a moderate PFD of 37.44 mol photons m^{-2} d^{-1}. Cultures were supplied with $NaNO_3$ and NaH_2PO_4 at 1*f* molar concentrations (Guillard and Ryther, 1962). Letters a, b, and c indicate significant differences (ANOVA, SNK; $P < 0.0001$). The experiment ran for 42 days.

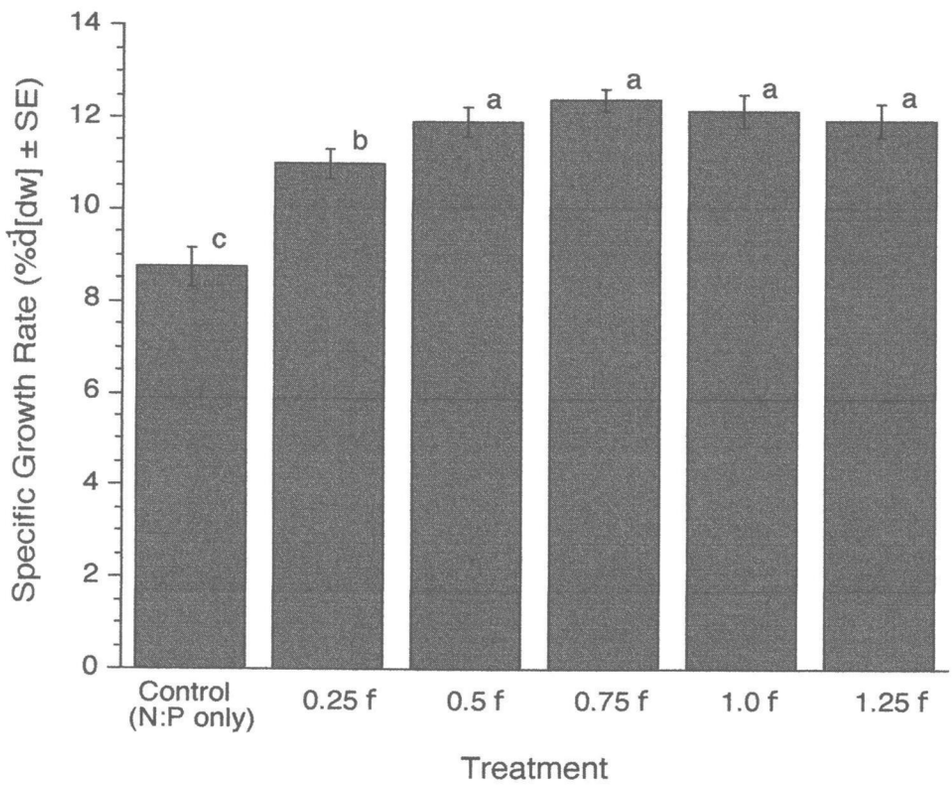


Figure 1.4: Specific growth rate of *Palmaria mollis* (± SE) as a function of pulse fertilization with either $NaNO_3$ (linear) or NH_4NO_3 (cubic spline) as the N source. The cubic spline curve represents a series of cubic polynomials connected together and has no single equation. Every three days, each treatment was supplied with 7059 μM – N as either $NaNO_3$ or NH_4NO_3. Cultures were supplied with NaH_2PO_4 and trace metals at 1*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}). A moderate PFD of 37.44 mol photons m^{-2} d^{-1} was employed. The experiment ran for 28 days.

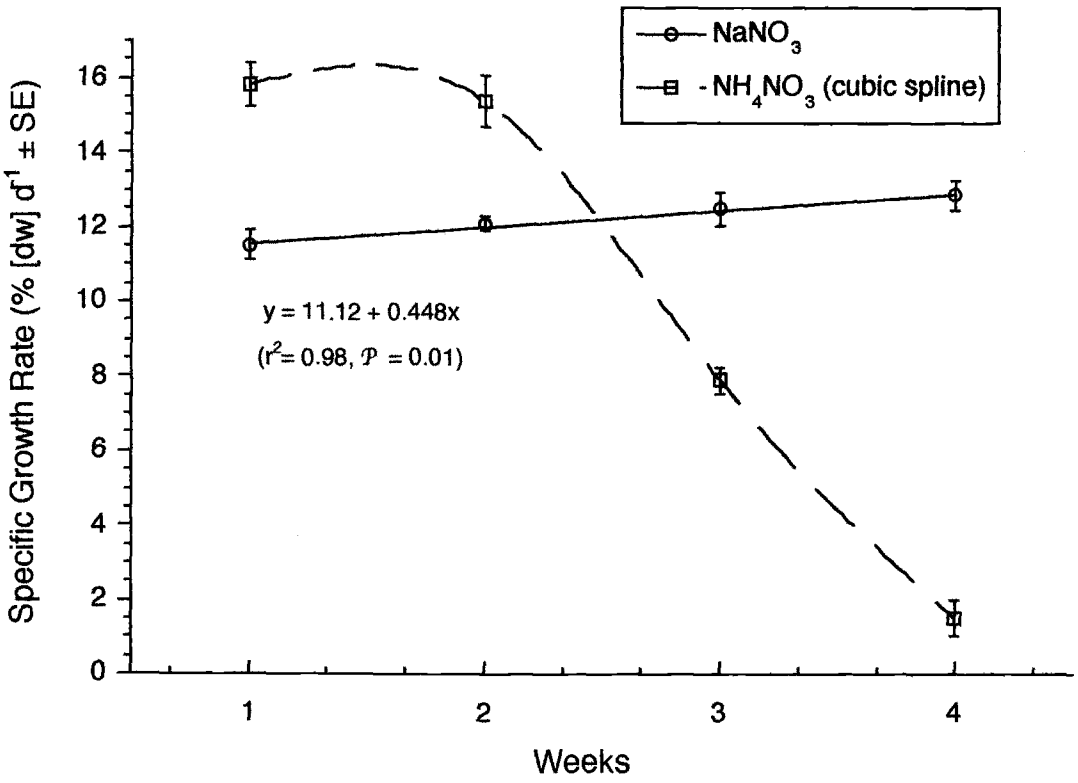
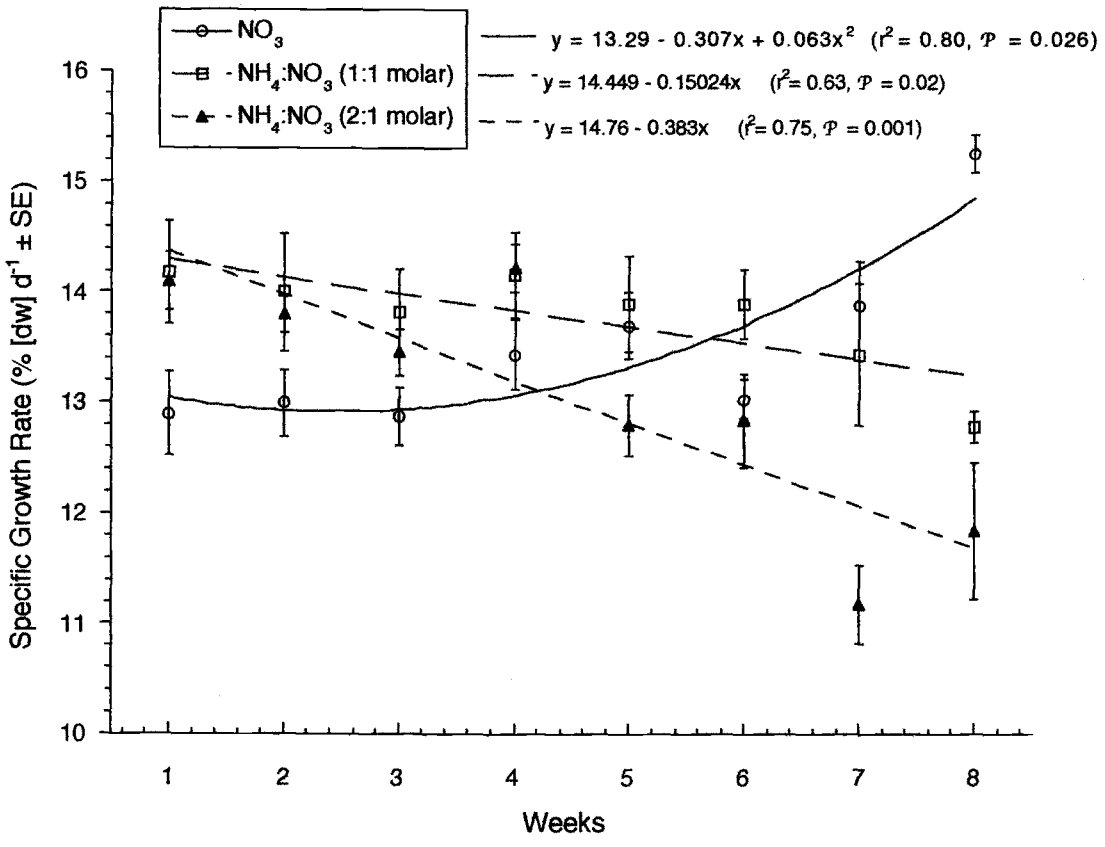


Figure 1.5. Specific growth rate of *Palmaria mollis* (± SE) as a function of continuous fertilization with either $NaNO_3$ or NH_4NO_3 as the N source. Cultures were fertilized daily with 2353 μM – N as either $NaNO_3$ or NH_4NO_3. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at 1*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}). A moderate photon flux density of 37.44 mol photons m^{-2} d^{-1} was employed. The experiment ran for 56 days.



Phosphate additions

The effects of phosphate addition on SGR's and tissue composition of *P. mollis* are shown in Table 1.2. Growth was hyperbolically related to the concentration of PO_4^{-3} additions. Background N and P (control) provided by 1 vol d^{-1} seawater exchange averaged 13.42±8.1 μM NO_3^- and 1.09±0.49 μM PO_4^{3-} d^{-1}, respectively, and supported an SGR of 2.4% d^{-1}, with the cultures degrading during the second week of the experiment. Under a low PFD (23.68 mol photons m^{-2} d^{-1}) a concentration of 42 μM – P d^{-1} (5 mg l^{-1} d^{-1} as NaH_2PO_4) provided the best growth. This was not significantly different (ANOVA; P = 0.32) from growth rates achieved with additions of 83 μM – P d^{-1} (10 mg l^{-1} d^{-1} as NaH_2PO_4). Under a high PFD (51.64 mol photons m^{-2} d^{-1}), a concentration of 83 μM – P d^{-1} (10 mg l^{-1} d^{-1} as NaH_2PO_4) provided the best growth, but was not significantly different (ANOVA; P = 0.096) from that achieved with 42 μM – P d^{-1} (5 mg l^{-1} d^{-1}). Phosphate concentrations higher than these not only depressed growth of *P. mollis* but also caused growth of macro-epiphytes, especially the chlorophytes, *Ulva* and *Enteromorpha*.

Under both light regimens tissue P concentrations of between 0.49 and 0.60 occurred in *P. mollis* cultures showing maximum growth rates but P tissue concentrations of 0.75% or greater were correlated with lower growth rates (Table 1.2). Tissue P values continued to rise in spite of declining growth, indicating 'luxury consumption' of P. Under both light levels there was a positive linear relationship between increasing ambient P and elevated tissue N (low light r^2 = 0.78; high light r^2 = 0.99).

Table 1.2: Specific growth rates of *Palmaria mollis* (%d^{-1} [dw] ± SE), tissue phosphorus (%dw), and protein content as a function of phosphate load d^{-1} under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}) after 49 days. Concentrations are daily averages for nutrients added once every 3 days. $NaNO_3$ was used as the source of N (mean conc. of 2942 μM d^{-1} provided as 250 mg l^{-1} d^{-1} $NaNO_3$). Protein content was calculated from 6.25 X total nitrogen content, with no residual nitrate detected in tissue samples. SW = seawater. * = no growth after 14 days. ND = not determined.

NaH_2PO_4 (mg l^{-1} d^{-1})	PO_4^- (μM d^{-1})	Light	SGR (%d^{-1}±SE)	N (%dw)	P (%dw)	N:P (molar)	Protein (%dw)
0.1 (SW)*	1.1	low	2.4±0.69	ND	ND	ND	ND
5	42	low	13.4±0.12	4.64	0.52	8.92	29.0
10	83	low	12.7±0.30	4.49	0.58	7.74	28.1
15	125	low	11.2±0.68	4.70	0.75	6.27	29.4
20	167	low	9.7±0.17	4.84	1.04	4.65	30.3
30	250	low	5.7±0.18	4.98	1.20	4.15	31.1
45	375	low	3.8±0.15	ND	ND	ND	ND
60	500	low	2.8±0.12	ND	ND	ND	ND
0.1 (SW)*	1.1	high	1.5±0.57	ND	ND	ND	ND
5	42	high	16.2±0.20	4.00	0.49	8.16	25.0
10	83	high	17.3±0.26	4.16	0.60	6.93	26.0
15	125	high	13.5±0.83	4.30	0.78	5.51	26.9
20	167	high	12.5±0.76	4.42	0.97	4.56	27.6
30	250	high	9.6±0.17	4.62	1.16	3.98	28.9
45	375	high	7.0±0.17	ND	ND	ND	ND
60	500	high	4.7±0.07	ND	ND	ND	ND

Nitrate additions

Background N and P concentrations (control) of seawater exchange at 1 vol d^{-1} averaged 15.5±6.37 μM NO_3^- and 1.63±0.36 μM PO_4^- d^{-1}, respectively, supporting an SGR of 2.28% d^{-1} under the low light factor (23.68 mol photons m^{-2} d^{-1}) and 2.07% d^{-1} under the high light factor (51.64 mol photons m^{-2} d^{-1}). The SGR's of *P. mollis* significantly increased with additions of NO_3^- up to a concentration of 2942 μM - N d^{-1} provided as 250 mg l^{-1} d^{-1} $NaNO_3$ (Fig. 1.6 and Appendix 2). Under low light SNK analysis showed a significant difference in SGR's between NO_3^- additions of 588 to 2942 μM – N d^{-1} (ANOVA, SNK; $P < 0.0001$). Under high light there was a significant difference in SGR's between NO_3^- additions of 1176 and 2942 μM – N d^{-1} (ANOVA, SNK; $P < 0.0001$). Concentrations higher than 2942 μM – N d^{-1} did not cause growth to decrease significantly, and caused no more epiphytism than lower levels of N. Slightly reduced water temperatures may have caused SGR's in this experiment to be lower than those observed in the phosphate experiment.

Under both low and high light, external NO_3^- had a significant positive linear affect on tissue concentrations of Fe, Zn, and Cu, but not Mn (Figs. 1.7, 1.8, 1.9, and 1.10). For the treatments, the average dry tissue concentrations ranged from 296 to 1220 μg Fe g^{-1}, 18 to 49 μg Zn g^{-1}, 6 to 11 μg Mn g^{-1}, and 12 to 15 μg Cu g^{-1}. Concentrations of metals in controls (no nutrients added) were typically higher than those of the other treatments and ranged from 1820 to 2400 μg Fe g^{-1}, 37 to 44 μg Zn g^{-1}, 45 to 53 μg Mn g^{-1}, and 19 to 23 μg Cu g^{-1}.

Figure 1.6: Specific growth rate of *Palmaria mollis* (± SE) as a function of nitrate additions (as $NaNO_3$) under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) and high light (PFD = 51.64 mol photons m^{-2} d^{-1}). Nutrient additions were made every 3 days. Cultures were supplied with NaH_2PO_4 and trace metals at the 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}). The experiment ran for 49 days.

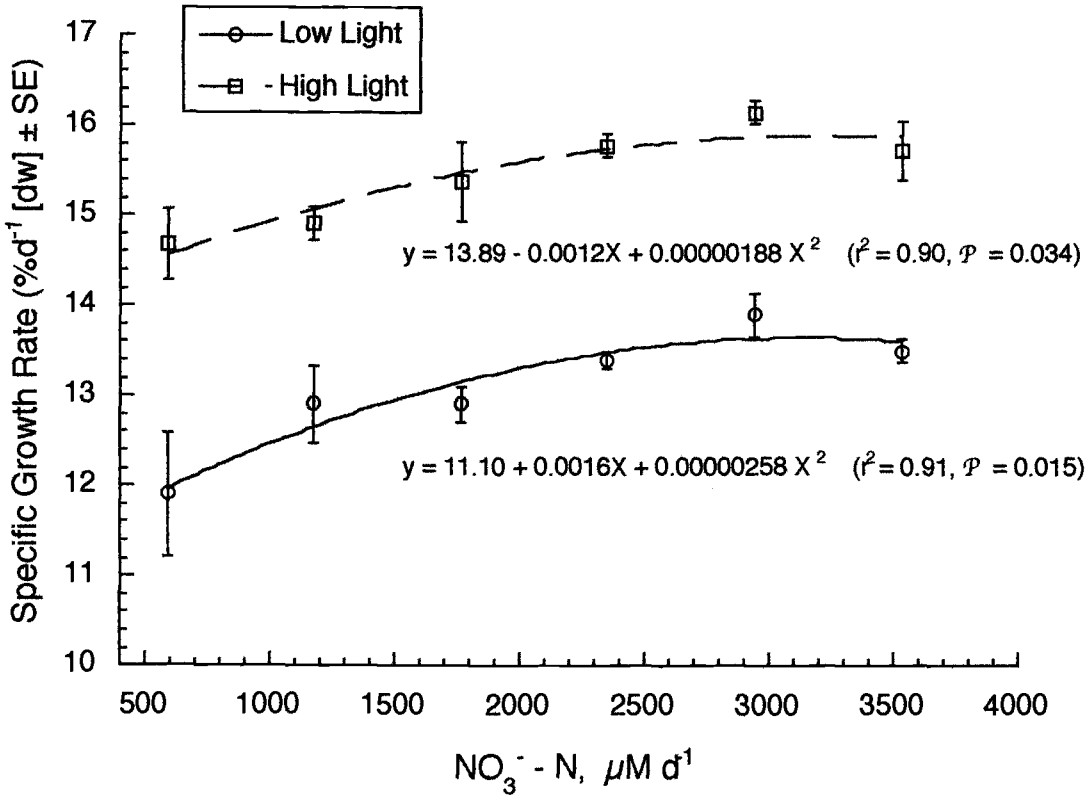


Figure 1.7: Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue iron concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis*. Regression lines represent best fit for photon flux densities of 23.68 (low light) or 51.64 (high light) mol photons $m^{-2}\ d^{-1}$. Nutrient additions were made every 3 days. Cultures were supplied with NaH_2PO_4 and trace metals at 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 $\mu M\ d^{-1}$).

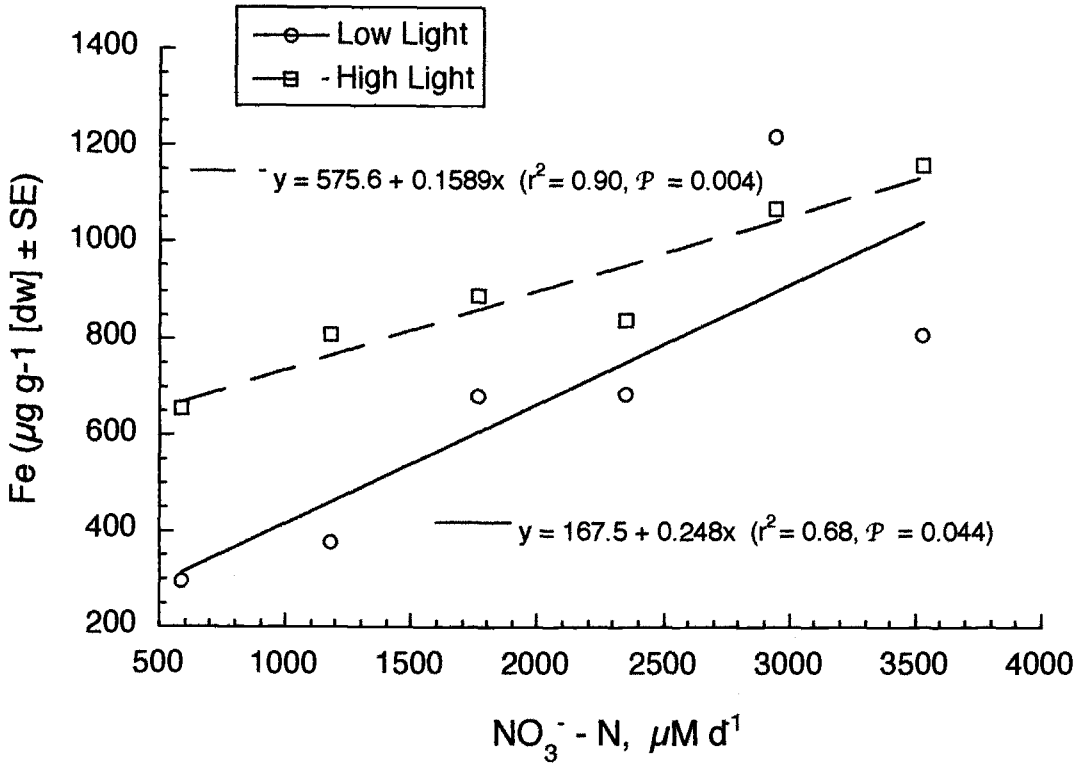


Figure 1.8: Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue zinc concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis*. Regression lines represent best fit for photon flux densities of 23.68 (low light) or 51.64 (high light) mol photons $m^{-2}\ d^{-1}$. Nutrient additions were made every 3 days. Cultures were supplied with NaH_2PO_4 and trace metals at 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 $\mu M\ d^{-1}$).

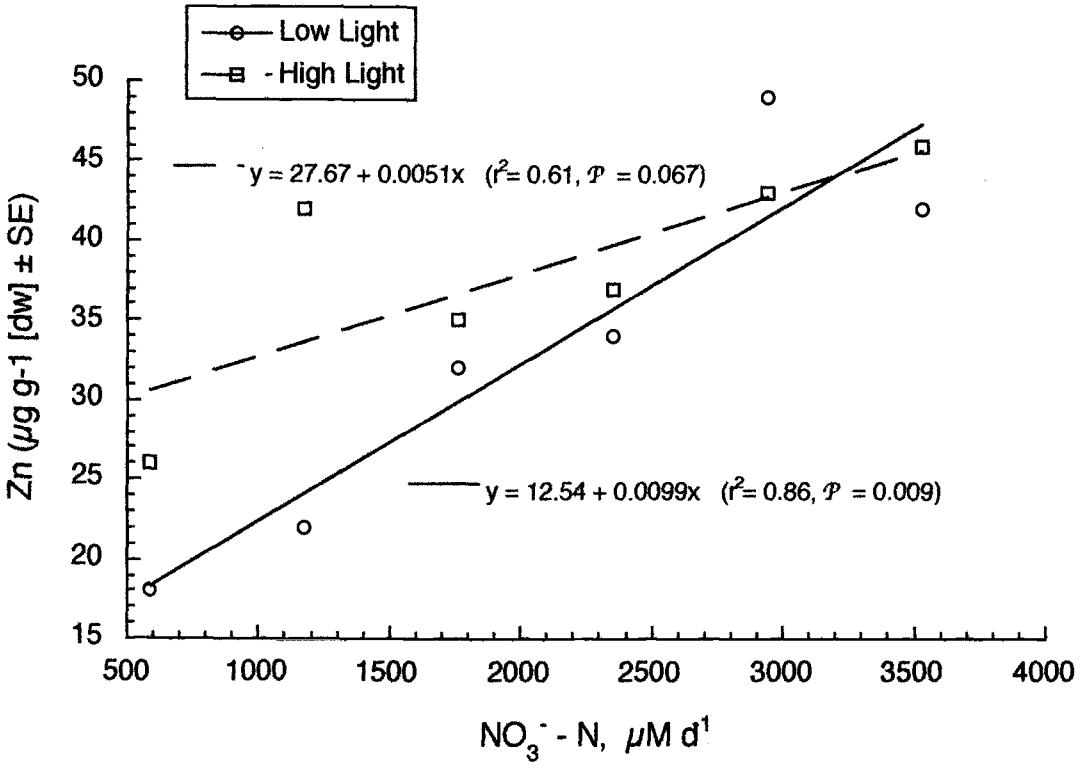


Figure 1.9: Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue manganese concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis*. Regression lines represent best fit for photon flux densities of 23.68 (low light) or 51.64 (high light) mol photons $m^{-2}\ d^{-1}$. Nutrient additions were made every 3 days. Cultures were supplied with NaH_2PO_4 and trace metals at 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 $\mu M\ d^{-1}$).

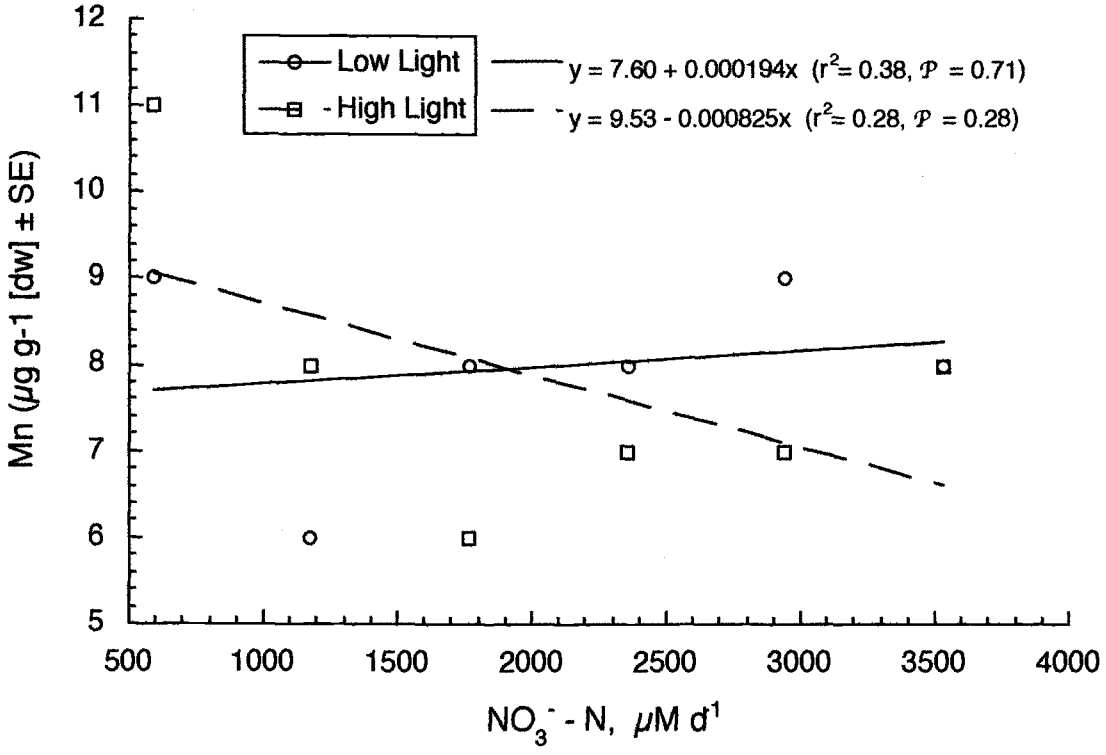
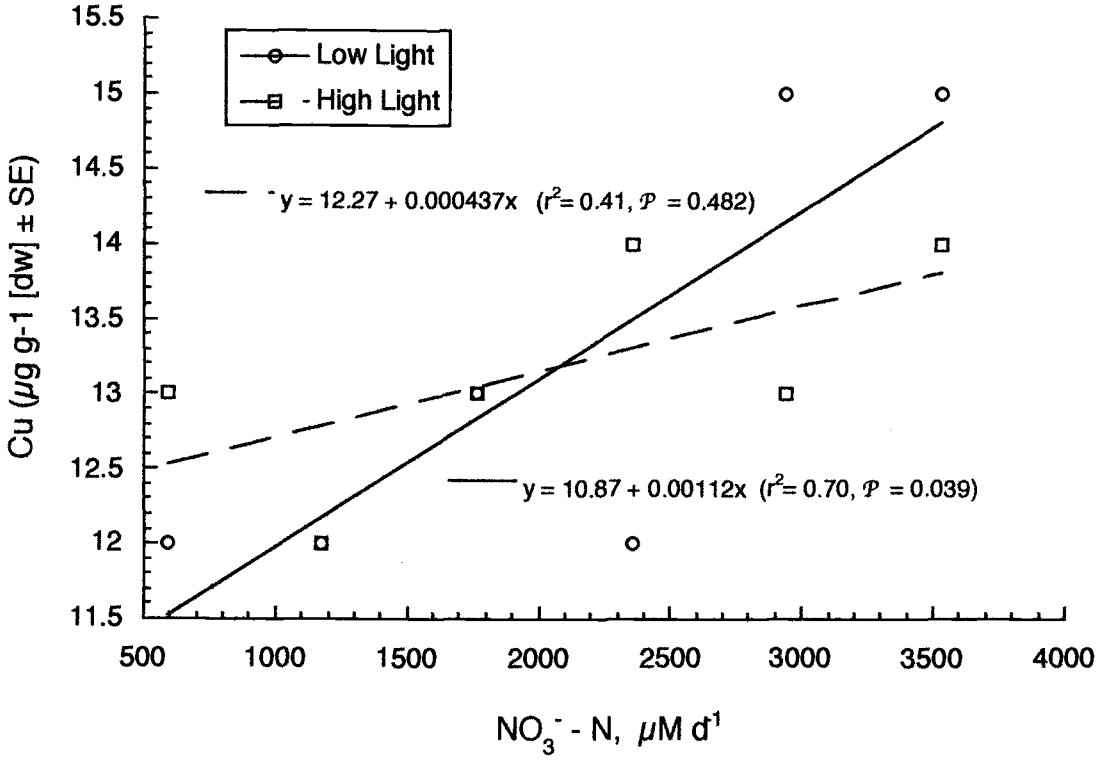


Figure 1.10: Relationship between external nitrate concentration (provided as $NaNO_3$) and tissue copper concentration ($\mu g\ g^{-1}$[dw] ± SE) for cultured *Palmaria mollis*. Regression lines represent best fit for photon flux densities of 23.68 (low light) or 51.64 (high light) mol photons $m^{-2}\ d^{-1}$. Nutrient additions were made every 3 days. Cultures were supplied with NaH_2PO_4 and trace metals at 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 $\mu M\ d^{-1}$).



The effects of NO_3^- concentration on growth, tissue C, N and P, and protein content under the different light and nutrient regimes are shown in Table 1.3. Tissue N for *P. mollis* from all seven treatments was nearly identical but tended to decline with increasing light. Tissue N values ranged from 4.35±0.52% dw to 5.27±0.61% dw with a mean of 4.72±0.25% dw for all treatments except the controls. Increasing NO_3^- had no effect on tissue P in this experiment (Table 1.3). *Palmaria mollis* grown under low light had a significantly (ANOVA, SNK; $P = 0.0017$) higher mean protein content (30.77±1.2) than under high light (28.03±1.03). The low-light cultures were also darker in color. There appeared to be no significant correlation between external N and protein content ($r^2 = 0.06$) within either light level.

Tissue C, N and P ratios

When tissue P levels for *P. mollis* from the phosphate and nitrate experiments were plotted together, the highest growth rates were observed when tissue P content was approximately 0.60% [dw] under low light conditions and approximately 0.65% [dw] under high light conditions (Fig. 1.11). By plotting data on tissue N:P ratios from both the nitrate and phosphate experiments, tissue N:P ratios correlated with high growth rates were approximately 8 for the low light and 7 for the high light conditions (Fig. 1.12).

There was no significant relationship ($P = 0.076$) between SGR and C:N for treatments (except controls) in the

nitrate experiment (Table 1.3); however, when these data were combined with the phosphate and nutrient application experiments, the relationship between SGR and tissue C:N was parabolic (r^2 = 0.75; P = 0.0047) (Fig. 1.13).

Table 1.3: Specific growth rates of *Palmaria mollis* (%d^{-1}[dw] ± SE) and tissue composition for carbon (C), nitrogen (N), phosphorus (P), molar ratios, and protein as a function of nitrate load d^{-1} (as $NaNO_3$) under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}) after 49 days. Nutrient concentrations are daily averages for additions every 3 days. NaH_2PO_4 was used as the source of P (mean conc. of 83.3 μM d^{-1} provided as 10 mg l^{-1} d^{-1} NaH_2PO_4) and the modified *f* medium (0.75*f* + Zn) provided the trace metals (cf. Trace metal additions experiment). SGR values represent means ± SE. Protein content was calculated from 6.25 X total nitrogen content, with no residual nitrate detected in tissue samples. SW = seawater. * = no growth after 28 days.

$NaNO_3$ (mg l^{-1} d^{-1})	NO_3^- – N (μM d^{-1})	Light	SGR (%d^{-1}±SE)	C (%dw)	N (%dw)	P (%dw)	C:N (molar)	C:P (molar)	N:P (molar)	Protein (%dw)
0 (SW)*	15.5	low	2.3±0.18	40.8	2.64	0.82	15.85	49.8	3.22	16.5
50	588	low	11.9±0.68	42.9	4.81	0.69	8.92	62.2	6.97	30.1
100	1176	low	12.9±0.43	42.6	4.92	0.64	8.66	66.6	7.69	30.8
150	1765	low	12.9±0.19	44.4	4.88	0.61	9.10	72.8	8.00	30.5
200	2353	low	13.4±0.10	43.5	5.27	0.63	8.25	69.1	8.37	33.1
250	2942	low	13.9±0.24	44.2	4.87	0.64	9.08	69.1	7.61	30.4
300	3530	low	13.5±0.12	43.0	4.75	0.61	9.05	70.1	7.79	29.7
0 (SW)*	15.5	high	2.1±0.15	40.6	2.97	0.84	13.7	48.3	3.54	18.6
50	588	high	14.6±0.25	44.2	4.57	0.75	9.67	58.9	6.09	28.6
100	1176	high	14.9±0.33	43.6	4.35	0.66	10.00	66.1	6.59	27.2
150	1765	high	15.4±0.44	41.7	4.54	0.69	9.83	60.1	6.58	26.5
200	2353	high	15.8±0.13	43.9	4.68	0.66	9.38	66.6	7.09	29.3
250	2942	high	16.2±0.12	44.3	4.47	0.68	9.91	65.2	6.57	28.0
300	3530	high	15.7±0.33	43.6	4.57	0.71	9.54	61.4	6.44	28.6

Figure 1.11: Tissue phosphorus concentration of *Palmaria mollis* as a function of specific growth rate under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}). Data were pooled from the phosphate and nitrate experiments. Nutrient additions were made every 3 days. Cultures were supplied trace metals at the 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}).

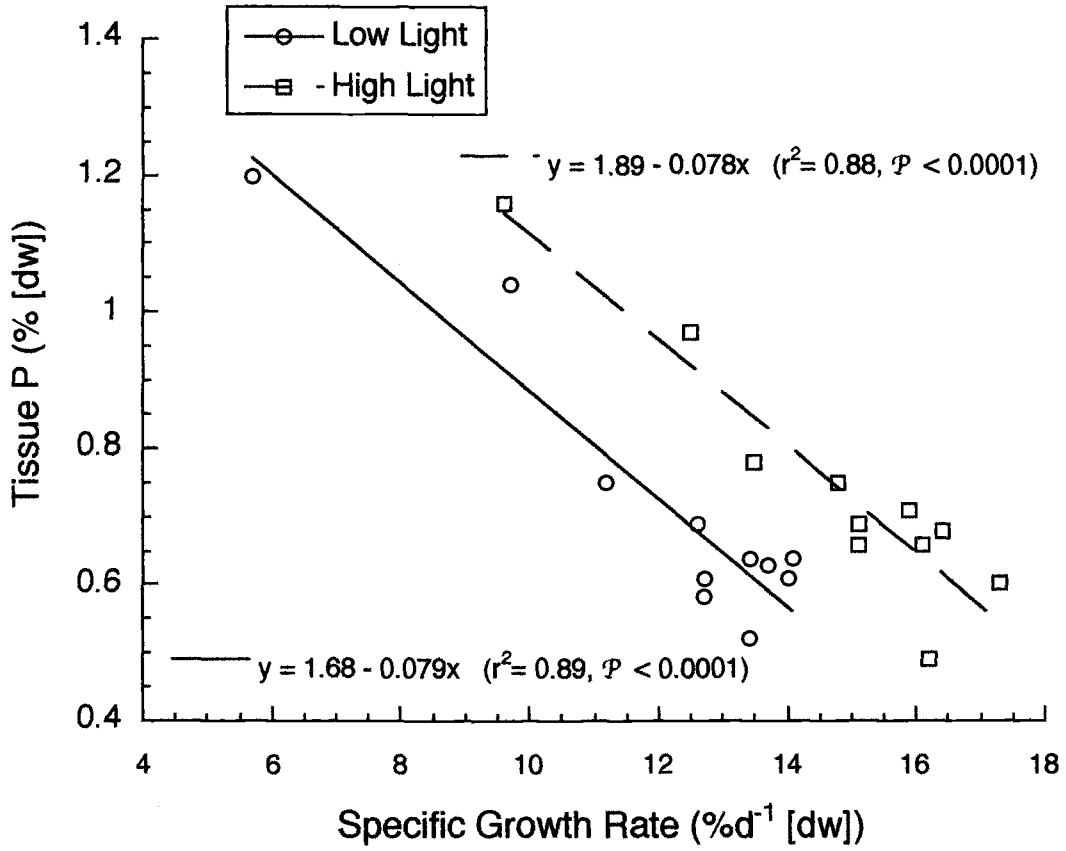


Figure 1.12: N:P ratio of *Palmaria mollis* as a function of specific growth rate under low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}). Data were pooled from the phosphate and nitrate experiments. Nutrient additions were made every 3 days. Cultures were supplied trace metals at the 0.75*f* molar concentrations (Guillard and Ryther, 1962) + Zn (total Zn = 1.82 μM d^{-1}).

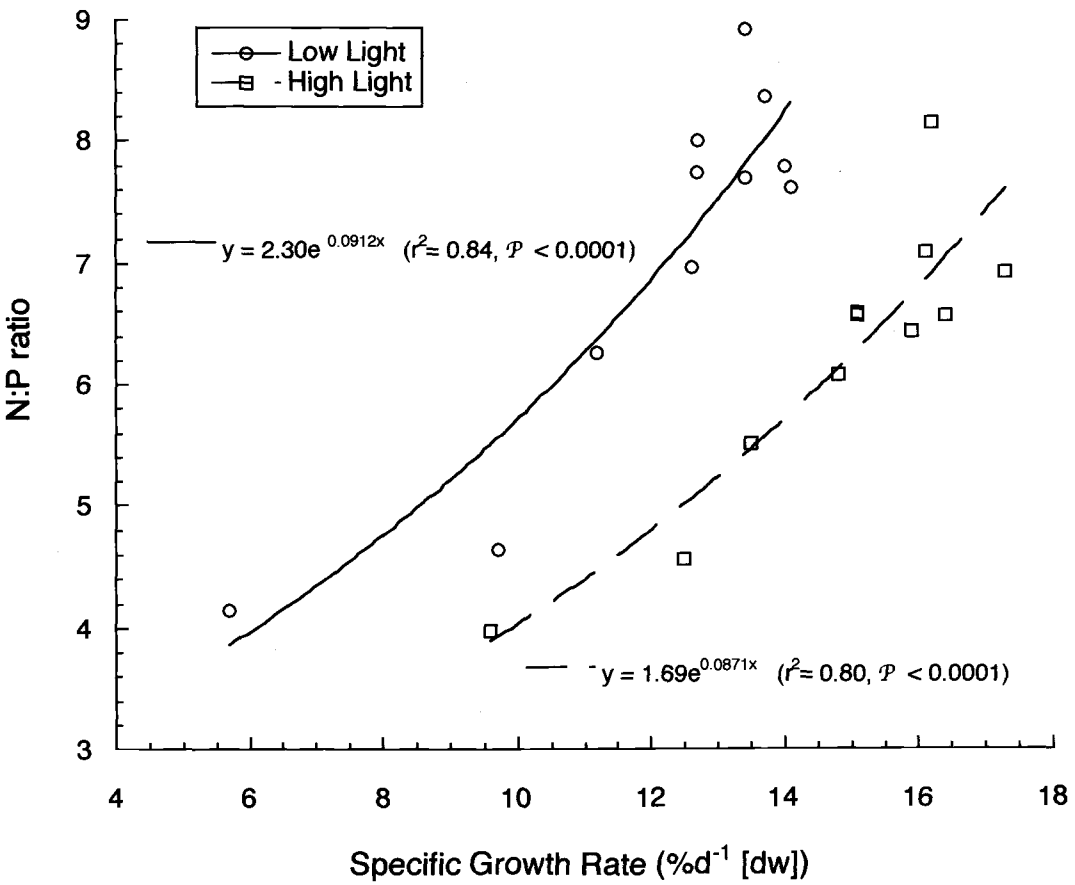
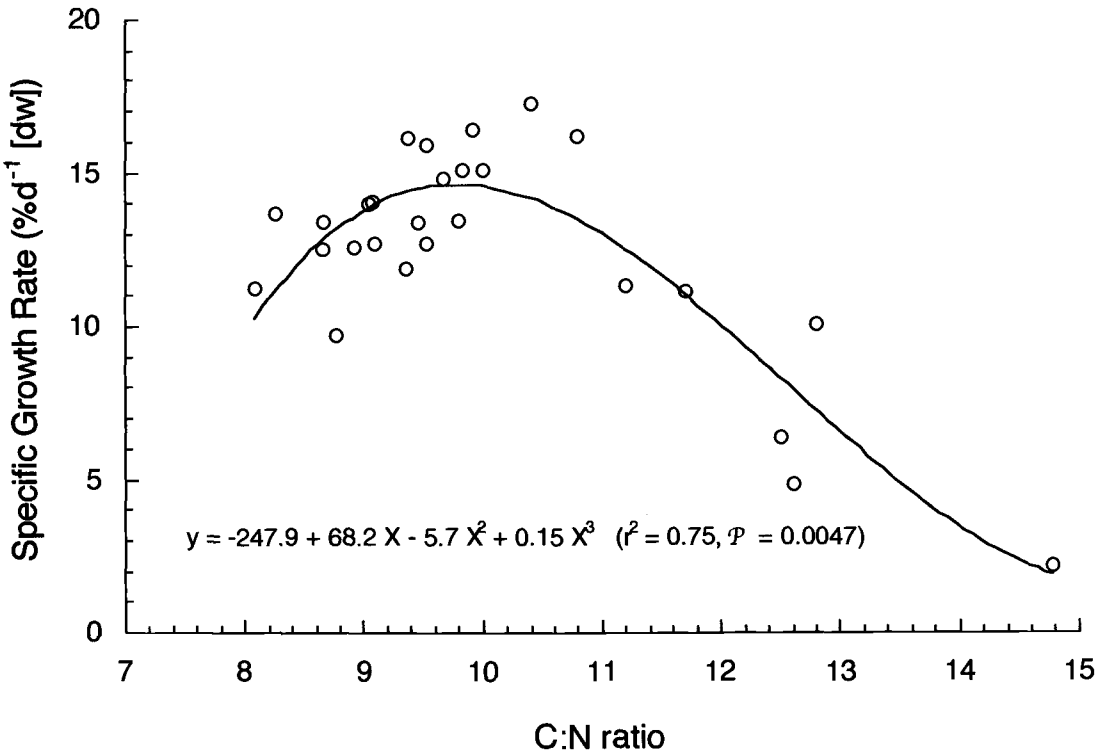


Figure 1.13: Specific growth rate of *Palmaria mollis* as a function of tissue C:N ratio under a photon flux density ranging from 22.5 to 51.64 mol photons m^{-2} d^{-1}. Data were pooled from the nutrient application rate and nitrate experiments. Nutrient additions were made every 1 to 7 days. Cultures were supplied trace metals at either 0.75*f* or 1*f* molar concentrations (Guillard and Ryther, 1962) both with and without Zn (total Zn = 1.82 μM d^{-1}).



DISCUSSION

Pulse fertilizing

Due to the large amount of apparent inter-cellular free space, macrophytes can store excess nutrients for use later when supplies are low (Thomas and Harrison, 1985). It is only when internal nutrient supplies are depleted that growth rates decline. This experiment showed *P. mollis* stored enough nutrients for a maximum of seven days under both light levels (Fig. 1.1) to maintain maximum growth rates. This finding agrees with the results of experiments with *P. palmata* (Morgan and Simpson, 1981a) and other Rhodophytes (Lapointe and Duke, 1984; Hanisak, 1990; Pickering et al., 1993) that showed pulse-fertilizing every 7 to 14 days was as effective as daily additions. Further, it is possible that the time interval between pulses could be lengthened if the soak time was increased beyond 12 hours (present study). An important advantage in periodic additions of nutrients was a dramatic reduction of epiphytes in treatments pulsed every 5 to 11 days (cf., Friedlander et al., 1991).

Ammonium

Generally, NH_4^+ is considered superior to NO_3^- for growth of most macrophytes, since NH_4^+ can be directly incorporated into amino acids and NO_3^- must be reduced prior use (Lobban and Harrison, 1997). However, the growth advantage of NH_4^+ vs. NO_3^- for cultured Rhodophytes varies, depending on

species and the NH_4^+ concentration being delivered. Under low NH_4^+ concentrations (50 μM – N d^{-1}), NH_4^+ is typically either equal to or superior to NO_3^- for most Rhodophyte species (Iwasaki, 1967; DeBoer, 1978; Levin, 1991). Under high NH_4^+ concentrations (500 to 2000 μM – N d^{-1}), and typically high light, NO_3^- is preferred by several Rhodophyte species, including *P. palmata* and *P. mollis* (Iwasaki, 1967; Lapointe and Ryther, 1979; Morgan and Simpson, 1981a; and this study).

In this study, long-term NH_4^+ additions to *P. mollis* cultures were toxic. However, at lower NH_4NO_3 concentrations (2353 μM – N d^{-1}) toxicity of NH_4^+ took 49 days to become apparent (Fig. 1.5). Use of NH_4^+ may result in higher short-term growth rates compared with those obtained with NO_3^- alone, provided it is supplied at relatively low concentrations (e.g., < 50 μM – N d^{-1}). Future work should examine the economic advantages of using combinations of NH_4^+ and NO_3^- in order to benefit from the low relative costs of NH_4^+. In addition, uptake and utilization by *P. mollis* of low concentrations of excreted NH_4^+ from abalone or fish in co-culture systems should be examined (cf., Evans and Langdon, 2000).

Trace metals

While little work has been carried out on trace metal requirements and metabolism in seaweeds, the importance of their role in phytoplankton productivity is well established (McLachlan, 1982; DeBoer and Whoriskey, 1983; Martin et al., 1990; Lobban and Harrison, 1997). Since Fe

is required for cell growth, respiration, and photosynthesis (Lobban and Harrison, 1997; Bidwell, 1979), additions of Fe are necessary under conditions of limited seawater exchange and high photosynthetic rates. Indeed, Fe is considered by some as a macronutrient and is reported as the fourth essential element in Irish moss aquaculture (Craigie and Shacklock, 1989). Studies indicate that Zn activates protein synthesis and Mn plays a principle role as an enzyme cofactor in photosynthesis (Lobban and Harrison, 1997). Like Fe, some researchers suggest Mn is a macronutrient which, along with Zn, is important in mitigating photodamage under high light conditions (Craigie, *pers. comm.*); however, not all Mn accumulated by a healthy plant is physiologically necessary for growth. In this study, *P. mollis* grew more slowly without additions of nutrients (N, P, and trace metals) but bio-accumulated high concentrations of Fe, Mn, Zn, and Cu in the seawater controls that were well above levels in the other treatments.

This study demonstrated enhancement of *P. mollis* growth by addition of Guillard and Ryther's *f* trace metal medium. Further improvement in growth was achieved by increasing Zn concentration to equal that of Mn (Figs. 1.2 and 1.3). Based on the relationship shown in Figure 1.3, 0.75*f* + Zn at 1.37 μM d^{-1} is likely the best trace metal concentration to support maximum growth rates over most natural PFD's.

Like other researchers (Hanisak, 1979; DeBoer, 1981), tissue nutrient status (i.e., N:P, C:N and trace metals) was correlated with *P. mollis* growth rates. Based on the results of this study and practical experience, tissue nutrient concentrations should be kept within the range set

forth in Table 1.4. These trace metal values compare favorably with those reported for wild-collected *P. palmata* (Munda, 1978; Morgan et al., 1980b) and other macrophytes (BeBoer, 1981). For example Morgan et al., (1980) reported ranges of tissue Fe from 153 to 4,400 μg g [dw], Mn from 11 to 110 μg g [dw], Zn from 41 to 200 μg g [dw] and Cu from 22 to 48 μg g [dw] in wild-collected *P. palmata*. Based on their work, Cu is the only tissue metal in our analysis that might be considered low. Cu supplementation is likely to be important for enhanced growth of *P. mollis* because as much as 99.7% of Cu in natural seawater of the northeastern Pacific may not be biologically available (Coale and Bruland, 1988).

For all treatments (except controls) and at both light levels, tissue concentrations of Fe, Zn, and Cu were significantly correlated ($P < 0.0001$) with increased culture NO_3^- concentrations (Figures 1.7, 1.8, 1.9, and 1.10). Under high light, more Fe was accumulated in *P. mollis* tissue than under low light, which was somewhat true for Zn and Cu. It is unclear whether or not there is a greater requirement for Fe, Zn and Cu with higher NO_3^- concentrations or whether accumulation was simply enhanced. Rice and Lapointe (1981) suggested that elevated trace metal concentrations in tissues may be due to trace metal complexation with organic nitrogen ligands.

Phosphate

Since tissue P concentrations were inversely correlated with *P. mollis* growth at PO_4^- seawater concentrations above 10 mg l^{-1} d^{-1}, there appears to have been some 'luxury consumption' of P. Thus, the amount of P in *P. mollis* tissue appears to be a good indicator of both growth and P availability, with high tissue P (> 0.70% [dw]) indicating excess P fertilization and potentially poor growth (cf., Tables 1.2, 1.3 and 1.4). In addition to lower SGR's, increasing external P beyond that required for maximum growth resulted in higher tissue N levels and hence a lower C:N ratio under both light levels.

Lapointe (1987) found a similar interaction between P and N enrichment for tissue N and P of *Gracilaria tikvahiae*. This result suggests that providing optimal levels of P is critical to producing high yields of *P. mollis*. Additionally, depending on the nature of upwelling or source of water, ambient seawater concentrations of PO_4^- may be high enough ($\geq$ 42 μM – P d^{-1}) to support high growth rates at seawater exchange rates of 7 vol d^{-1} or higher (Mencher et al., 1983; Demetropoulos, *pers. obs.*).

Table 1.4: Predicted optimal tissue [dw] N, P, N:P, C:N and trace metal concentrations (μg g^{-1}[dw] ± SE) for maximum growth of *Palmaria mollis* as a function of low light (PFD = 23.68 mol photons m^{-2} d^{-1}) or high light (PFD = 51.64 mol photons m^{-2} d^{-1}). Values used came from means ± SE that were not significantly different in growth rate (ANOVA, SNK; P = 0.378), pooled from the nitrate and phosphate experiments.

Light	N (%dw)	P (%dw)	N:P (molar)	C:N (molar)	Fe (%dw)	Zn (%dw)	Mn (%dw)	Cu (%dw)
23.68	4.9±0.2	0.63±0.02	7.8±0.3	8.8±0.4	753±305	36±10	8±1	13±2
51.64	4.6±0.1	0.69±0.02	6.7±0.3	9.7±0.3	990±151	40±50	7±1	14±1

Nitrate

Maximum growth rates of *P. mollis* occurred at NO_3^- concentrations of 2942 μM – N d^{-1}. Additions above this level caused *P. mollis* growth to decline slightly but not significantly. Tissue % N [dw] content was the same for all treatments (except controls) under a given light level and it was not possible to identify tissue N contents that would indicate growth-limiting N concentrations in the culture medium apart from those associated with *P. mollis* from the seawater control (no NO_3^- additions). We would expect the critical tissue N level lies somewhere between 2.97% [dw] (seawater control; no growth after 28 days) and 4.35% [dw] (lowest tissue N sustaining high growth for 49 days) under light conditions of 23.68 to 51.64 mol photons m^{-2} d^{-1} (Table 1.3).

While Bjornsater and Wheeler (1990) have suggested that tissue N contents greater than 4% [dw] can indicate N surplus, *P. mollis* may have greater N requirements than those suggested by the literature (cf., Morgan and Simpson, 1981 a,b; Levin, 1991). In support of this argument, total %N[dw] has been reported as "high" for *P. palmata* in comparison to other seaweeds (Morgan et al., 1980), with the highest recorded values in wild *P. palmata* at 5.7% [dw] (Chaumont, 1978, reviewed in Morgan et al., 1980). A value of 5.7% N [dw] would place protein content at a maximum of approximately 35.6%[dw] and compares favorably with our highest measure of 36.4%[dw] (Table 1.1). Based on SGR's in the nitrate and phosphate experiments, optimal tissue N for *P. mollis* (after 3 days of tissue NO_3^- purging) for maximal growth appears to be

4.9% for the low light and 4.6% for the high light conditions (Table 1.4), resulting in a mean protein content of approximately 30.6% and 28.75% respectively. Providing *P. mollis* with high levels of N (as NO_3^-) appears to result in high SGR's rather than increasing protein content because tissue N values were not statistically different over the range of nitrate concentrations tested within each light level.

Results from this study also suggest an inverse relationship between tissue N and light at this culture temperature (cf., Morgan and Simpson, 1981b,c; Levin, 1990). This relationship may be due to lower concentrations of proteins associated with protein-based photosynthetic pigments under high PFD's (Lobban and Harrison, 1997). If *P. mollis* protein content plays a role in the nutrition of herbivores, such as abalone, it is possible that *P. mollis* cultured under high light conditions may not be as nutritious as plants cultured under low light. Davison (1991) also suggests there may be a requirement for increased N to support the need for increased photosynthetic pigment content under higher temperatures, which are generally coupled with high PFD's. Thus, maintaining high nitrogen concentrations (2353 to 2942 μM d^{-1} NO_3^- - N) in *P. mollis* cultures is probably the best strategy to maintain high growth rates (cf., Demetropoulos and Langdon, *this volume*).

N:P

The intent of the N and P fertilization experiments was to determine nutrient requirements for growth in the context of two different PFD's and low seawater exchange conditions. Our results indicate the best nutrient addition molar N:P for optimal *P. mollis* growth is between 14.2 to 35.5 under low PFD's (23.68 mol photons m^{-2} d^{-1}) and 21.3 to 35.5 under high PFD's (51.64 mol photons m^{-2} d^{-1}) (Tables 1.2 and 1.3). The common nutrient N:P ratio for both of these light levels represented N and P concentrations of 2942 μM – N d^{-1} and 83 μM – P d^{-1}, resulting in an optimal tissue N:P of between 6.57 and 8.37 for the fastest growing cultures under both light regimens of the nitrate experiment (Fig. 1.12 and Table 1.3). When data from the phosphate and nitrate experiments are combined, optimal tissue N:P ratios ranged from 6.7±0.3 (4.6% N, 0.69% P) for high light to 7.8±0.3 (4.9% N, 0.63% P) for low light conditions (Table 1.4).

C:N

Based on the parabolic function generated in Fig. 1.13, the highest growth rates under both low and high light conditions occurred with C:N tissue ratios ranging from 9.4 to 10.4, with the optimal ratio being approximately 10 (cf., Lapointe and Duke, 1984; Lobban and Harrison, 1997). C:N ratios less than 10 are generally an indication of surplus nitrogen and a lack of light saturation (Lobban and Harrison, 1997). For example, Levin

(1991) found a C:N of between 4.7 and 5.25 for *P. mollis* cultured exclusively on salmon effluent under low light (< 18 mol photons m^{-2} d^{-1}). Above a C:N of 10, nitrate limitation under saturating light plays a more important role in decreasing SGR's of *P. mollis*. For example, Rosen et al., (2000) reported C:N ratios of 13 and 14 for *P. mollis* cultured under moderate to high light (30 to 52 mol photons m^{-2} d^{-1}) and low seawater exchange (1 vol d^{-1}), which resulted in relatively low SGR's of between 2.6 and 4.9% d^{-1}.

Conclusions

Reduced exchange rates coupled with addition of nutrients is likely to be a more favorable economic strategy than pumping large volumes of seawater through a facility to provide sufficient nutrients for high seaweed productivity.

One of the most important goals of this study was to develop a nutrient regime and management protocol that would optimize *P. mollis* growth under limited seawater exchange conditions while simultaneously limiting epiphytism and the occurrence of weed species. In this regard, the strategy of adding nutrients during the dark cycle every 5 to 7 days has several advantages: 1) at night, water can more easily be shut off for many hours, such that nutrients are not lost to flushing yet culture temperatures can remain relatively constant, 2) the cost associated with nutrient additions is reduced because nutrient uptake is more efficient, 3) epiphyte growth is

better controlled, and 4) Chlorophyte species are not provided with light necessary for their nutrient uptake (cf., Hanisak, 1987, Lobban and Harrison, 1997).

The tendency of the seaweed culturist is generally to supply nutrients such that they slightly exceed demand. However, this can easily produce both depressed growth (e.g., phosphate depressed growth as shown here) and blooms of weed species such as *Ulva*, *Enteromorpha*, and filamentous diatoms (Schramm, 1991). Thus, it is best to supply nutrients on a schedule that takes advantage of the physiology of red macroalgae and the demand for nutrients based on growth. When the concentration of a nutrient is low in the tissue of *P. mollis* relative to physiological or structural demands, that nutrient limits growth. Tissue nutrient assays, similar to those used by terrestrial farmers, provide the most reliable indicator of macroalgal health and by extension, yields as a function of incident light (Hanisak, 1979; Hanisak 1982; DeBoer, 1981). Tissue analysis should be adopted as a standard method for determining the nutritional status of *P. mollis*. However, it is important to stress that tissue nutrient status needs to be tracked closely since nutrient demand is a function mainly of temperature and incident light, which often vary over short periods of time.

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ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF SEAWATER EXCHANGE, PH, AND INORGANIC CARBON CONCENTRATION ON GROWTH

ABSTRACT

Addition of carbon dioxide to cultured algae is generally considered necessary to maintain high yields and healthy cultures. However, this represents a major cost for commercial production. This study was conducted to determine requirements for dissolved inorganic carbon (DIC) to maintain high growth rates of *Palmaria mollis* or Pacific dulse. Methods for introducing DIC included additions of $NaHCO_3$, CO_2, and variable seawater exchange rates. Specific growth rates of *P. mollis* were positively correlated with ambient concentrations of DIC and cultures supplied with both CO_2 and $NaHCO_3$ resulted in higher growth rates than those supplied with either inorganic carbon sources alone. A pH of 8.9 resulted in growth rates that were not significantly different from those at pH 8.3. Adopting a seawater flushing regime that increased flushing during the light cycle (seawater exchange rate of 7.5 vol d^{-1}) but lowered flushing during the dark cycle (0.65 vol d^{-1}) resulted in growth rates equal to those obtained by maintaining pH at 8.5 by sparging with CO_2 at a daily exchange rate of 7.5 vol d^{-1}.

INTRODUCTION

Palmaria mollis (= *Rhodymenia palmata* var. *mollis*, Setchell and Gardner 1903) (Rhodophyceae, Bangiales) is found in the low intertidal and upper subtidal rocky shore from Southeastern Alaska to San Luis Obispo County, California. While some studies have been published describing the role of dissolved inorganic carbon (DIC) and growth for its close relative, *P. palmata* (dulse) (Robbins, 1978; Morgan et al., 1980; Kübler and Raven, 1994, 1995; 1996), no work has been reported on DIC utilization by *P. mollis*.

Primary productivity of terrestrial and aquatic plants is dependent on fixation of inorganic carbon through the process of photosynthesis. For most seaweeds, inorganic carbon is typically available as either dissolved CO_2 or HCO_3^-. Because CO_2 dissolves slowly in water, inadequate DIC can quickly limit productivity of land-based seaweed cultures (Bidwell et al., 1985; McLachlan et al., 1986). Craigie and Shacklock (1989) have suggested that the first element to be depleted in tank culture of seaweeds is DIC. Aeration alone is not sufficient to maintain commercially viable yields under mass culture conditions (Bidwell, 1981). For example, Hanisak and Ryther (1984) found that it took 9 days of aeration to increase values of DIC from 0.4 to 1.1 mM in tanks that had been previously stripped of inorganic carbon.

Seawater at equilibrium with the atmosphere has a pH of 7.8-8.2 and DIC (CO_2 + HCO_3^- + CO_3^{2-}) of about 2.2 – 2.5 mM (Dring, 1982). As outlined by Craigie and Shacklock (1989), the equilibrium of DIC in seawater is pH

dependent, with a rise in pH indicating a loss of DIC from the system and potential DIC malnutrition (cf., Debusk and Ryther, 1984; Bidwell et al., 1985, Braud and Amat, 1996). To re-establish a more optimal pH, DIC is typically introduced into seawater through sparging of CO_2 gas (Bidwell et al., 1985; Craigie and Shacklock, 1989), with $NaHCO_3$ (Simpson et al., 1978) or with inorganic/organic acids (Debusk and Ryther, 1984; Amat and Braud, 1993). The required addition of DIC is often automatically controlled by use of pH-activated solenoid valves (Bidwell et al., 1985; Craigie and Shacklock, 1989).

At a seawater pH of 8.0, HCO_3^- represents approximately 90% of the DIC, exceeding that of CO_2 by a factor of 200 (Bidwell, 1981). With increasing pH the ratio of HCO_3^- to CO_2 continues to rise (Lyman, 1956). Thus, it is not surprising that many macrophytes appear to utilize HCO_3^- (in addition to CO_2) as a carbon source (Sand-Jensen and Gordon, 1984; Bidwell and McLachlan, 1985; Lignell and Pedersén, 1989), including *Palmaria sp.* (Bidwell and McLachlan, 1985; Kubler and Raven, 1995). However, DIC uptake rates of macrophytes in culture (including *P. mollis*) are often much higher than CO_2 absorption rates by seawater from the air (Bidwell, 1981).

Under moderate photon flux densities (PFD's) of 200-400 μmol photons m^{-2} s^{-1} it has been shown that increasing total dissolved CO_2 concentrations to 3.0 mM (Johnston et al., 1992) or perhaps as high as 6.0-7.0 mM (Robbins, 1978) can stimulate photosynthetic rates almost 3 fold in *P. palmata*. Therefore, DIC enrichment is likely to be necessary to achieve economically viable yields of *P. mollis* under high PFD's. Further, determination of

economic methods for DIC delivery is an important commercial concern since CO_2 injection can represent as much as 36.9% of the operational costs for mass culture (Braud and Amat, 1966).

The purpose of this investigation was to examine both requirements and methods of delivering DIC to cultures of *P. mollis* under moderate to high, natural PFD's. Unlike previous studies, our goal was to investigate DIC utilization and management under conditions that begin to approach commercial scale production. All of the experiments were conducted at the aquaculture park established by the Natural Energy Laboratory of Hawaii (NELH) located north of Kailua-Kona on the Big Island of Hawaii.

MATERIALS AND METHODS

All *P. mollis* cultures were obtained from the Hatfield Marine Science Center of Oregon State University (OSU, C-3 strain; Demetropoulos and Langdon, 2001). Cultures were enriched with Guillard and Ryther's (1962) *f* medium, by pulse-fertilizing three times per week at night when water temperature allowed the tanks to be shut off for several hours (Demetropoulos and Langdon, *this volume*). The temperature was held between 12° and 17° C over the course of the experiments by adjusting the relative supplies of cold and warm seawater provided by the aquaculture park.

Cultures were not subjected to sterile techniques in order to approach the "commercial reality" of air/water born contamination. All enrichments were added during the dark phase of the diurnal cycle so as to offset chlorophyte and epiphyte growth (Hanisak, 1987; J. McLachlan, *pers comm.*). The contents of the culture vessels were damp-weighed weekly using the spin cycle of a washing machine and thinned to their original weight. Specific growth rate was calculated according to the equation: $SGR = 100\ \ln(W_f/W_i)/t$ (DeBoer, et al., 1978), where SGR is the % change in dry weight d^{-1}, W_f and W_i are the final and initial algal dry weights, and t is time (days), The equation assumes-steady state exponential growth (DeBoer et al., 1978) based on final dry weights. Samples from each experiment were analyzed for dry weight and crude protein (Lowry method; Lowry, et al., 1951) at the University of Hawaii Analytical lab. Ambient photosynthetically active radiation (PAR) was quantified using a LI-192S (2 π) sensor placed near the tanks. Tanks

were tumble-cultured under a natural Hawaiian photon flux density (PFD) of 26.4 to 52.6 mol photons m^{-2} d^{-1} PAR.

DIC sources and concentrations

Since *Palmaria sp.* is reported to use both HCO_3^- and CO_2 (Bidwell and McLachlan, 1985) it seemed reasonable to compare CO_2 and HCO_3^- enrichment. The experiment was conducted to determine if additions of DIC above ambient levels would improve growth rates of *P. mollis* and if there was a growth advantage to supplying DIC to *P. mollis* cultures as either HCO_3^- alone or as CO_2 in combination with HCO_3^-.

Rosettes of *P. mollis* were placed in 140 l, opaque tanks at a density of 7g l^{-1} supplied with continuous flow of 14° C seawater, obtained by mixing surface and deep water, at a seawater exchange rate of 3.5 vol d^{-1}.

Three treatment concentrations (2 replicates each) of HCO_3^- (2.0, 4.0 and 6.0 mM HCO_3^-) at two levels of CO_2 (with and without) were tested against two controls. Additions of $NaHCO_3$ five times during the 14 hr light period were used to produce the three HCO_3^- concentrations at the first treatment level (no CO_2 sparaging). For the second treatment level, CO_2 was sparaged via pH-stat valves (Bidwell et al., 1985) through the tanks to maintain a pH of 8.0 in combination with the three concentrations of HCO_3^-. The two controls included: 1) seawater supplemented with CO_2 sparaged at a rate to maintain the pH between 7.9 and 8.2 (average DIC ≈ 2.0 mM) (Morgan, et al., 1980; Johnston et al., 1992) and 2) non-DIC supplemented

seawater (average DIC ≈ 1.6 mM). In the second control, DIC was allowed to rise and fall diurnally, and limited DIC supplies were introduced by seawater exchange.

Temperature and pH were measured daily during the course of the experiment. After a 21 day acclimation period under DIC and flow conditions described above, *P. mollis* specific growth rate (SGR) was determined by measuring the weekly increase in dry weight yields over a subsequent 28-day period.

Seawater exchange rates

Based on the estimated costs of adding CO_2 and HCO_3^- to mass cultures (Braud and Amat, 1996) and the difficulty in maintaining culture temperatures < 18° C under low exchange rates and high PFD's, an attempt was made to provide adequate DIC through seawater exchange alone. This approach for supplying DIC was evaluated in two experiments. First, a threshold exchange rate was determined, below which SGR's of fast growing cultures were significantly reduced, then this was tested against higher exchange rates. Second, the effects on SGR's of *P. mollis* were determined when exchange rates were adjusted diurnally to maximize DIC supply when photosynthetic demand was highest during the light cycle.

These experiments were conducted in 125 l translucent, culture tanks, laid out on a southeast/northwest line such that mutual tank shadowing during the morning and afternoon was eliminated. Due to translucency of the tanks, it was necessary to quantify total PFD using a

combination of LI-192S (2 π) and LI-193S (4 π) sensors. Two tanks in each experiment were fitted with three sensors. A 2 π sensor on a black disk at the tank surface was used to measure surface light. The lower half of the same tank was blackened out and the tank was fitted with a 4 π sensor in the center of the top half to measure light available though the side walls in the top portion of the tank. The top half of the second tank was blackened out and fitted with a 4 π sensor in bottom center to measure light available though the side walls in the bottom portion of the tank. Both tanks were supplied with air and water and run without algae for the course of the experiments. Light measurements were integrated daily.

Palmaria mollis was stocked at an initial density of 8 g l^{-1} (damp wt) in both experiments. Triplicate treatments were set up in a randomized block design. After a seven-day acclimation period, *P. mollis* growth rates were determined by measuring the weekly increase in dry weight yields over a 28-day period for both experiments.

Constant seawater exchange rates: Preliminary experiments showed that under stocking densities of 8 to 15 g l^{-1} (damp wt) and moderate to high PFD's (42 to 48 mol photons m^{-2} d^{-1}), the pH of *P. mollis* cultures decreased almost linearly with increased seawater exchange rates up to 24 tank volume exchanges d^{-1} (C.L. Demetropoulos, *unpublished data*). To better understand the growth response of *P. mollis* to DIC (as measured by pH) over a broad range of seawater exchanged rates, and under saturating PFD's, *P. mollis* growth was measured as function of four exchange rates [7.5 vol d^{-1} (0.65 l min^{-1}), 15 vol d^{-1} (1.3 l min^{-1}), 30 vol d^{-1} (2.6 l min^{-1}), and 60

vol d^{-1} (5.2 l min^{-1})]. An exchange rate of 7.5 vol d^{-1} was chosen initially because it was high enough to maintain an optimal temperature (16±1° C) for *P. mollis* in mass culture throughout most of the year and low enough to be of potential economic interest (C.L. Demetropoulos, *unpublished data*). Triplicate treatments were set up in a randomized block design.

Variable seawater exchange vs. CO_2 additions: This experiment was conducted to determine the effects of varying seawater exchange rates according to the light cycle and to compare its effectiveness with sparging CO_2 as a means of reducing pH and providing DIC. As in the previous experiment, a seawater exchange rate of 7.5 vol d^{-1} was chosen as the control because it balanced both biological needs and economic costs. A total of six treatments were tested; three seawater exchange treatments and three pH levels. The seawater exchange treatments were, 1) 7.5 vol d^{-1} constant throughout the light/dark cycle (0.65 l min^{-1}), 2) 7.5 vol d^{-1} variable (1.3 l min^{-1} day and 0.32 l min^{-1} night), 3) 15 vol d^{-1} constant throughout the light/dark cycle (1.3 l min^{-1}). The three levels of CO_2 sparging (as measured by pH) were 8.1, 8.5, and 8.9 - all at a constant exchange rate of 7.5 vol d^{-1} (0.65 l min^{-1}) constant throughout the light/dark cycle. The pH of the latter treatments were controlled by sparging CO_2 gas through the cultures via pH-stat valves.

Statistics

The first experiment was designed as a fully randomized factorial with three HCO_3^- treatments at two levels of CO_2 addition (with and without) plus two controls. Data were log transformed to correct for heterogeneous variances and two-factor ANCOVA (light as the covariant) was used to test for the interaction of light and treatment (homogeneity of slopes) on SGR's of *P. mollis*. Subsequently, ANOVA was used to test for significant differences between treatments ($\alpha = 0.05$). The second and third experiments were set up in a randomized block design. Two-factor ANOVA was used to test for significant treatment effects. Significant differences among treatments for all experiments were compared by the Student-Newman-Keuls (SNK) multiple comparison test ($\alpha = 0.05$).

Results

DIC sources and concentrations

Once the data were log-transformed, ANCOVA showed no significant interaction between light and treatment (P = 0.001). Subsequently, ANOVA followed by SNK showed significant differences ($P < 0.0001$) between several treatments and the highest DIC treatment used (CO_2 + 6 mM HCO_3^-). Increasing concentrations of DIC resulted in higher growth rates of *P. mollis*, especially at the highest DIC treatment level of CO_2 + 6 mM HCO_3^- (Fig. 2.1). For all but the 2 mM DIC treatments (which was linear), an exponential function provided the best fit for the data. The relationship between the effect of increasing DIC on SGR's can be seen on mean SGR's during the last 28 days of the experiment (Fig. 2.2). During this period significant differences (ANOVA, SNK; $P < 0.0001$) in SGR's were found between three distinct DIC groups; 1) (seawater control, 2 mM HCO_3^-), 2) (4 mM, 6 mM HCO_3^-, CO_2 alone, CO_2 + 2 mM HCO_3^-, CO_2 + 4 mM HCO_3^-), and 3) (CO_2 + 6 mM HCO_3^-). Growth reached a maximum with the addition of CO_2 + 6 mM HCO_3^-.

The pH for the deep water supplied to the NELH site was typically 7.6 in contrast to a pH of 7.8 to 8.0 that is usually found in coastal waters where *P. mollis* naturally occurs. Measures of pH in the cultures showed that additions of CO_2 and additions of HCO_3^- at all tested concentrations were sufficient to maintain an average pH of 8.1±0.2 during the light cycle. While additions of CO_2 and HCO_3^- to *P. mollis* cultures maintained a pH < 8.3

during the day, pH often decreased to 6.7 by 23:00 hours in the evening as a result of respiration.

Seawater temperature of the cultures ranged between 13° (dark phase) to 16° C (light phase). The only exception to this was in the 5th week of the experiment where temperatures reached 18.5° C for several hours during each of 4 successive days. Instantaneous measures of light using the 2 π sensor at the surface showed the tanks received a maximum PFD of 2051 μmol photons m^{-2} s^{-1} with a daily PFD ranging from 22.25 to 30.5 mol photons m^{-2} d^{-1}.

While biochemical analysis showed no significant differences (ANOVA; P = 0.371) in protein content of *P. mollis* among treatments (ca. 27 to 30% [dw]), *P. mollis* from the highest DIC treatment (CO_2 + 6 mM HCO_3^-) had the lowest (27% [dw]) protein content.

Seawater exchange rates

Ambient sunlight in Hawaii resulted in a total average PFD of between 42.1 and 51 mol photons m^{-2} d^{-1} (mean = 43 mol photons m^{-2} d^{-1}) at the tank's water/air interface during these experiments. Instantaneous PFD measures using the 2 π sensor and the two 4 π sensors in the control tanks showed total PFD was greater by a factor of approximately 2.6 compared with surface illuminated tanks.

Constant seawater exchange: ANOVA showed there was no significant block effect within treatments (P = 0.682). The analysis indicated that there were significant (ANOVA, SNK; $P < 0.0001$) differences in average pH's among the

treatments during the light phase. Increasing seawater exchange rates from 7.5 to 60 vol d^{-1} and consequently, lowering pH during the light phase, significantly (ANOVA, SNK; $P < 0.0001$) increased SGR's (Fig. 2.3 and Table 2.1); however, the log-function generated by the data (Fig. 2.3) shows seawater exchange had less of an effect on growth once an exchange rate of 15 vol d^{-1} or greater was reached.

Figure 2.1: Specific growth rate of *Palmaria mollis* (± SE) taken weekly as a function of seven dissolved inorganic carbon (DIC) additions and a seawater control under photon flux densities ranging from 26.4 to 36.2 mol photons m^{-2} d^{-1}. Cultures were allowed a 21-day acclimation period followed by a 28-day data collection period. Seawater exchange was 3.5 vol d^{-1}. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at 1*f* molar concentrations (Guillard and Ryther, 1962).

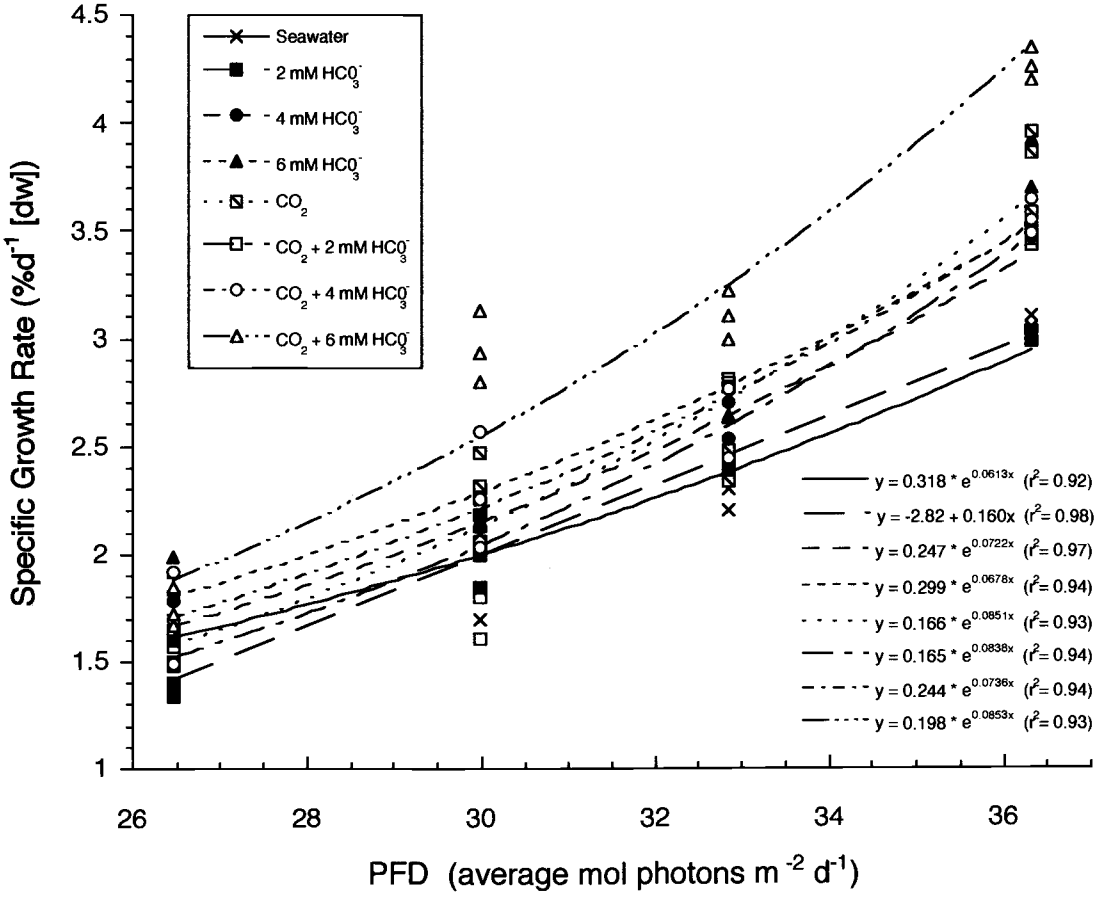


Figure 2.2: Specific growth rate of *Palmaria mollis* (± SE) as a function of seven dissolved inorganic carbon (DIC) treatments and a seawater control under photon flux densities ranging from 26.4 to 36.2 mol photons m^{-2} d^{-1} during a 28-day experiment. Seawater exchange was 3.5 vol d^{-1}. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at 1*f* molar concentrations (Guillard and Ryther, 1962). Letters a, b and c indicate significant differences (ANOVA, SNK; $P < 0.0001$).

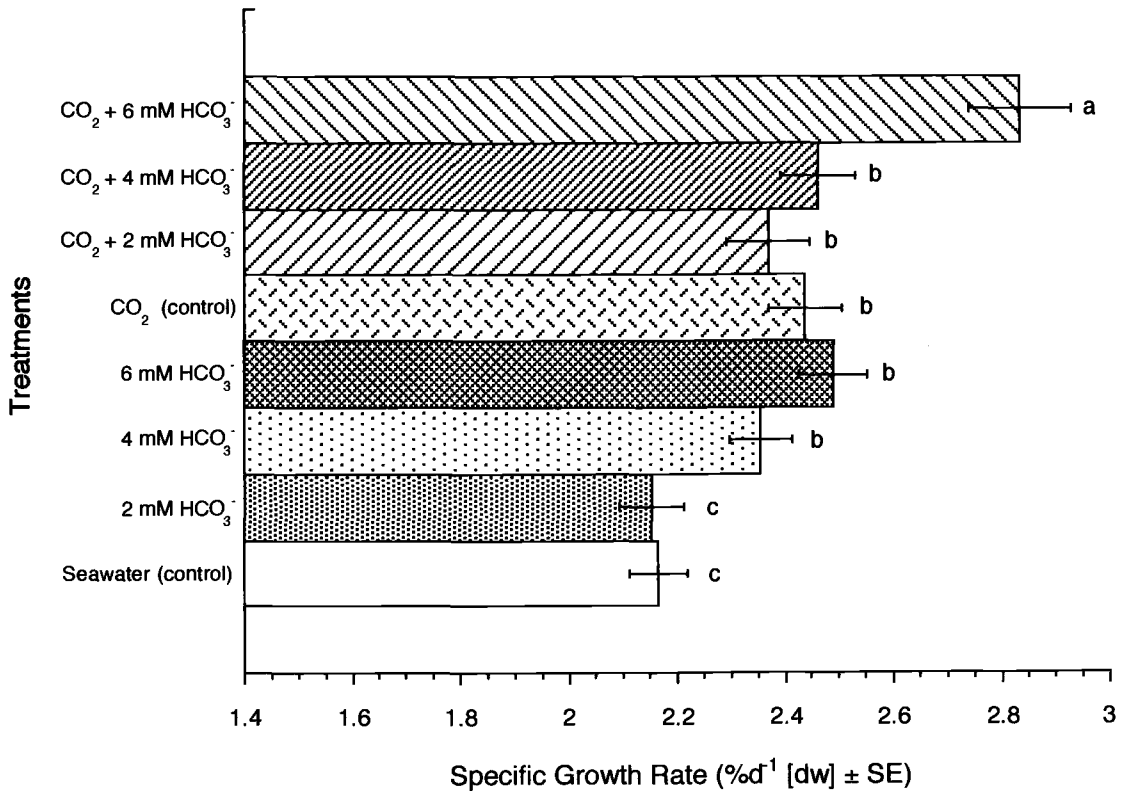


Figure 2.3: Specific growth rate of *Palmaria mollis* (± SE) and pH (± SE) as a function of exchange rate under natural light (mean PFD = 43 mol photons m^{-2} d^{-1}) in translucent tanks. Measurements of SGR and pH were taken daily over a 28-day experimental period during the light phase. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at 1f molar concentrations (Guillard and Ryther, 1962).

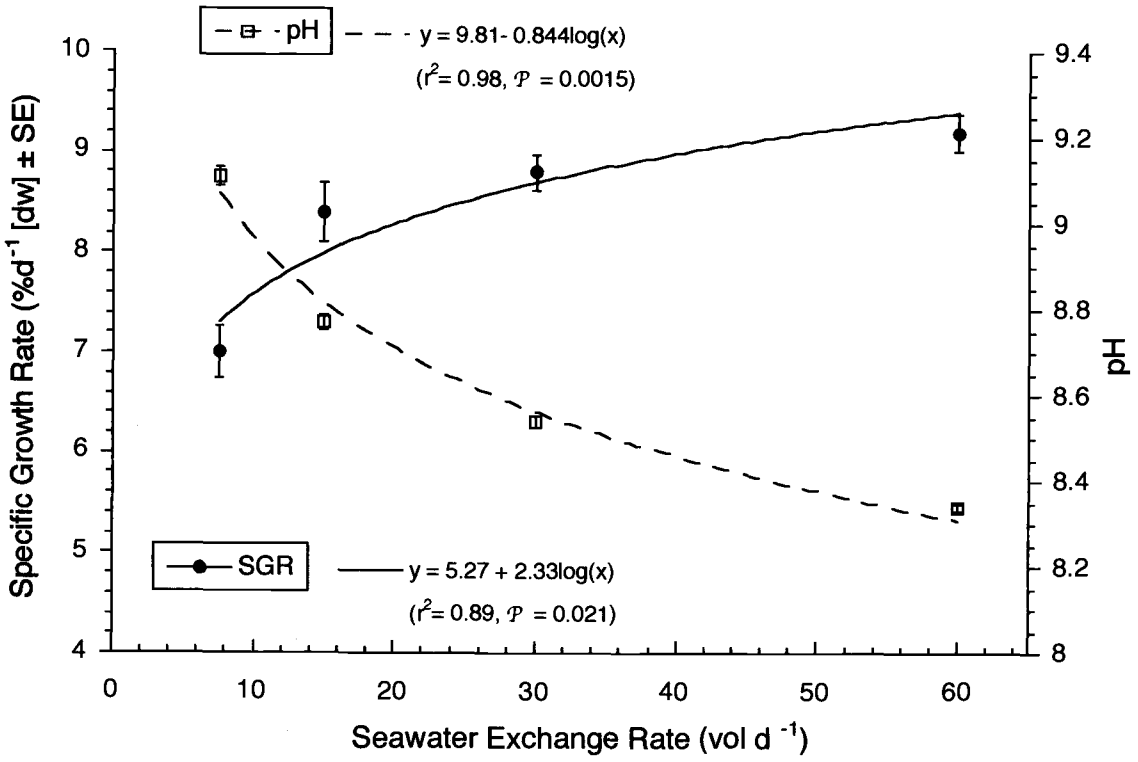


Table 2.1: Mean pH's (± SE) and SGR's (± SE) of *Palmaria mollis* cultured under different seawater exchange rates over a 28-day period. Letters a, b and c indicate significant differences (ANOVA, SNK; $P < 0.0001$).

Exchange Rate (vol d^{-1})	Mean pH (±SE)	Mean SGR (%[dw]d^{-1}±SE)	Sig. Diff. in SGR's (ANOVA, SNK; $P < 0.0001$)
7.5	9.11±0.022	7.0±0.26	a
15	8.77±0.017	8.4±0.30	b
30	8.54±0.013	8.8±0.18	b c
60	8.34±0.010	9.2±0.19	c

Seawater temperatures during the experiment ranged between 12° (dark phase) and 17° C (light phase). Oxygen saturation ranged from 72% (night) to 145% (day) over all of the experiments. Biochemical analysis showed no significant differences (ANOVA; $P = 0.27$) in protein content among treatments (ca. 28.4 to 30.3% [dw]).

Peak seawater exchange vs. CO_2 additions: Holding pH at 8.1 with CO_2 sparging together with a constant exchange rate of 7.5 vol d^{-1} resulted in cultures with a significantly higher (ANOVA, SNK; $P < 0.0001$) average SGR (10.39±0.09 $\%d^{-1}$[dw]) than cultures where pH varied between 8.5 and 9.1 (Table 2.2, Appendix 3, and Fig. 2.4). There were no significant differences among treatments when pH ranged between 8.5 and 8.9, irrespective of whether pH was controlled by exchange rate or through CO_2 sparging. A variable exchange rate of 1.3 l min^{-1} (equivalent to the exchange rate for 15 vol d^{-1}) during the day and 0.32 l min^{-1} (equivalent to the exchange rate for 3.75 vol d^{-1}) during the night, resulting in daily average exchange rates of 7.5 vol d^{-1}, supported an SGR that was not significantly different from that obtained with a constant exchange rate of 15 vol d^{-1}. In spite of receiving the same amount of water during a 24-hour period, cultures with the variable exchange rate had a mean SGR that was significantly (ANOVA, SNK; $P < 0.0001$) higher than those receiving a constant seawater exchange of 7.5 vol d^{-1}.

Figure 2.5 provides a summary of SGR as a function of pH from the seawater exchange experiments. A third degree polynomial equation provided the best fit for the data ($y = 5304.8 - 1844.6X + 214.18X^2 - 8.29X^3$; $r^2 = 0.72$). Results indicate that SGR's were similar over a pH of 8.3

to 8.9. Higher growth occurred below pH 8.3 whereas growth decreased as pH increased from 8.9 to 9.13 (the highest mean recorded).

Table 2.2: Mean pH's (± SE) during the light phase, and SGR's of *Palmaria mollis* (± SE) cultures receiving either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 to control pH at constant seawater exchange (7.5 d^{-1}). Letters a, b and c indicate significant differences (ANOVA, SNK; $P < 0.0001$).

Exchange Rate (vol d^{-1})	pH Range (light phase)	Mean pH (±SE) (light phase)	Mean SGR (% d^{-1} [dw]±SE)	Sig. Diff. in SGR's
7.5 constant	8.57 to 9.23	9.13±0.03	8.5±0.17	a
15.0 constant	8.40 to 9.08	8.72±0.02	9.2±0.05	b
7.5 variable	8.14 to 8.98	8.73±0.02	9.4±0.18	b
7.5 constant	8.24 to 9.10	8.90±0.10	9.5±0.11	b
7.5 constant	7.99 to 8.61	8.50±0.10	9.4±0.07	b
7.5 constant	7.78 to 8.15	8.10±0.10	10.4±0.09	c

Figure 2.4: Specific growth rate of *Palmaria mollis* (± SE) as a function of either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 sparaging to control pH at constant seawater exchange (7.5 d^{-1}). The experiment ran under natural light (mean PFD = 47.1 mol photons m^{-2} d^{-1}) for 28 days. Cultures were supplied with $NaNO_3$, NaH_2PO_4, and trace metals at 1*f* molar concentrations (Guillard and Ryther, 1962). Letters a, b and c indicate significant differences (ANOVA, SNK; $P < 0.0001$).

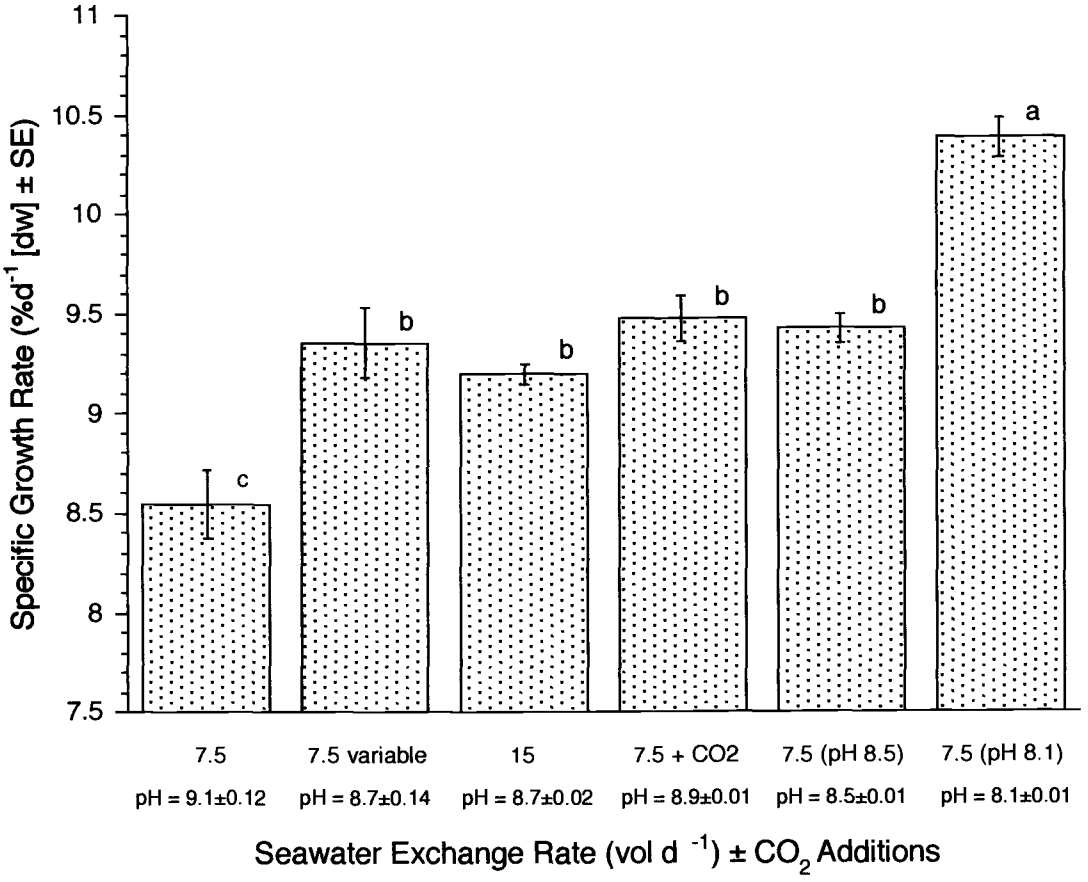
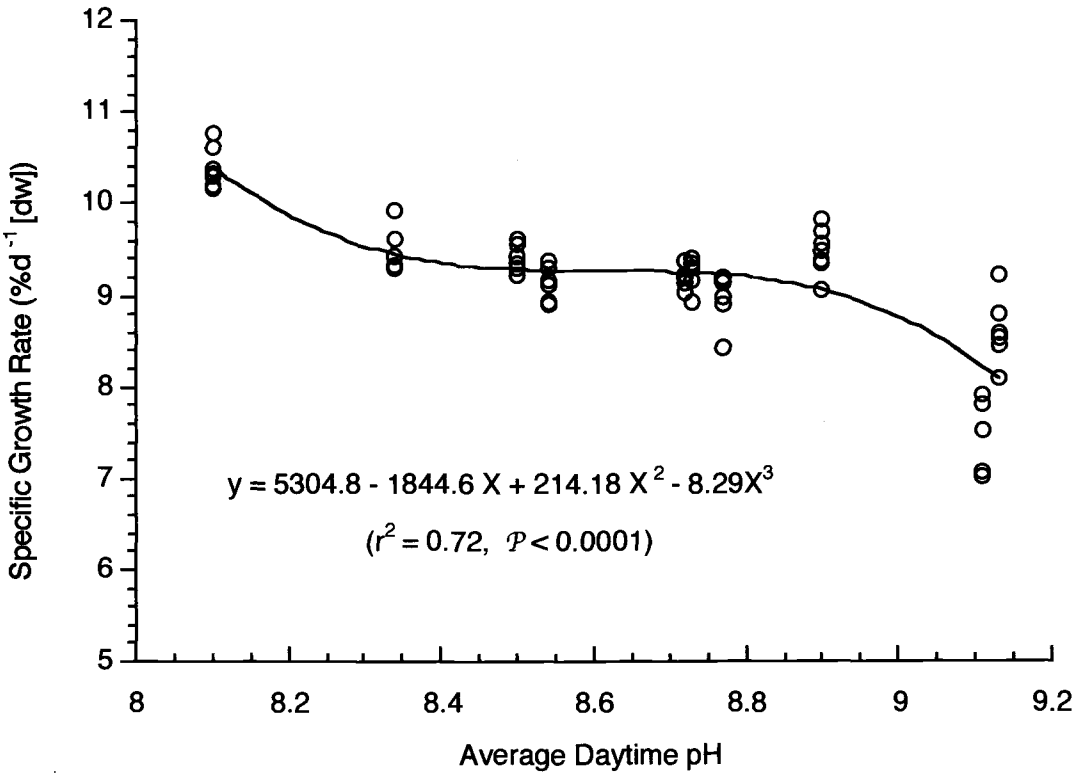


Figure 2.5: Summary of specific growth rate of *Palmaria mollis* as a function of average daytime pH under natural Hawaiian light (mean PFD ≈ 48 mol photons m^{-2} d^{-1}) from the last two weeks of the two seawater exchange rate experiments.



DISCUSSION

DIC sources and concentrations

This study shows that a combination of CO_2 sparging and addition of up to 6 mM HCO_3^- can produce an SGR that is significantly greater than those produced by adding less DIC in the form of HCO_3^-, CO_2 or combinations of both at lower HCO_3^- concentrations. This was particularly apparent under exposure of *P. mollis* to higher PFD's of 30 to 36.2 mol photons m^{-2} d^{-1} (Fig. 2.1). Like *P. palmata*, *P. mollis* is probably DIC-limited under most culture conditions. Growth can be enhanced by increasing availability of DIC (Kübler and Raven, 1996) and saturating more uptake sites.

Mechanisms of HCO_3^- use in seaweeds are much debated (Haglund and Pedersén, 1992; Lobban and Harrison, 1997). Like *P. palmata*, *P. mollis* is apparently able to utilize CO_2 directly as well as HCO_3^- indirectly after dehydration to CO_2 by extracellular CA (cf., Robbins, 1978; Johnston et al., 1992; Kübler and Raven, 1994). In the case of utilization of HCO_3^-, elevated levels of extracellular carbonic anhydrase (CA) are necessary (Bidwell and McLachlan, 1985). A threshold value of ambient HCO_3^- may be required to stimulate enhanced CA production in *P. mollis*. This may explain similar *P. mollis* growth rates over the middle range of DIC additions, whereas SGR increased dramatically when either 4mM HCO_3^- or CO_2 + 6 mM HCO_3^- was added to the cultures (Fig. 2.2). The addition of CO_2 + 6 mM HCO_3^- possibly exceeded a threshold value of ambient DIC necessary for increased CA production. This would involve adequate disturbance of the surface boundary

layers (i.e., via strong aeration in this case) (W.N. Wheeler, *pers. comm.*).

Utilization of each DIC form may vary with light intensity. For example, under light-limiting conditions *P. palmata* is less likely to use HCO_3^- vs. CO_2 (Kübler and Raven, 1994). Under high light conditions both HCO_3^- and CO_2 can be utilized, with HCO_3^- being used preferentially over CO_2 (Bidwell and McLachlan, 1985; Johnston et al., 1992; Maberly et al., 1992; and Kübler and Raven, 1995). Furthermore, HCO_3^- use in association with CA activity may allow *P. mollis* to mitigate the damaging effects of photorespiration under high light, as suggested by Reiskind et al. (1989) for Rhodophytes in general.

While Bidwell et al. (1985) point out that it is DIC and not pH per se that is the more important parameter controlling photosynthetic rate, pH can be used to track the relative concentrations of CO_2 and HCO_3^- (Craigie and Shacklock, 1989). Optimal pH for growth of *P. palmata* has been reported to range from 6.5 (Robbins, 1978) to 8.5 (Morgan et al., 1980). Under commercial stocking densities and high natural light conditions of this study we found a pH of 8.1 or less resulted in the highest growth rates for *P. mollis*. While SGR's decreased at pH values greater than 8.1, there were no significant differences in growth rates of cultures held at pH values from 8.3 to 8.9, demonstrating a range of pH values in which *P. mollis* growth was independent of pH (Fig. 2.5). As pH increases from 8.0 to 8.9 the relative proportion of DIC present as CO_2 is reduced by over 73% while the relative proportion of HCO_3^- decreases by only 13% (Lyman, 1956). CA activity increases in macroalgae in response to

low concentrations of CO_2, dehydrating HCO_3^- to release CO_2 and OH^- ions and causing a rise in pH (Bidwell and McLachlan, 1985; Hagland and Pedersén, 1992).

At a pH of 9 or greater, free CO_2 is almost completely unavailable (Ryther and DeBusk, 1982; Hanisak and Ryther, 1984), and most of the DIC (> 60%) is in the form of CO_3^{2-}, which is unusable as a carbon source (Reiskind et al., 1989). *Palmaria mollis* grown at pH 9.13 is likely dependent on the use of a combination of HCO_3^- as well as limited daily CO_2 supply from both exchange water and respiration at night.

Seawater exchange

Previous work at NELH had shown that under high light (> 50 mol photons m^{-2} d^{-1}) supplying *P. mollis* with seawater exchange rates < 7.5 vol d^{-1} produced inadequate growth and deterioration of cultures (Demetropoulos, *unpublished data*), in spite of pH control (pH = 8.0). The present experiment showed that raising exchange rates from 7.5 to 60 vol d^{-1} substantially increased SGR's under high PFD's and kept pH (DIC) within acceptable limits. Moreover, a strategy of increased exchange rates can also enhance growth of *P. mollis* by improving other flow-dependent parameters such as culture temperature, supply of nutrients, removal of epiphyte spores and growth-inhibiting metabolites.

A seawater exchange rate of 7.5 vol d^{-1} was considered a mininum rate for economic commercial *P. mollis* production (Demetropoulos, *unpublished data*). Thus, an

attempt was made to develop a seawater exchange strategy that took into account both the higher SGR's associated with increased exchange (up to 15 vol d^{-1}), and cost savings associated with low exchange rates. This strategy was designed to provide high seawater exchange during periods of high photosynthetic rates in the light and reduced exchange during the dark phase.

Growth of cultures under a variable seawater exchange rate (high during the day and low at night; daily average of 7.5 vol d^{-1}) was similar to that of cultures supplied with a constant exchange rate of 15 vol d^{-1} with or without additions of CO_2. High exchange rates during the day had the effect of offsetting DIC limitation, removing metabolic wastes and O_2, as well as decreasing culture temperature. Further, reducing culture temperatures by increasing exchange rates during the day may be economically advantageous when the temperature of the supplied seawater cannot be easily cooled by mixing with cold deep water, as is possible at the NEHL site.

Cultures grown under variable seawater exchange conditions had a significantly higher SGR (by 11%) than those grown with a constant seawater exchange rate of 7.5 vol d^{-1} (control), in spite of receiving the same average amount of water during a 24 hour period. This suggests that increasing DIC or removal of metabolic wastes during high photosynthetic rates is primarily responsible for better yields under conditions of high exchange rates rather than improved micro-nutrient supply.

In conclusion, carbon limitation does not produce any obvious symptoms except slow growth (Bidwell, 1981). DIC additions to land-based *P. mollis* culture are essential in

one form or another. DIC sources generally used include sparged CO_2, HCO_3^-, and increasing seawater exchange rates; however, in terms of growth our work suggests that a combination of CO_2 + 6 mM HCO_3^- is most effective under high natural light (PFD > 30 mol photons m^{-2} d^{-1}) and at an exchange rate of 7.5 vol d^{-1}.

The best strategy to maintain optimal daytime pH in commercial cultures of *P. mollis* also depends on economic factors. An economic pH optimum for *P. mollis* culture is likely to be found between pH 8.5 to 8.8, due to the fact that SGR's vary little over the pH range of 8.3 to 8.9 and that direct costs associated with CO_2 injection decrease dramatically as pH increases from 8.1 to 8.75 (Braud and Amat, 1996).

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ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF STOCKING DENSITY, LIGHT, SALINITY, AND TEMPERATURE ON ALGAL GROWTH RATES

ABSTRACT

Palmaria mollis or Pacific dulse is used as both a biofilter and feed in temperate, land-based marine aquaculture of abalone and finfish. Land-based tumble-culture techniques capable of producing commercial quantities of *P. mollis* are still in development. In this study the effects of stocking density, light, salinity, and temperature requirements on growth rates and yields of *P. mollis* were examined.

Under a natural photon flux density (PFD) of 39 to 52 mol photons m^{-2} d^{-1} an aerial stocking density of 3 to 4 kg m^{-2} provided the best yield. At a specific light density (SLD) of 0.0048 to 0.036 mol photons g^{-1} [fresh wt.] d^{-1}, specific growth rates of *P. mollis* were highly correlated with natural and artificial light and no light saturation was evident up to an SLD of 0.036 mol photons g^{-1} [fresh wt.] d^{-1} at a culture temperature of 16±1° C.

Growth of *P. mollis* was best at a salinity of 30±1‰, whether municipal tap water or brackish well water was used for diluting seawater, and at a temperature of 12° C at low light intensities (SLD = 0.010 mol photons g^{-1} d^{-1}) and 14° to 18° C at high light intensities (SLD = 0.021 mol photons g^{-1} d^{-1}). Growth at higher temperatures was positively correlated with increased light. Lowering culture temperature at night from 15° C to 11° C

significantly increased growth for cultures that were not supplied with supplemental inorganic carbon.

INTRODUCTION

Palmaria mollis (= *Rhodymenia palmata* var. *mollis*, Setchell and Gardner 1903) (Rhodophyceae, Bangiales) is found in the low intertidal and upper subtidal rocky shore from Southeastern Alaska to San Luis Obispo County, California. It is closely related to *Palmaria palmata*, which is harvested and cultured in France, the British Isles, and the northeastern seaboard of the U.S. and Canada. In the Pacific, *P. mollis* is of interest mainly to the abalone industry where it supports high abalone growth rates (Evan and Langdon, 2000; Demetropoulos and Langdon, 2002d).

Optimal growth rates for mass culture of seaweeds such as *P. mollis* are typically achieved by adjusting ambient parameters such as light, temperature, salinity, pH (DIC), water turbulence, and nutrient availability. Laboratory-based photosynthetic and growth experiments used to determine these parameters are generally conducted under highly controlled conditions. While this type of experimentation is important in understanding fundamental physiological processes, drawbacks to this approach for application to long-term, commercial production include: use of low light levels, short duration (hours in some cases), and lack of an adequate acclimation period to experimental culture conditions such as light (cf., Gantt, 1990; Beach and Smith, 1996a).

Although some studies have been published describing the role of stocking density, light, salinity, and temperature on *P. palmata* (Robbins, 1978; Morgan et al., 1980; Morgan and Simpson, 1981), these culture parameters

have not been studied with *P. mollis* (cf., Davis, 1980). A wide range of light intensities (6 to 43 mol photons m^{-2} d^{-1}) appear to be adequate for growth in *P. palmata* (Morgan et al., 1980) and perhaps for *P. mollis* (Levin, 1991; Evans and Langdon, 2000). Further, light saturation has been reported to occur at 212 mol photons m^{-2} s^{-1} for both *P. palmata* (Robbins, 1978) and *P. mollis* (Davis, 1980). The optimal salinity for photosynthesis in *P. palmata* has been reported to be 32 ppt (Morgan et al., 1980). The genus *Palmaria* is considered to be cold temperate (Lindstrom et al., 1996). Studies with *P. palmata* have shown an optimal temperature range of 6-14° C for growth (Morgan and Simpson, 1981) with significant reductions in growth rate at or above 18° C.

The purpose of this study was to determine optimal stocking densities, light, salinity, and temperature conditions for large-scale production of *P. mollis*. We undertook some of the experiments on a large-scale at the Natural Energy Laboratory of Hawaii (NELH) aquaculture park located north of Kailua-Kona on the Big Island of Hawaii.

MATERIALS AND METHODS

Experiments were conducted at either the Hatfield Marine Science Center (HMSC) of Oregon State University or the Natural Energy Laboratory of Hawaii (NELH). Initial inocula of *P. mollis* cultures were provided by HMSC (OSU, C-3 strain; Demetropoulos and Langdon, 2001). Since commercial operations are often subject to air/water born epiphyte contamination, epiphytes were not eliminated from cultures. However, all nutrient enrichments were added during the dark cycle every three to four days so as to offset chlorophyte and epiphyte growth (Hanisak, 1987; J. McLachlan, *pers comm.*, Demetropoulos and Langdon, 2002a). Cultures were enriched with Guillard and Ryther's (1962) *f* medium, by pulse-fertilizing two times per week at night when water temperature allowed flow to the tanks to be shut off for four to six hours (Demetropoulos and Langdon, 2002a). Culture temperatures were maintained by adjusting relative proportions of cold and warm seawater supplied by NELH or by use of temperature-controlled seawater baths at HMSC. Relative concentrations of photosynthetically available inorganic carbon were estimated several times a week by measuring pH midway through the light and dark cycle.

Unless otherwise stated, all experimental treatments were tested in triplicate. Cultures were provided with adequate time (minimum two weeks) for acclimation to treatment conditions prior to experiments. This process took several months in Hawaii before growth rates became constant under treatment conditions. Metal halide lamps (1000 W) on a 16:8 photoperiod, were used in laboratory

experiments at HMSC. These were adjusted to produce photosynthetic active radiation (PAR) within a range of 400 to 900 μmol photons m^{-2} s^{-1} at the surface of culture vessels, resulting in average daily photon flux densities (PFD's) between 22.5 and 51.64 mol photons m^{-2} d^{-1}. One advantage in the use of these lamps was that they produced a significant amount of UV (UV spectral power = 0.03 W nm^{-1} 1000 $lumens^{-1}$; Osram Sylvania *pers. comm.*), and better-simulated outdoor conditions than other light sources such as fluorescent or high-pressure sodium lamps. Cultures at NELH were grown in outdoor tanks under natural light.

Typically, algae from culture vessels were damp-weighed weekly using either the spin cycle of a washing machine or a salad spinner, thinned, and returned to culture tanks at their original stocking densities. This method of measuring live weight *P. mollis* was found to be highly repeatable (±0.03).

Aerial stocking density

This experiment determined the affect of aerial or two dimensional stocking density (kg m^{-2}) on yield and SGR of *P. mollis* under moderate natural light at NELH (39 to 52 mol photons m^{-2} d^{-1}). Weekly yields and SGR's of *P. mollis* were recorded at six initial aerial stocking densities (0.6, 2, 3, 4, 5, and 6 kg m^{-2}) over a 35-day period in commercial (15,000 l) tanks. The tanks had a water depth of 70 cm. Each tank received approximately 10 vol d^{-1} of 16±1° C seawater to supply adequate dissolved inorganic carbon. Aeration was provided by a central line that

created two tumble-culture cells. The experiment was fully randomized and replicated in time.

Light

Since light represents one of the most important governing factors for land-based seaweed cultivation (Bidwell et al., 1985; McLachlan, 1991), the affect of light on growth was determined in order to develop a predictive equation for *P. mollis* production. This analysis was designed to allow prediction of growth rates as a function of total light available per fresh weight of *P. mollis*, and was expressed as specific light density (mol photons g^{-1}[fresh weight] d^{-1}). Dry-weight specific growth rates (SGR's) and yields of *P. mollis* were pooled from eight laboratory experiments and six large-scale experiments conducted under artificial and natural light at NELH and HMSC and plotted as a function of specific light density.

Pooling data across experiments was based on the following assumptions: 1) artificial light provided comparable growth to natural light at the same PFD, 2) dissolved inorganic carbon (DIC) was not limiting (pH = 8.1 to 8.5 across all experiments), 3) cultures were not nutrient limited (1*f* [Guillard and Ryther, 1962] was used in all cultures as described by Demetropoulos and Langdon, *this volume*), and 4) differences in algal density (g l^{-1}) resulted in only light-related effects on growth. Aeration was provided at 1.8 to 2.6 l min^{-1} resulting in an algal velocity relative to the water of > 10 cm s^{-1} [cf.,

Gonan et al., 1993; Demetropoulos, *unpublished data*]), Culture systems included 15,000 l tanks (depth, 70 cm), opaque 140 l tanks (depth, 70 cm), translucent 125 l tanks (depth, 65 cm), and opaque 3.8 l pitchers (depth, 28 cm). All cultures were provided with similar conditions (i.e., temperature, inorganic carbon, nutrients, aeration, etc.) and received between 1 and 15 vol d^{-1} of seawater. The mean density of *P. mollis* (g^{-1}) over a 7 day period was determined by log-transforming initial and final weights, calculating means, and anti-logging these values. Mean densities were used to calculate specific light densities (mol photons g^{-1}[fresh weight] d^{-1}).

Ambient PAR, expressed in terms of photon-flux density or PFD (mol photons m^{-2} d^{-1}) was quantified using a LI-192S (2 π) logged on a Licor LI-1000 data logger at HMSC and NELH. In experiments that used 125 l translucent tanks, it was necessary to quantify the total PFD using a combination of LI-192S (2 π) and LI-193S (4 π) sensors in order to include light transmitted through the tank's side wall. Two 125 l tanks were fitted with three sensors. A 2 π sensor on a black disk at the tank surface was used to measure surface light. The lower half of the same tank was blackened and a 4 π sensor was placed in the center of the top half to measure light passing through the wall of the top half portion. The surface and top half of a second tank was blackened and a 4 π sensor was placed in bottom center to measure light passing through the side walls in the bottom portion of the tank. Light measurements from these tanks over the course of the experiments were taken when supplied with air and water but with no additions of algae. Side walls of the

remaining tanks were opaque and light coming into these tanks was measured with a 2 π sensor on a black disk at the surface of the tanks. Specific light density (mol photons g^{-1} [fresh wt.] d^{-1}) was calculated as:

$$SLD = (PFD*SA/FW) \quad (1)$$

where SLD was specific light density, PFD was photon-flux density in mol photons, SA was the area of illuminated surface in m^2, FW was fresh weight of *P. mollis* in grams. SLD was expressed in terms of *P. mollis* fresh weight (in grams) for practical use in daily calculations. Growth rates and yields were calculated as:

$$SGR = 100 \text{ X } (\ln W_f - \ln W_i)/t \quad (2)$$

and

$$Yield = (W_f - W_i)/(SA \text{ X } t) \quad (3)$$

where SGR was specific growth rate (% [dw] d^{-1}), W_f and W_i were final and initial algal dry weights in grams, SA was the surface area of tanks exposed to light in m^2, and t was time (days).

Salinity

The effects of reduced salinity on the growth of *P. mollis* was studied because the salinity of seawater at NELH was 35‰ or approximately 3‰ higher than the reported optimal salinity for *P. mollis* (Davis, 1980) and 7‰ higher than optimal for cultures being grown in Tomales Bay, CA (Tim Fleming, *pers comm.*). The first experiment was conducted to validate the effectiveness of lowering culture salinity within the range proscribed by others for *P. mollis*. The second experiment was conducted to evaluate the use of brackish well water for maintaining lower salinity in mass culture.

Salinity adjustments using municipal fresh water: *P. mollis* rosettes were randomly placed in triplicate 140 liter, opaque tanks at a density of 7g l^{-1} (6.5 kg m^{-2}) supplied with continuous flow of 16±1° C seawater (by mixing surface and deep water) at an exchange rate of 10 vol d^{-1}. Tanks were tumble-cultured under a natural Hawaiian PFD of 31 to 43 mol photons m^{-2} d^{-1}. The high salinity treatment (35‰) was obtained using deep seawater provided by NELH. The low salinity treatment (30±1‰) was obtained by addition of chlorine-free tap water mixed with deep seawater. Carbon dioxide was sparged through the cultures to achieve a daytime pH = 8.1±0.1 (Demetropoulos and Langdon, *this volume*). The experiment was carried out for 35 days beyond a 14-day acclimation period.

Salinity adjustments using brackish well water: In the second experiment, the growth effects two salinities (30±1‰ and 35‰) were evaluated. Salinities were obtained by mixing a combination of brackish well water, warm

surface seawater, and cold deep seawater. The treatments were each randomly assigned to triplicate 125 l translucent tanks. A randomized block design was used to account for possible temperature and light differences due to the position of the tanks. Rosettes were stocked at a density of 8 g l^{-1}. Each tank received approximately 60 vol d^{-1} (at 16±1° C) to supply dissolved inorganic carbon and maintain pH (daytime pH = 7.9±0.2). Total PFD was quantified using a combination of 2 π and 4 π sensors as described above. The experiment was carried out for 28 days beyond a 14-day acclimation period.

Temperature and light

Temperature: This experiment was designed to determine the effect of temperature on the growth of *P. mollis* under high natural light at NELH (45 to 54 mol photons m^{-2} d^{-1}). Specific growth rates of *P. mollis* in duplicate cultures at three temperatures (14±1° C, 16±1° C and 18±1° C) were determined. Temperatures were generated using a combination of warm surface and cold deep seawater sources. A randomized block design was used to account for possible temperature and light differences among cultures. Rosettes were stocked at an initial density of 8 g l^{-1} in 125 l translucent-walled tanks. Light was measured in two separate tanks using the same combination of 2 π and 4 π sensors described in the light methods above. Each tank received approximately 60 vol d^{-1} of seawater to supply adequate dissolved inorganic carbon.

The experiment ran for 28 days after a 14-day acclimation period.

Interaction between temperature and light: Possible interaction effects between temperature and light on *P. mollis* growth were investigated in the following experiment at HMSC. The experiment was designed as a 6 X 2 factorial, with six temperature treatments (12±1° C, 14±1° C, 16±1° C, 18±1° C, 20±1° C, and 22±1° C) at two light levels (13 and 29 mol photons m^{-2} d^{-1}, equivalent to 0.010 and 0.021 mol photons g^{-1} d^{-1}, respectively. Triplicate 3.8 l vessels with opaque walls were placed in seawater baths and aerated vigorously at a rate of 1.8 l min^{-1}. In order to ensure adequate supplies of inorganic carbon under low exchange rates with no additions of inorganic carbon, vessels were stocked with low densities of *P. mollis* at 0.5 Kg (fresh wt) m^{-2} or 2.6 g l^{-1} under a seawater flow rate of 1 vol d^{-1}. Salinity ranged from 30 to 32‰. A hexagonal "box" covered with mylar film was placed around the vessels to block out ambient light and evenly distribute artificial light intensities. Submersible heaters in the water baths were used for temperature control. The experiment was carried out for 21 days beyond a 14-day acclimation period.

Manipulation of diurnal temperature: This experiment was conducted to determine if lowering temperature at night could increase *P. mollis* growth under high natural light at NELH (43 to 50 mol photons m^{-2} d^{-1}). It was designed as a 2 X 2 factorial experiment with two temperature treatments - either (1) 15±1° C over 24 hr, or (2) 15±1° C from 0800 to 1700 hr and 11±1° C from 1700 to 0800 hr, and two levels of CO_2 (with additions of CO_2 to

maintain pH 8.1, and no additions of CO_2 resulting in pH values that diurnally ranged from 6.7 to 8.8). A randomized block design was used to account for possible temperature and light differences according to culture position. Initial stocking density was 8 g l^{-1} in 125 l translucent-walled tanks with a seawater exchange rate of 15 vol d^{-1}. The experiment ran for 28 days beyond a 14-day acclimation period.

Chemical analysis

Water chemistry and tissue nutrient analyses were performed by Fruit Growers Lab, Santa Paula, CA. All tissue samples were frozen and were ground into a fine powder. Seawater nitrate concentrations and *P. mollis* tissue N were measured using a LECO, CNS 2000 elemental analyzer. Trace metals were determined after ashing (Jarrall Ash method) followed by measurement with an inductively-coupled argon plasma spectrophotometer.

Statistics

Experiments were set up as either fully randomized factorial or as randomized block designs, as noted above. Single factor ANOVA, two-factor ANOVA, or two-factor ANCOVA (with light as the covariant) were used to test for significant ($\alpha = 0.05$) treatment effects. Significant differences among treatments were compared by the Student-Newman-Keuls (SNK) multiple comparison test ($\alpha = 0.05$).

RESULTS

Aerial stocking density

Figure 3.1 shows yield and SGR as a function of initial aerial density. Under a natural Hawaiian PFD ranging from 39 to 52 mol photons m^{-2} d^{-1} the relationship between yield, SGR and initial aerial density (light availability) of *P. mollis* was described by the general equations:

$$\text{Yield} = 8.96 + 25.35\ x - 2.32\ x^2 - 0.185\ x^3 \quad (4)$$

$$(r^2 = 0.75,\ n = 67)$$

$$\text{SGR} = 20.517 * e^{-0.25847x} \quad (5)$$

$$(r^2 = 0.91,\ n = 67)$$

where x was the density of *P. mollis* per unit area. A third degree polynomial provided the best fit for yield (Fig. 3.1, Eq. 4) while SGR declined exponentially with increasing density (Fig. 3.1, Eq. 5). ANCOVA (light = covariant) followed by SNK showed stocking densities of 3 to 4 kg m^{-2} supported significantly (ANCOVA, SNK; $P < 0.0001$) higher yields of *P. mollis* than all other stocking densities under this range of PFD's.

Light

Specific growth rates (SGR) of *P. mollis* as a function of natural and artificial specific light density (SLD) in mol photon g^{-1} [fresh wt.] d^{-1} are summarized in Figure 3.2. SGR's were a logarithmic function of SLD (Eq. 6) up to an SLD of 0.036 mol photon g^{-1} [fresh wt.] d^{-1} (Fig. 3.2).

$$SGR = 40.896 + 16.107 \log (\text{mol photon } g^{-1}[\text{fw}]\ d^{-1}) \qquad (6)$$

($r^2 = 0.92$, $n = 139$, $P < 0.0001$)

Figure 3.1: Specific growth rate and yield of *Palmaria mollis* (± SE) measured weekly over a 35-day period as a function of six aerial densities (0.6, 2, 3, 4, 5, and 6 kg m^{-2}) under natural light (PFD = 39 to 52 mol photons m^{-2} d^{-1}). Tanks were 15,000 l, 70 cm deep and received approximately 10 vol d^{-1} of 16±1° C seawater. Cultures were enriched with Guillard and Ryther's (1962) *f* medium by pulse-fertilizing two times per week at night.

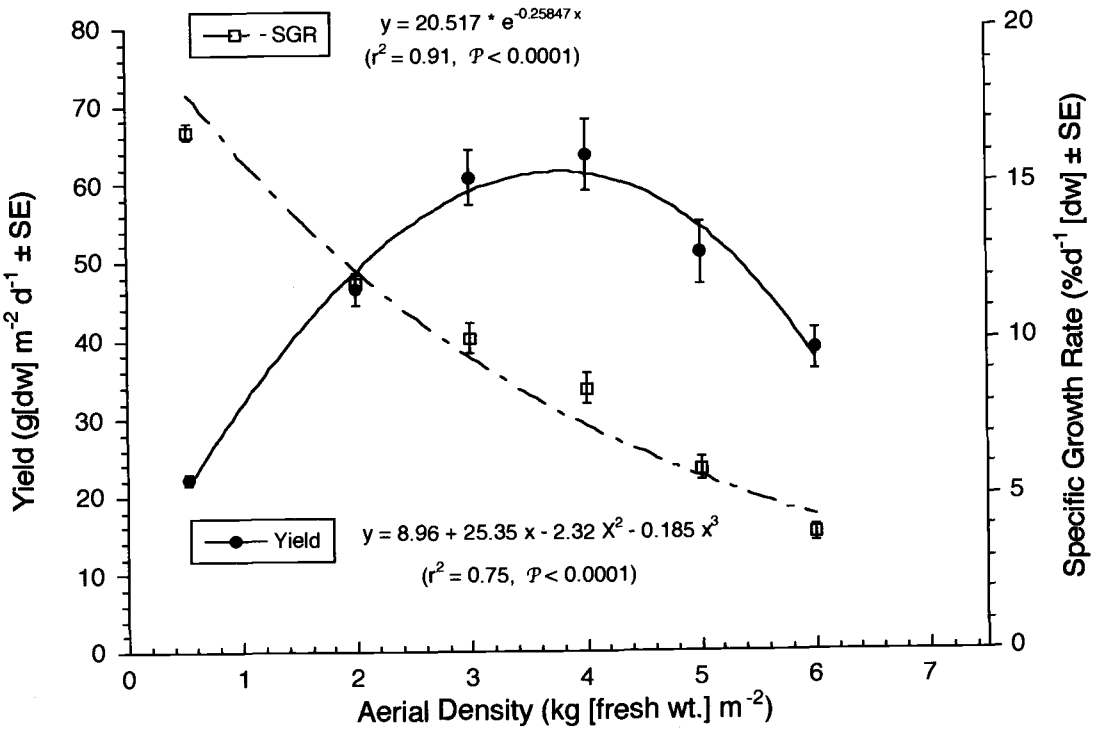
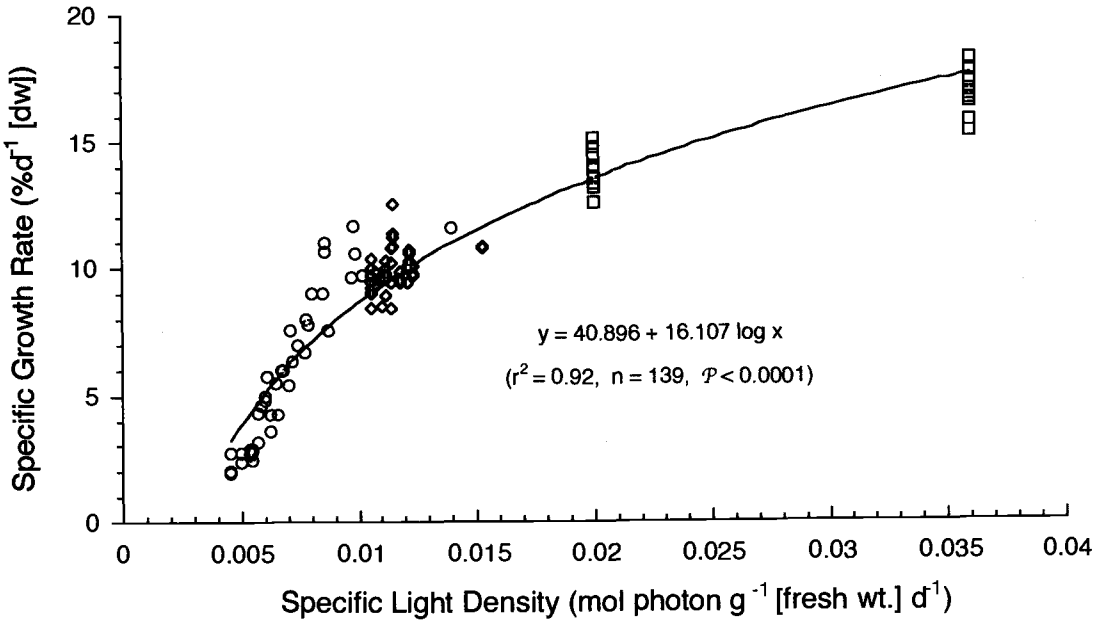


Figure 3.2: Specific growth rate of *Palmaria mollis* as a function of specific light density (SLD). Values are pooled from eight laboratory experiments and six large-scale experiments conducted under artificial and natural light. Culture conditions included: O = opaque 140 and 15,000 l tanks (depth, 70 cm) with natural light, ◊ = translucent 125 l tanks (depth, 65 cm) with natural light, and □ = opaque 3.8 l pitchers (depth, 28 cm) with artificial light. All cultures were provided with similar growth parameters; temperature = 16±1° C, pH = 8.1 to 8.5, nutrients = Guillard and Ryther's (1962) *f* medium, pulse-fertilized two times per week at night. Cultures received between 1 and 15 vol d^{-1} of seawater.



Salinity

Salinity adjustments using municipal fresh water: *Palmaria mollis* grew better at the lower salinity of 30±1‰ than the higher salinity of 35‰ (Fig. 3.3). The differences between these two treatments tended to widen with increasing PFD. Under the lowest PFD recorded (31.6 mol photons m^{-2} d^{-1}; SLD = 0.0046 mol photons g^{-1} [fresh wt.] d^{-1}) there was no significant difference in SGR's between the two treatments (ANOVA; P = 0.880). Under increasing PFD up to 42.5 mol photons m^{-2} d^{-1}; SLD = 0.0062 mol photons g^{-1} [fresh wt.] d^{-1} growth at a salinity of 30±1‰ was significantly higher (ANOVA, P < 0.0001), producing SGR's up to 24% higher than at a salinity of 35‰.

Salinity adjustments using brackish well water: Lowering salinity from 35‰ to 30±1‰ with the use of brackish well water at NELH significantly (ANOVA; P < 0.0001) improved growth of *P. mollis*, resulting in SGR's that were 8.8 to 14.3% higher than treatments where surface/deep seawater was used. Across all light levels the lower salinity produced a mean SGR of 10.92 ± 0.64% d^{-1} vs. 9.61 ± 0.74% d^{-1} with the high salinity treatment (Fig. 3.4). Light levels for this experiment were much higher than in the first salinity experiment (48 to 52 mol photons m^{-2} d^{-1}; SLD = 0.01168 to 0.01237 mol photons g^{-1} [fresh wt.] d^{-1}).

Figure 3.3: Specific growth rate of *Palmaria mollis* (± SE) as a function of specific light density (SLD) and photon flux density (PFD) for two salinity treatments (30±1‰ and 35‰) under natural light over a 35-day period. The 30±1‰ treatment was generated by combining non-chlorinated municipal freshwater with deep seawater provided by NELH and the 35‰ treatment was generated with deep seawater alone. Tanks were opaque, 140 l and received approximately 10 vol d^{-1} of 16±1° C seawater. Cultures were enriched with Guillard and Ryther's (1962) *f* medium by pulse-fertilizing two times per week at night.

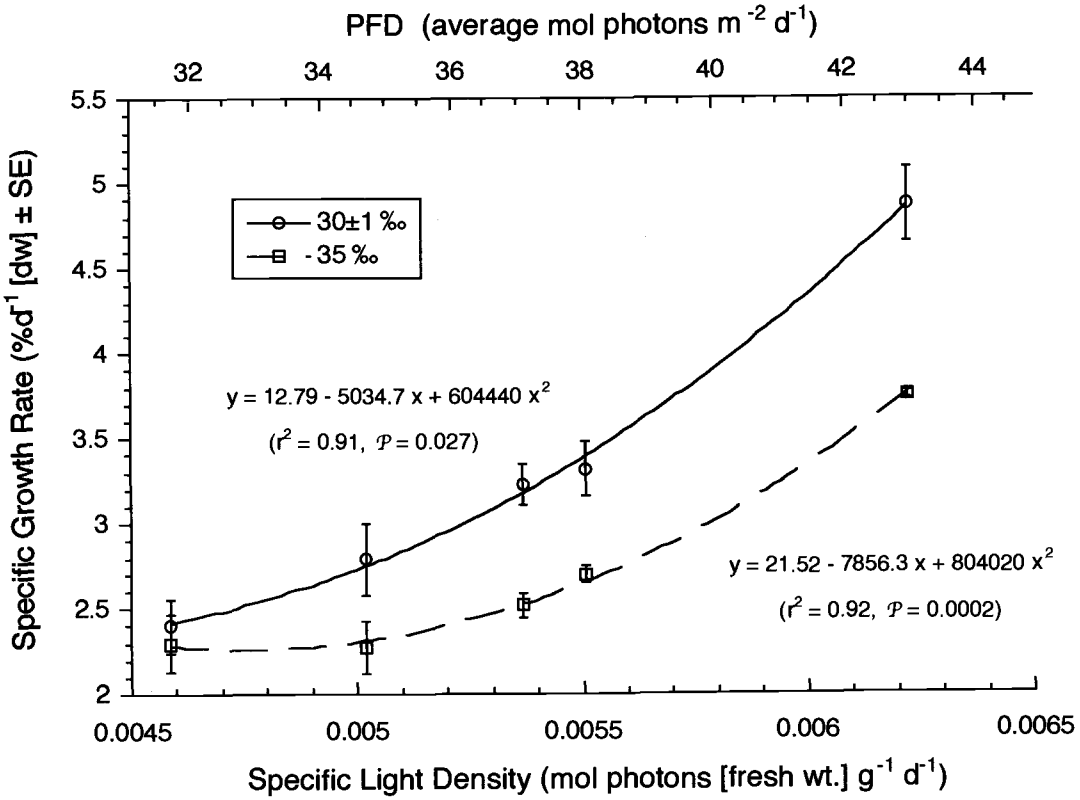
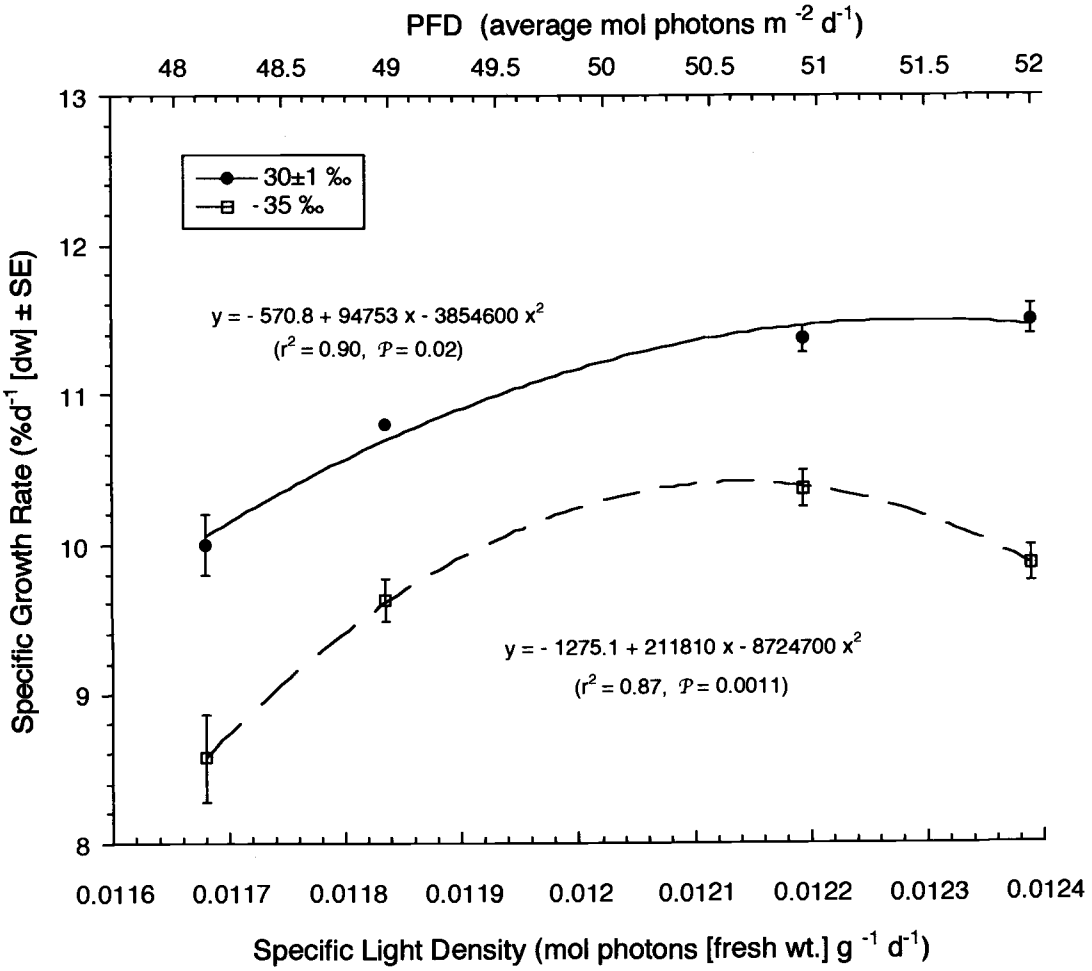


Figure 3.4: Specific growth rate of *Palmaria mollis* (± SE) as a function of specific light density (SLD) and aerial photon flux density (PFD) for two salinity treatments (30±1‰ and 35‰) under natural light over a 28-day period. The 30±1‰ treatment was generated by combining brackish well water with deep/surface seawater provided by NELH and the 35‰ treatment was generated with deep/surface seawater alone. Tanks were translucent, 125 l and received approximately 60 vol d^{-1} of 16±1° C seawater. Cultures were enriched with Guillard and Ryther's (1962) *f* medium by pulse-fertilizing two times per week at night.



Analysis of the brackish water showed that it had a slightly lower pH (7.60) than the mixtures of warm surface and deep cold water supplied by NELH (pH = 7.94). Brackish water was slightly higher in NO_3^- than deep cold water (42.06 μM compared to 39.03 μM) and PO_4^- was twice that found in deep cold water (6.39 μM compared to 2.89 μM). The warm surface water had far less NO_3^- (0.24 μ M) and PO_4^- (0.15 μM) than either brackish water or deep water. No other significant differences were observed in the nutrient content of the brackish water and seawater at NELH. Algal tissue analysis showed both of the salinity treatments resulted in comparable crude protein contents (21%), but different levels of carbohydrate (30±1‰ treatment = 29.23% vs. 35‰ treatment = 43.53%). *Palmaria mollis* from the brackish water treatment had twice the level of Mg and approximately three times the levels of Zn and B than *P. mollis* from the 35‰ treatment. These nutrients may be important to the growth of *P. mollis* (Lobban and Harrison, 1997).

Temperature and light

Results of experiments conducted at NELH, Hawaii, showed there were no significant differences (ANOVA; P = 0.063) between growth rates of *P. mollis* cultured at temperatures of 14±1 to 18±1° C (SGR = 11.1±0.54% d^{-1} and 9.2±0.5% d^{-1} respectively), although there was a decreasing growth trend with increasing temperature (Fig. 3.5). These treatments produced yields of approximately 173 to 197 g [dw] m^{-3} d^{-1}. Apart from a slight browning in color

there were no morphological differences among treatments. Ambient sunlight resulted in a total average PFD of approximately 55 mol photons m^{-2} d^{-1} or approximately 0.015 mol photons g^{-1} [fresh wt.] d^{-1}. Epiphytes did not present a problem in this experiment.

Interaction between temperature and light: Growth rates as a function of temperature and artificial light at HMSC are shown in Figure 3.5 and Appendix 4. Growth of *P. mollis* was maximum under low light (13 mol photons m^{-2} d^{-1} or 0.010 mol photons g^{-1} d^{-1}) between 12±1° to 14±1° C (SGR = 8.69±0.29% d^{-1}) and under high light (29 mol photons m^{-2} d^{-1} or 0.021 mol photons g^{-1} d^{-1}) between 14±1° to 18±1° C (SGR = 10.5±0.12% d^{-1}). Growth above these temperatures was significantly reduced and deterioration of the thallus took place above 18±1° C for the low light and above 20° C for the high light treatments after 21 days. Across all treatments there was a significant interaction (ANOVA, $P < 0.0001$) between light and temperatature (Appendix 4). Under high light conditions, treatments could be categorized into three significantly different groups from highest to lowest growth effect: (14±1 to 18±1° C), (12±1° C), and (20±1° to 22±1° C) (ANOVA, SNK; $P < 0.0001$). Under low light there was no difference in growth between 12±1° C and 14±1° C, while growth at higher temperatures were significantly different from one another and decreased with increasing temperature (ANOVA, SNK; $P < 0.0001$) (Fig. 3.5 and Appendix 4). Typically, necrosis of apices occurred during the third week under high temperatures (≥20±1° C) at both light levels and epiphytes were more of a problem at 18±1° C or higher.

Manipulation of diurnal temperature: Lowering culture temperature at night provided a mixed result for growth rates of *P. mollis* (Fig. 3.6). Without CO_2 additions (daytime pH = 8.58 to 8.99) lowering culture temperature to 11±1° C at night resulted in a significantly higher SGR (ANOVA, SNK; $P = 0.025$) compared to that of cultures held at a constant temperature of 15±1° C. However, when CO_2 was sparged through the cultures to maintain a daytime pH of 8.1±0.1 there was no significant difference in daily growth between the two temperature treatments (Fig. 3.6). Ambient sunlight resulted in a total average daytime PFD of approximately 49 mol photons m^{-2} d^{-1} or approximately 0.01186 mol photons g^{-1} [fresh wt.] d^{-1}.

Figure 3.5: Specific growth rate of *Palmaria mollis* (± SE) as a function of temperature at an average natural PFD of 55 mol photons m^{-2} d^{-1} (0.016 mol photons g^{-1} [fresh wt.] d^{-1}) and an artificial PFD of 13 or 29 mol photons m^{-2} d^{-1} (0.010 and 0.021 mol photons g^{-1} [fresh wt.] d^{-1}, respectively), from the temperature and temperature/light interaction experiments. Culture conditions included: ▲ = natural light, translucent 125 l tanks (depth, 65 cm) receiving 60 vol d^{-1} of seawater at NELH, O and □ = artificial light, opaque 3.8 l pitchers (depth, 28 cm) receiving 1 vol d^{-1} of seawater at HMSC. Cultures were enriched with Guillard and Ryther's (1962) *f* medium by pulse fertilizing two times per week at night. Values represent means for the last 28 days of the natural light experiment and the last week of the 21-day artificial light experiment. For both experiments there were no significant differences in *P. mollis* growth between 14 and 18° C under the high PFD (ANOVA; $P = 0.063$ at NELH and ANOVA; $P = 0.059$ at HMSC).

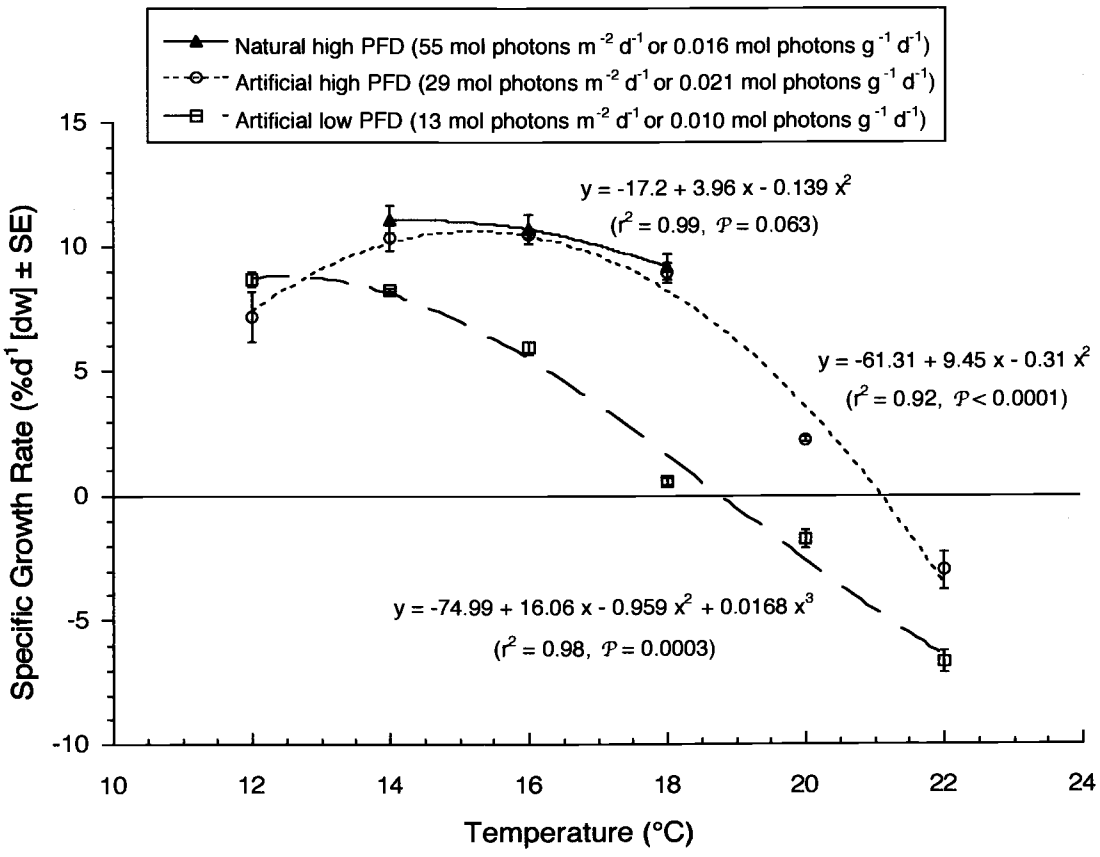
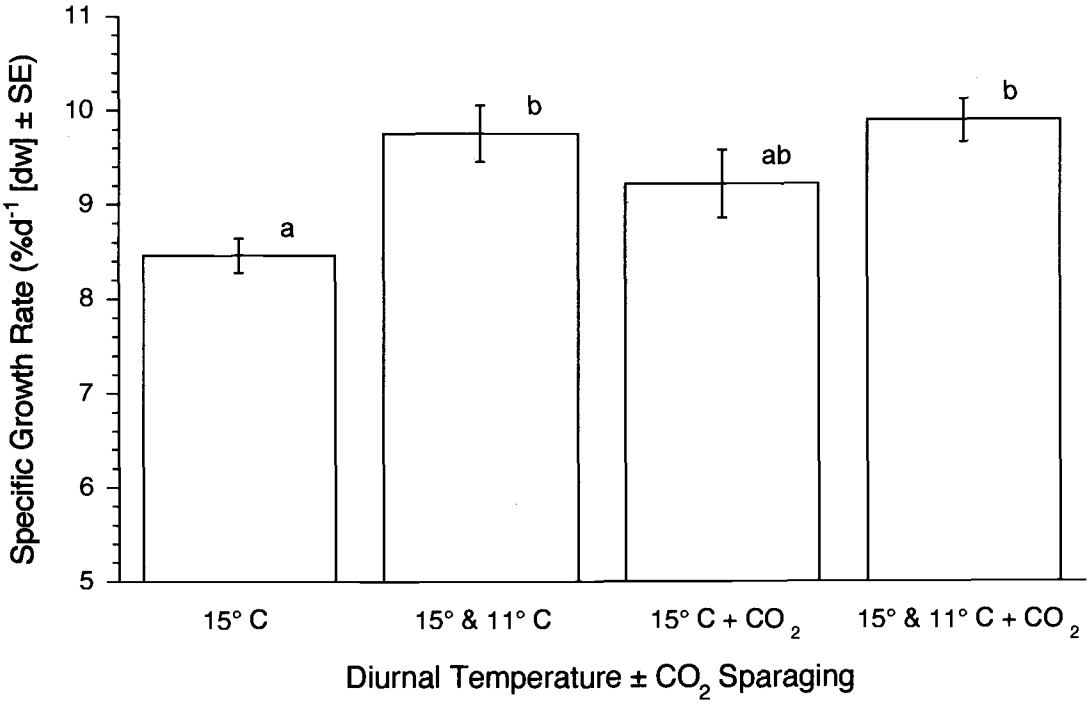


Figure 3.6: Specific growth rate of *Palmaria mollis* (± SE) as a function of lowering temperature from 15±1° C during the day to 11±1° C during the night both with and without CO_2 sparging. The average PFD was 49 mol photons m^{-2} d^{-1} or approximately 0.01186 mol photons g^{-1} [fresh wt.] d^{-1}. Tanks were translucent 125 l and received approximately 15 vol d^{-1} of seawater. Cultures were enriched with Guillard and Ryther's (1962) *f* medium by pulse-fertilizing two times per week at night. Values represent means for the last 28 days of a 35-day experiment. Letters a and b indicate significant differences (ANOVA, SNK; $P = 0.025$).



DISCUSSION

Stocking density

Figure 3.1 shows yield and SGR as a function of *P. mollis* initial fresh weight aerial densities (kg m^{-2}) under a natural PFD of 39 to 52 mol photons m^{-2} d^{-1}. The figure shows SGR decreased exponentially while yield initially increased and then decreased as both direct and collateral light become limiting. While yield is typically the product of SGR and density, the plot shows yields that are slightly higher than predicted due to increasing biomass over 7 days of growth. Under this PFD (39 to 52 mol photons m^{-2} d^{-1}), an initial stocking density of between 3 and 4 kg m^{-2} provided the best yield.

Light

The production of seaweeds in land-based systems is dependent on the intensity and duration of light exposure. Optimum levels of these two factors vary widely from species to species. Provided all other factors are not limiting, production will ultimately become light-limited as a result of self-shading and absorption of light by water and water-born contaminants. However, one of the difficulties in studying light as a parameter is comparing growth rates (both from the literature and in a practice) under different tank culture conditions such as depth and stocking density because light intensity is affected by these factors. Understanding species-specific light

requirements under culture conditions and designing systems that supply adequate light are among the most important challenges in managing land-based seaweed culture systems.

Both *P. palmata* and *P. mollis* are considered low intertidal to subtidal species with similar physiological characteristics. Light saturation or P_{max} for *P. palmata* is reported to occur at approximately 212 μmol photons m^{-2} s^{-1} PAR (Robbins, 1978) and may be the same for *P. mollis* (Davis, 1980). This is much lower than the lowest PFD recorded (approximately 553 μmol photons m^{-2} s^{-1}) from 0900 to 1500 hr in the center of 125 l translucent vessels at NELH, and indicates that *P. mollis* in these cultures (and those under higher light at HMSC) were receiving PFD's well beyond reported saturation limits, with the result of increasing growth rates. Under the highest SLD supplied, *P. mollis* showed growth rates of up to 18% d^{-1} (Fig. 3.2, artificial light).

Our results indicate that with optimal stocking density, DIC, adequate supply of macro and micro nutrients, and water motion, *P. mollis* can grow well for sustained periods of time (months) under PFD's which are considerably higher than PFD's typical of temperate regions (i.e., 2400 μmol photons m^{-2} s^{-1} in Hawaii vs. 1800 μmol photons m^{-2} s^{-1} from central California to Alaska) (cf., Guist, et al., 1982).

Improved nutrient conditions, espiecally the supply of Fe, Zn, and Mn, provided in this study may have allowed high P_{max} values (cf., Guist et al., 1982; Demetropoulos and Langdon, *this volume*). Further, cycling plants between light and dark in air-generated culture cells may

also allow *P. mollis* cultures to grow under high PFD conditions (cf., Bidwell et al., 1985; Terry, 1986; Kübler and Raven, 1996a, 1996b). High growth rates are also likely to be due to photoadapation of *P. mollis* to high PFD's resulting in increased carotenoid and phycobillin pigment ratios, and increases in UV-A absorbing substances (absorbance between 320-400nm) from undetectable levels to approximately 28 $\mu g\ g^{-1}$[fw] (Demetropoulos, *unpublished data*). Similary, Beach and Smith (1996a, 1996b) found higher carotenoid and UV-absorbing pigments and greater photosynthetic performance for naturally occurring tropical rhodophytes growing as a canopy (high light) compared with those from understory (low light) microhabitats. Thus, while some have suggested a P_{max} of 212 μmol photons $m^{-2}\ s^{-1}$ may preclude growth of *P. mollis* under high natural PFD's (Davis, 1980), long-term physiological adjustments under optimal mass culture conditions outlined in this research may allow photoacclimation of *P. mollis* to high natural PFD's.

Figure 3.2 indicates SGR's of *P. mollis* can be estimated as a function of specific light density expressed in terms of mol photons g^{-1} [fresh wt.] d^{-1}, independently of culture vessel type or light source (artificial vs. natural light). The SLD log function predicts light-saturation above 0.036 mol photons g^{-1} [fresh wt.] d^{-1} at a culture temperature of 16±1° C (the average temperature of the experiments). Under lower temperatures light-saturation may be lower as evident in the temperature experiment conducted as part of this study (Fig. 3.5). The SLD log function provides information for adjusting density to match ambient light in *P. mollis* mass

culture in order to produce higher yields. Potential yield may be calculated by taking the product of SGR (as a function of light) and the stocking density. Further, while most land-based tank culture systems that receive light only from the surface (two dimensionally) will operate at lower SLD's of the model (< 0.01 mol photons g^{-1} [fresh wt.] d^{-1} based on 140 and 15,000 l opaque walled tanks at NELH) these data suggest *P. mollis* is suitable for use in photobioreactors where light from three dimensions can easily exceed 0.04 mol photons g^{-1} [fresh wt.] d^{-1}.

Salinity

Lowering salinity to 30±1‰ with the use of either municipal water or brackish water significantly improved growth of *P. mollis* in both salinity experiments and under the two light conditions (SLD = 0.0046 to 0.0124 mol photons g^{-1} [fresh wt.] d^{-1}). The magnitude of difference in SGR's between salinity treatments in the two experiments was about the same (SGR differed by 1 to 1.5% d^{-1}). However, growth rates decreased at a salinity of 35‰ while they slightly increased at a salinity of 30±1‰ at the highest PFD recorded in the second experiment (SLD = 0. 0124 mol photons g^{-1} [wet wt.] d^{-1}), indicating a negative interaction between high salinity and high light (Fig 3.4). This can be compared with with Figure 3.2 where high growth rates were associated with high SLD's under the lower salinity of HMSC treatments. Salinities above 32‰ are known to increase the energy required for

osmotic adjustment, negatively affecting the ability to utilize available DIC for growth (Lobban and Harrison, 1997). Our result is consistent with the work of Davis (1980) who showed an optimal salinity between 30 to 32‰ for *P. mollis* cultured in Puget Sound and with Robbins (1978) who showed a sharp decline in photosynthetic and respiratory rates when *P. palmata* was exposed to salinities above 32‰. The lower salinity treatments in both experiments were also remarkably free of epiphytes, supporting work carried out by Friedlander (1992) who showed a similar result with *Gracilaria conferta* cultures in Israel at salinities of 40‰ compared to those of 30‰.

Temperature and light

While temperature requirements for *P. mollis* have not been previously reported, our results agree with those of Robbins (1978) and Morgan et al. (1980) who indicated *P. palmata* grew best at 14° to 15° C under similar light conditions (20 to 35 mol photons m^{-2} d^{-1}). The NELH temperature experiment showed that the yield of *P. mollis* (ca. approximately 173 g [dw] m^{-3} d^{-1}) grown at 18±1° C was not significantly different from that of *P. mollis* cultured at 14±1° to 16±1° C (Fig. 3.5). The data also show optimal temperature for SGR is a function of light for *P. mollis*, with increased light supporting higher growth rates at a higher temperature due to an interaction between light and temperature (Fig. 3.5 and Appendix 4). This result is similar to that reported by Mathieson and Norall (1975) for *Chondrus crispus* who showed

photosynthesis of winter plants was maximum at a lower temperature than for summer plants.

The high yields and SGR's at NELH were due to the use of translucent tanks in combination with moderate *P. mollis* stocking densities (SLD = 0.015 mol photons g^{-1} [fresh wt.] d^{-1}), high seawater flushing (60 vol d^{-1}), and high natural light (55 mol photons m^{-2} d^{-1}). Lignell et al. (1987) reported similar high SGR's for *Gracilaria secundata* by submerging lights in the culture vessels.

Manipulation of diurnal temperatures showed that reducing culture temperature to 11±1° C at night increased yields by as much as 13.4% in the treatment with no CO_2 additions. Such a temperature reduction can be as beneficial as maintaining a constant temperature of 15±1° C and pH ≤ 8.0 with CO_2 additions during the day (Fig. 3.6). Increased growth rates with cooler night temperatures are likely due to enhanced carbon fixation. Conserved energy and fixed carbon during dark respiration at a lowered culture temperature (Davidson, 1991) could be made available for growth during the following photoperiod. This phenomenon (known as thermal periodicity [Noggle and Fritz, 1983; Lobban and Harrison, 1997]) has a parallel in flowering plants, which can grow better when subjected to reduced night temperatures.

In summary, by making adjustments to the culture parameters described above, high *P. mollis* SGR's can be attained. In particular, when cultures are provided with proper nutrition (N,P, trace metals, and DIC) and adequate water turbulence (> 10 cm s^{-1}; Demetropoulos and Langdon, 2000) as previously described, there is no light saturation for *P. mollis* up to an SLD of 0.036 mol photons

g^{-1} [fresh wt.] d^{-1}. Adjustment of stocking density to light level can be made in order to maximize yields, typically resulting in an optimal stocking density of between 2 to 4 kg m^{-2}. This optimal aerial stocking density generally agrees with work reported for *Gracilaria sp.* and other macrophytes in culture (Lapointe and Ryther, 1978; McLachlan 1991; Ugarte and Santelices, 1992; Friedlander and Levy, 1995). Salinities of *P. mollis* cultures should be adjusted to 30±1‰. *Palmaria mollis* is a temperate species and requires cool (≤ 16° C) water to grow well. However, the *P. mollis* OSU C-3 strain used in this study (Demetropoulos and Langdon, 2001) may be able to tolerate and grow well at warmer temperatures (18±1° C in these experiments) for many weeks (Demetropoulos, *unpublished data*). Finally, where it is economically feasible, water temperature should be lowered at night to further increase yields.

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ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH ABALONE IN A LAND-BASED SYSTEM: EFFECTS OF *PALMARIA MOLLIS* NUTRIENT ENRICHMENT AND BIOCHEMICAL COMPOSITION ON ABALONE GROWTH AND CONDITION

ABSTRACT

Two growth experiments were conducted with juvenile red abalone, *Haliotis rufescens* and Japanese abalone, *Haliotis discus hannai*. The first experiment was set up to determine if nutrient levels used for co-culture of the Rhodophyte, Pacific dulse (*Palmaria mollis*) directly affected abalone growth. No significant differences (ANOVA; $P = 0.117$) were found in the growth of abalone cultured in either ambient seawater alone or seawater supplemented with nutrients (Fe, Zn, Mn, Cu, Mo, and CO), phosphate, and nitrate additions (1176, 1765, 2353, and 2942 μM d^{-1} NO_3^- - N) as a modified version of Guillard and Ryther's (1962) *f* medium.

The second experiment was designed to determine the effect of different nutrient levels on the nutritional value of *P. mollis* when fed to abalone. *Palmaria mollis* was cultured with different $NaNO_3$ loads (1176, 1765, 2353, and 2942 μM d^{-1} NO_3^- - N) and NaH_2PO_4 (83.3 μM d^{-1} PO_4^-) in the presence or absence of trace metals (Fe, Zn, Mn, Cu, Mo, and Co). *Haliotis discus hannai* showed the highest linear shell growth and SGR when fed on *P. mollis* supplemented with nitrate loads ranging from 1176 to 2353 μM d^{-1} NO_3^- - N. In contrast, *H. rufescens* showed the highest linear shell growth when fed on *P. mollis* supplemented with higher nitrate loads of 2353 to 2942 μM

d^{-1} NO_3^- - N, while nitrate load had no significant effect on specific growth rates. Both abalone species grew better on *P. mollis* supplied with 0.75*f* + Zn (d^{-1}) metal solution (Guillard and Ryther, 1962) compared with those without trace metal additions, with *H. discus hannai* showing a further improvement in FCE.

The present work shows that understanding and manipulating the culture environment of *P. mollis* can significantly affect growth, FCE, and DFC of *H. discus hannai* and *H. rufescens*.

INTRODUCTION

The major limiting factor in expansion of abalone cultivation continues to be the availability of suitable and inexpensive diets. Farm-reared abalone have not been produced in sufficient quantity and quality to meet the world market demand with the result that farm-gate prices for shell-on, 80 to 100mm red abalone (*Haliotis rufescens*) have reached $10.35/kg (U.S.) as of February, 2002.

Traditional commercial abalone culture systems in the U.S. are based on supplying abalone with harvested kelp (*Macrocystis* sp. and *Nereocystis luetkeana*). Unfortunately, the nutritional quality of kelp varies seasonally and kelp degrades after a few days in the culture system, leading to deterioration in water quality and accumulation of ammonia unless high water exchange rates are maintained.

Research at the Hatfield Marine Science Center (HMSC), Oregon State University, has focused on developing a high yield method for co-culturing abalone and seaweed using low seawater exchange rates (1 vol d^{-1}). The fundamental goal was to help create a more profitable abalone culture industry over a larger geographic range, including areas with good water quality remote from kelp beds. Levin (1991) first demonstrated the effectiveness of using the Rhodophyte, *Palmaria mollis* (= *Rhodymenia palmata* var. *mollis*, Setchell and Gardner 1903) in removing ammonia, nitrate, and phosphate from aquaculture effluents and subsequently feeding the algal biofilter to abalone.

Recent studies have shown that both *Palmaria palmata* and *P. mollis* can support similar or better growth

compared to artificial diets or other macro-algal species for *Haliotis tuberculata*, *H. discus hannai* and *H. rufescens* (Mercer et al., 1993; Mai et al., 1994, 1995, 1996; Rosen et al., 2000; Evans, *unpublished data*; Demetropoulos and Langdon, *in prep.*). Work by Evans and Langdon (2000) demonstrated that *P. mollis* could be maintained in co-culture with *H. rufescens* as a reliable supply of nutritious food while simultaneously ensuring high water quality through the uptake of excreted NH_4^+. Rosen et al. (2000) showed that culture conditions of *P. mollis* could have significant effects on its nutritional value for *H. rufescens*. Demetropoulos and Langdon (*this volume*) provided methods for enhanced production of *P. mollis* through manipulation of nutrients, inorganic carbon, stocking density, temperature, salinity, and light.

The present study was designed to determine if nutrient levels used to optimize the culture of *P. mollis* directly affected abalone growth and to determine the effects of different nutrient enrichments on the nutritional value *P. mollis* when fed to abalone.

MATERIALS AND METHODS

Macroalgae

Cultured *P. mollis* rosettes (OSU, C-3 strain; Demetropoulos and Langdon, 2001) from HMSC were used for all experiments. Inoculla of 750 g were tumble-cultured in 110 l vessels supplied with 1 vol d^{-1} of filtered seawater at 15±2° C. Metal halide lamps (1500 W) on a 16:8 h photoperiod provided a photon flux density (PFD) of 750 μ mol photons m^{-2} s^{-1} or 43 mol photons m^{-2} d^{-1} [PAR]. Since the mean relative water velocity of macroalgae under tumble-culture conditions can influence nutrient and inorganic carbon uptake (Gonen et al., 1993; C. L. Demetropoulos, *unpublished data*), an average tumbling velocity of between 10 and 15 cm sec^{-1} was provided to cultures by aeration. A pH of 7.7 to 8.4 was maintained by sparaging CO_2 gas through the cultures via pH-stat valves (cf., Demetropoulos and Langdon, *this volume*).

Previous work had indicated that the best yields of *P. mollis* were obtained by using a modified version of Guillard and Ryther's (1962) *f* medium (0.75*f* + Zn [d^{-1}]), where the concentration of P was doubled to 83.3 μM d^{-1} (as NaH_2PO_4) and the concentration of Zn was adjusted to match that of Mn (1.37 μM d^{-1}) (see Demetropoulos and Langdon, *this volume*). For the present experiment, eight *P. mollis* diets were produced by varying the $NaNO_3$ load and presence or absence of trace metals supplied to the cultures. Nitrate was supplied at 1176, 1765, 2353, and 2942 μM d^{-1} NO_3^- - N, with or without addition of 0.75 *f* + Zn trace metals (d^{-1}). Nutrients were added by pulse-fertilizing

twice a week in total darkness for six to eight hours so as to offset nutrient uptake by epiphytes, resulting in a nutrient concentration three times that described above (Hanisak, 1987; J. McLachlan *pers. comm.*; Demetropoulos and Langdon, *this volume*).

The nutritional value of *P. mollis* cultured under the eight nutrient regimes was evaluated by feeding it to *H. rufescens* and *H. discus hannai* and measuring its effect on growth, daily food consumption (DFC) and food conversion efficiency (FCE). Background levels of nitrate and phosphorus in influent water were measured by ion chromatography (U.S. EPA, Newport, OR). Oxygen and pH were recorded either weekly or bi-weekly throughout the experiments.

Abalone

Juvenile *H. rufescens* (25 mm SL ± 0.4 mm; 3.14 g [damp wt.] ± 1.61 g), previously reared on kelp (*Macrocystis pyrifera* and *Nerocystis leutkena*), were supplied by Abalone International of Crescent City, California. Juvenile *H discus hannai* (24 mm SL ± 0.3 mm; 3.14 g [damp wt.] ± 1.61 g) were reared from wild brood stock obtained from Japan. *Haliotis discus hannai* were reared on *P. mollis* and animals used in the experiments were sampled from the middle of the size frequency distribution. Both species were acclimated to temperature and flow conditions of the two experiments for two weeks prior to initiating the experiment.

Nutrient bioassay experiment

The experiment consisted of seven nutrient factor levels testing abalone growth against ambient phosphate, nitrate and trace metals. Triplicate groups of *H. discus hannai* (10/group) were placed in 13 cm diameter plastic mesh socks and randomly assigned to 3.8 l vessels arrayed in a 600 l water bath maintained at a temperature of 18±0.5° C. A metal halide lamp (1000 W) on a 16:8 photoperiod was used to simulate a diurnal cycle that provided a PFD of 46 mol photons m^{-2} d^{-1} [PAR]. Light was evenly delivered across abalone treatments by the use of an aluminized octagon placed around the vessels.

Nutrient additions were made for eight hours during the dark cycle every three and four days (Table 4.1). The added amounts corresponded to those required by *P. mollis* on a daily basis to sustain optimal growth under moderate to high PFD's (cf., Demetropoulos and Langdon, *this volume*). To preclude phosphate and metal interactions, nitrate and phosphate were introduced into each vessel on days preceding the addition of trace metals.

Other than periods of nutrient additions, 1.0 μ filtered seawater (18±1° C) was passed through the vessels at an exchange rate of 19 to 20 vol d^{-1} at a salinity of 31 to 32‰. Aeration in the vessels was vigorous (1.8 l min^{-1}). Each replicate of abalone were fed weekly with 45 g (damp wt.) of *P. mollis* that had been fertilized daily-equivalent with 250 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4, and 0.75f + Zn metal enrichment as described in the feed experiment below.

Standard length (SL) and tissue dry weight were recorded on days 1 and 61. Abalone growth rates (μm d^{-1}) were determined by measuring (to 0.1 mm) the longest axis of the shell with calipers. On day 61, animals were measured for standard length, shucked, body tissues dried (60° C for 48 hr) and weighed. Growth rates were determined as follows:

$$\Delta SL = (L_f - L_i)/t \quad (1)$$

and

$$SGR = 100 \text{ X } (\ln W_f - \ln W_i)/t \quad (2)$$

where ΔSL was the shell length increase (μm d^{-1}), L_f and L_i were final and initial lengths (μm), SGR was the specific growth rate (% d^{-1}), W_f and W_i were the final and initial dry weights (g), and t was time in days.

Table 4.1: Summary of the seven average daily-equivalent nutrient loads applied to each bioassay treatment with *H. discus hannai*. Nutrients additions were made every three or four days resulting in concentrations that were three or four times the daily average loads given in this Table. Culture vessels were 3.8 l, vigorously aerated, and abalone were fed once wk^{-1} with *Palmaria mollis* (C-3 strain) enriched with 250 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4, and 0.75*f* + Zn metal enrichment (d^{-1}). Vessels were flushed with seawater at an exchange rate of 12 to 15 vol d^{-1} (except during the eight hour nutrient addition).

Nutrient additions (nitrate, phosphate, and trace metals d^{-1})	
1.)	No nutrients (SW control)
2.)	0.75*f* + Zn
3.)	50 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4 and 0.75*f* + Zn
4.)	100 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4 and 0.75*f* + Zn
5.)	150 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4 and 0.75*f* + Zn
6.)	200 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4 and 0.75*f* + Zn
7.)	250 mg l^{-1} $NaNO_3$, 10 mg l^{-1} NaH_2PO_4 and 0.75*f* + Zn

Feed experiment with H. discus hannai and H. rufescens

A randomized block design was employed consisting of 48 seven cm diameter plastic mesh socks (3 mm), with two abalone species in different socks, eight *P. mollis* diets (as described above) and three replicates per diet. Triplicate groups (8 abalone per group) of *H. rufescens* and *H. discus hannai* were randomly assigned (separately) to the eight *P. mollis* diets in the mesh socks. An initial commercial stocking density of one abalone per 32 cm^2 (Chris Van Hook, *pers. comm.*; Ebert and Houk, 1984; Demetropoulos, *unpublished data*) was adopted by compressing the abalone into the lower third of the mesh socks with mesh plungers. The socks were placed in 3.8 l culture vessels (three $vessel^{-1}$), arrayed in a 600 l water bath maintained at 18±0.5° C. Filtered seawater (18±1° C) was passed through the vessels at a rate of 3 to 12 vol d^{-1} (salinity = 31 to 32‰). Aeration in the vessels was vigorous (1.8 l min^{-1}). Abalone were fed *ad libitum* on a weekly basis throughout the experiment. Each week, all uneaten *P. mollis* was removed and replaced with fresh rosettes. The experiment ran for 100 days in near dark conditions (< 5 μ mols m^{-2} d^{-1}). After 67 days, treatments without addition of 0.75 *f* + Zn trace metals (d^{-1}) were dropped from the experiment due to their poor performance.

Standard lengths (SL) were recorded on days 1, 67 and 100 by measuring the longest axis of the shell (to 0.1 mm) with calipers. Damp weights were recorded on days 1 and 67 by blot drying abalone (Rosen et al., 2000). Dry weights were recorded on day 1 (by sacrificing an additional 25 abalone of each species) and day 100.

Abalone linear growth rates (ΔSL) were recorded as μm d^{-1} and specific growth rates (SGR) as % [dw] d^{-1} (see above), with damp weight being used for determination of SGR on day 67.

At eight time intervals (approx. every 2 weeks) during the experiment all *P. mollis* was removed after four days in the mesh socks to determine food conversion efficiencies (FCE's) and daily feed consumption rates (DFC). FCE is a measure of how efficiently the diet is used for growth and DFC is simply the amount of *P. mollis* ingested by the abalone d^{-1}. *Palmaria mollis* was damp weighed to remove excess seawater by use of a washing machine on the spin cycle. Control mesh socks with algae only were included to determine changes in algal weight and adjustments were made in FCE and DFC accordingly. FCE was calculated from the formula:

$$FCE = 100*(W_f - W_i)/(F_g - F_u) \quad (3)$$

where W_f and W_i were the final and initial whole abalone damp weights (g), F_g was the damp weight of food given (g) and F_u was the damp weight of food uneaten during the experimental period. DFC was calculated from the formula:

$$DFC = (F - R)/(W*t) \quad (4)$$

where DFC was the daily consumption ($g_{P.\ mollis}\ g^{-1}_{abalone}\ d^{-1}$), F was the damp weight (g) of *P. mollis* offered during the experimental period, R was the damp weight of *P. mollis* uneaten during the experimental period, W was the mean

damp weight (assuming linear growth) of abalone during the experimental period, and t was time in days.

Biochemical analysis

Samples of each diet were rinsed with seawater, 0.5 M ammonium formate, and distilled water to remove salt and living organisms. Subsequently, samples were ground to a fine powder and stored at - 80° C under nitrogen gas until analysis. Tissue N was measured using a LECO, CNS 2000 elemental analyzer (Fruit Growers Lab, Santa Paula, California). Crude protein was determined by multiplying tissue N by 6.25. Since high NO_3^- load and subsequent storage in intracellular NO_3^- pools were likely to interfere with this determination, plants were starved for 3 days prior to analysis (cf. Demetropoulos and Langdon, *this volume*).

Carbohydrate and total lipid of *P. mollis* diets fertilized with or without 0.75f + Zn metals were compared using pooled samples from different nitrate treatments. Total carbohydrate was determined by the phenol sulfuric acid method and total lipid by chloroform and methanol method (Southern Testing and Research Labs, Wilson, North Carolinia).

Statistical analysis

To account for differences in initial size, two-factor ANCOVA ($\alpha = 0.05$) was used to test for significance of

factors in both the nutrient bioassay treatments and diet treatments on growth and feeding measurements, using initial length or whole damp weight as a covariate. A paired t-test was used to determine the effects on abalone growth of adding 0.75*f* + Zn trace metal enrichment to *P. mollis* cultures compared with no trace metal additions. A paired t-test was also used to determine differences in tissue N of *P. mollis* when adding 0.75*f* + Zn trace metal enrichment to *P. mollis* cultures compared with no trace metal additions. Student-Newman-Kuels (SNK; $\alpha = 0.05$) multiple range test was used to test differences among individual treatments. All error terms are presented as SE.

RESULTS

Bioassay experiment with H. discus hannai

When compared to the control (seawater addition only) the addition of phosphate, various nitrate loads, and 0.75 *f* + Zn (d^{-1}) trace metals had no significant affect on linear growth (SL) (ANCOVA; $P = 0.117$) or SGR (ANCOVA; $P = 0.195$) of *H. discus hannai* over the 61-day experiment (Table 4.2 and Appendix 5). Survival during the experiment was 100% and all animals appeared healthy.

Feed experiment with H. discus hannai and *H. rufescens*

The experimental system supported a healthy environment for both abalone species with oxygen levels ranging from 85 to 105% saturation and pH ranging from 7.5 to 8.2. Abalone survival across all treatments was 100%. There was no significant difference among blocks (ANOVA; $P = 0.331$). The control vessel with *P. mollis* alone showed no significant (ANOVA; $P = 0.635$) change in algal biomass over the 4-day period used to determine FCE.

Growth of *H. discus hannai*

Over the first 67 days there was a significant negative effect (ANCOVA, SNK; $P = 0.047$) of increasing NO_3^- load and a significant positive effect (paired t-test; $P = 0.001$) of 0.75f + Zn trace metal additions to *P. mollis*

culture on linear growth rates and SGR's of *H. discus hannai* (Figs. 4.1, 4.2, and Appendix 5). Linear growth rates ranged from 110.5±3.3 μm SL d^{-1} for abalone fed on *P. mollis* fertilized with 2942 μM NO_3^- - N d^{-1} to 140.5±3.6 μm SL d^{-1} for abalone fed *P. mollis* fertilized with 1176 μM NO_3^- - N and 0.75f + Zn metals d^{-1}. SGR's ranged from 0.94% d^{-1} for abalone fed on *P. mollis* fertilized with 2353 μM NO_3^- - N d^{-1} to 1.09% d^{-1} for abalone fed *P. mollis* fertilized with 1176 μM NO_3^- - N and 0.75f + Zn metals d^{-1}.

Final linear growth rates for remaining treatments (treatments with no trace metal additions were dropped on day 67) after 100 days ranged from 94.9 μm SL d^{-1} for abalone fed on *P. mollis* fertilized with 2942 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} to 103.59 μm d^{-1} for abalone fed on 2353 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} (Fig. 4.1). There were no significant differences among either linear growth rates (ANCOVA; $P = 0.341$) or SGR's (ANCOVA; $P = 0.548$) of *H. discus hannai* fed on *P. mollis* supplied with 1176 to 2942 μM NO_3^- - N and 0.75*f* + Zn trace metals d^{-1} (Figs. 4.1 and 4.2).

DFC and FCE of *H. discus hannai*

For the first 67 days, daily feed consumption (DFC) of *H. discus hannai* ranged from 5.02±0.4% for abalone fed *P. mollis* fertilized with 1765 μM NO_3^- - N d^{-1} to 6.63±0.4% for abalone fed *P. mollis* fertilized with 2942 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} (Fig. 4.3). While there was little correlation between increasing NO_3^- in *P. mollis* cultures and DFC (Fig. 4.3), there was a significant difference

(ANCOVA, SNK; $P < 0.0001$) in DFC for animals fed *P. mollis* provided with 0.75*f* + Zn metals d^{-1} compared to treatments where *P. mollis* was grown on N and P alone (DFC = 5.02±0.4 to 5.29±0.3 for N and P alone as compared to DFC = 6.45±0.3 to 6.63±0.4 for 0.75*f* + Zn metals d^{-1}). For the remaining treatments from day 68 to 100, DFC's were 4.42±0.2% (2353 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}) to 5.16±0.5% (1176 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}) with no significant differences (ANCOVA; $P = 0.321$) in DFC found among the treatments (Fig. 4.3).

Table 4.2: Survival, linear growth rate (μm SL d^{-1} ± SE), and specific growth rate (%[dw] d^{-1} ± SE), of *Haliotis discus hannai* (SL ≈ 24mm to 34mm) after 61 days of exposure to various nutrient regimens differing in nitrate load and the addition of 0.75 *f* + Zn (d^{-1}) trace metals. There was no significant difference in either SL (ANCOVA; $P = 0.117$) or SGR (ANCOVA; $P = 0.195$) among treatments.

Treatment Nitrate load (μM d^{-1})	NO_3^- – N	0.75*f* + Zn (d^{-1})	Survival (%)	Linear growth (μm SL d^{-1}±SE)	SGR (%[dw]d^{-1}±SE)
Control (SW)	none	No	100	155±9.8	1.32±0.09
Metals only	none	yes	100	148±9.7	1.23±0.05
50 + metals	588	yes	100	148±3.2	1.23±0.05
100 + metals	1176	yes	100	142±4.3	1.22±0.03
150 + metals	1765	yes	100	165±4.9	1.33±0.03
200 + metals	2353	yes	100	161±8.2	1.31±0.10
250 + metals	2942	yes	100	163±3.2	1.42±0.10

Figure 4.1: Shell length increase of *Haliotis discus hannai* (μm SL d^{-1} ± SE) feed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.1-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, c, and x, indicate significant differences within each sampling period (ANCOVA, SNK; P = 0.047).

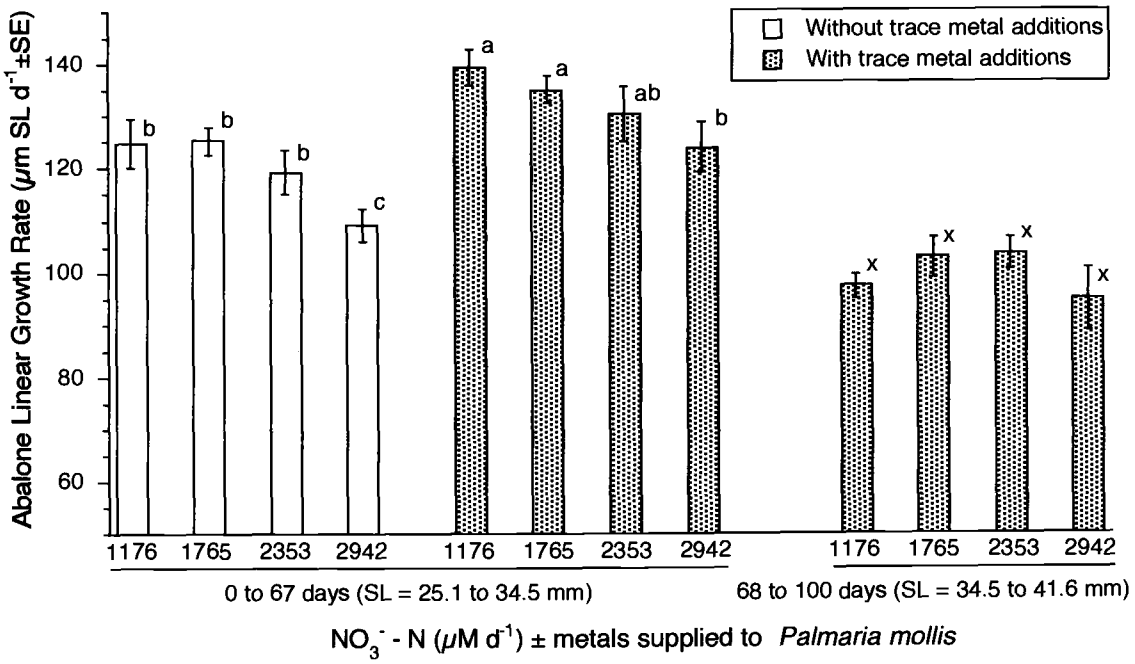


Figure 4.2: Specific growth rate of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.1-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, c, and x indicate significant differences within each sampling period (ANCOVA, SNK; P = 0.0393).

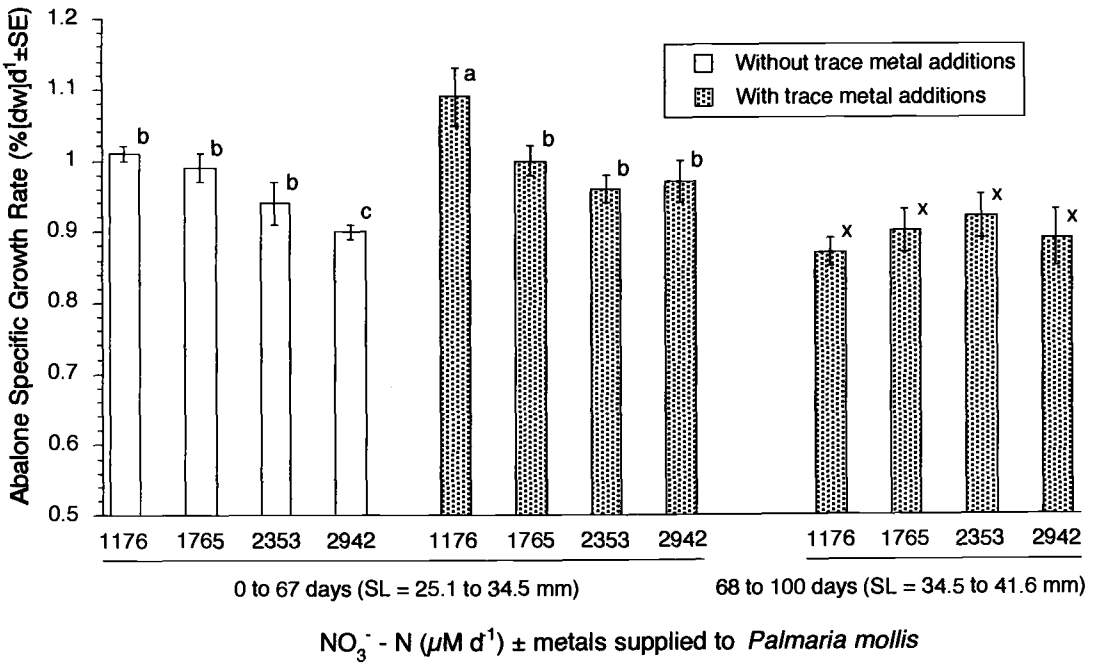
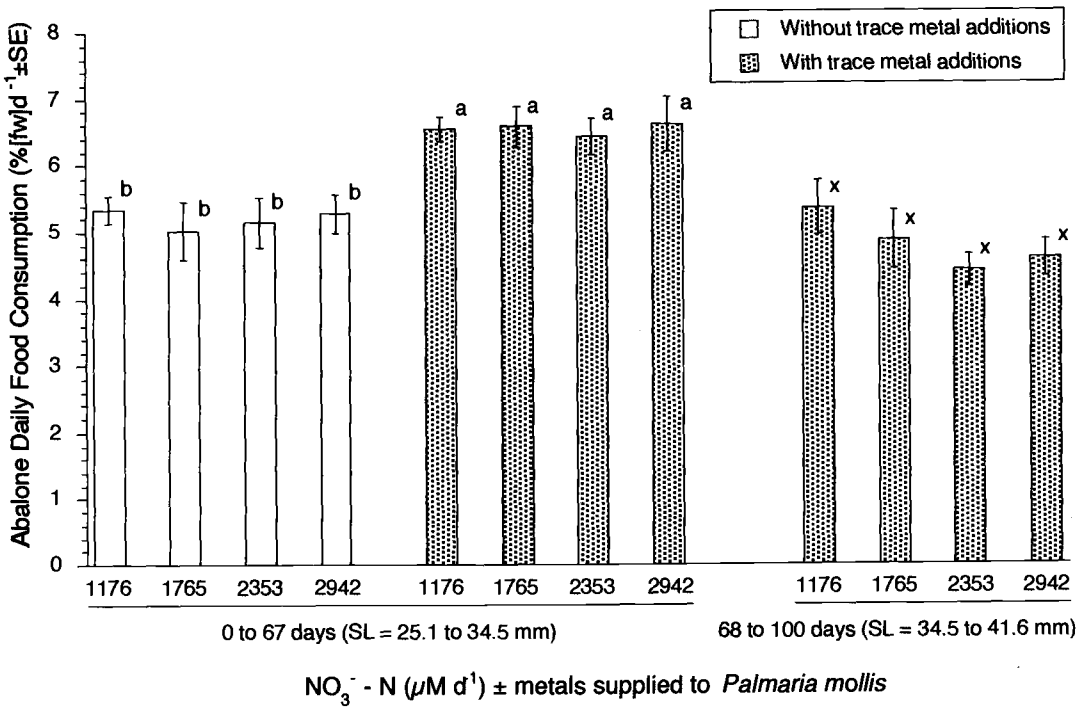


Figure 4.3: Daily food consumption of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.1-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, and x indicate significant differences within each sampling period (ANCOVA, SNK; $P < 0.0001$).



During the first 67 days feed conversion efficiencies (FCE's) among the *P. mollis* treatments ranged from 5.8±0.04% (2942 μM NO_3^- - N d^{-1}) to 8.83±0.4% (1176 μM NO_3^- - N with 0.75*f* + Zn metals d^{-1}) (Fig. 4.4). FCE of *H. discus hannai* was significantly different (ANCOVA, SNK; P = 0.042) for animals fed *P. mollis* provided with 0.75*f* + Zn metals d^{-1} compared to treatments where *P. mollis* was grown on N and P alone (FCE = 5.8±0.04 to 8.05±0.4% without 0.75*f* + Zn metals d^{-1} as compared to FCE = 7.34±0.4 to 8.83±0.3% with 0.75*f* + Zn metals d^{-1}). Generally, there was a negative correlation between increasing NO_3^- and FCE for the nitrate treatments tested from day 0 to 67 (Fig. 4.4). For the nitrate + trace metal treatments tested from day 68 to 100 FCE's ranged from 13.93±1.8% (2942 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}) to 16.17±1.1% (2353 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}) with no correlation (ANCOVA; P = 0.728) between increasing NO_3^- and FCE (Fig. 4.4).

Growth of *H. rufescens*

A positive and significant effect of additions of NO_3^- and 0.75*f* + Zn trace metals on the nutritive value of *P. mollis* for *H. rufescens* was seen on linear growth rates (ANCOVA, SNK; P = 0.04) during the first 67 days (Fig. 4.5). Linear growth rates ranged from 169.0±3.8 μm SL d^{-1} for abalone fed on *P. mollis* fertilized with 1176 μM NO_3^- - N d^{-1} to 198.1±2.7 μm d^{-1} for abalone fed on 2942 μM NO_3^- - N + 0.75*f* + Zn trace metals d^{-1}. SGR's ranged between 1.47% d^{-1} for abalone fed on *P. mollis* fertilized with 1176 μM

NO_3^- - N d^{-1} to 1.72% d^{-1} for abalone fed on *P. mollis* fertilized with 2353 μM NO_3^- - N + 0.75*f* + Zn trace metals d^{-1}. However, NO_3^- load supplied to *P. mollis* cultures had less effect on *H. rufescens* SGR's. A significant difference (ANCOVA, SNK; $P = 0.047$) was detected between 2942 μM NO_3^- d^{-1} without trace metals and other nitrate loads without trace metals (Fig. 4.6). As with *H. discus hannai*, both linear growth and SGR were significantly higher (paired *t*-test; $P = 0.0003$) during this period for *H. rufescens* fed on *P. mollis* fertilized with NO_3^- + 0.75*f* + Zn metals as compared to abalone fed *P. mollis* fertilized with phosphate and nitrate alone (Figs. 4.5 and 4.6).

Final linear growth rates for the remaining treatments (nitrate only treatments were eliminated on day 67) ranged from 157.9±1.8 μm SL d^{-1} for abalone fed on *P. mollis* fertilized with 1765 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} to 163.6±2.1 μm d^{-1} for abalone fed on 2942 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} (Fig. 4.5). There were no significant differences in either linear growth rates (ANCOVA; $P = 0.761$) or SGR's (ANCOVA; $P = 0.711$) of *H. rufescens* fed on *P. mollis* fertilized between 1176 to 2942 μM NO_3^- - N with the addition of 0.75*f* + Zn metals d^{-1} (Figs. 4.5 and 4.6).

DFC and FCE of *H. rufescens*

During the first 67 days, DFC's of *H. rufescens* ranged from 8.02±0.4% for abalone fed *P. mollis* fertilized with 2353 μM NO_3^- - N d^{-1} to 9.78±0.5% for abalone fed *P. mollis*

fertilized with 2942 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} (Fig. 4.7). There was no correlation between increasing NO_3^- load in *P. mollis* cultures and DFC. The only significant difference (ANCOVA, SNK; $P = 0.021$) in DFC's for *H. rufescens* during the first 67 days was between treatments where *P. mollis* was fertilized with 0.75*f* + Zn trace metals d^{-1} and those that were not (Fig. 4.7). For the remaining treatments between day 68 and 100 of the experiment DFC of *H. rufescens* ranged from 5.17±0.7% (1176 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}) to 5.82±0.5% (1765 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}), with no significant difference (ANCOVA; $P = 0.836$) in DFC among treatments (Fig. 4.7).

Figure 4.4: Food conversion efficiency of *Haliotis discus hannai* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.1-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, c, and x indicate significant differences within each sampling period (ANCOVA, SNK; P = 0.042).

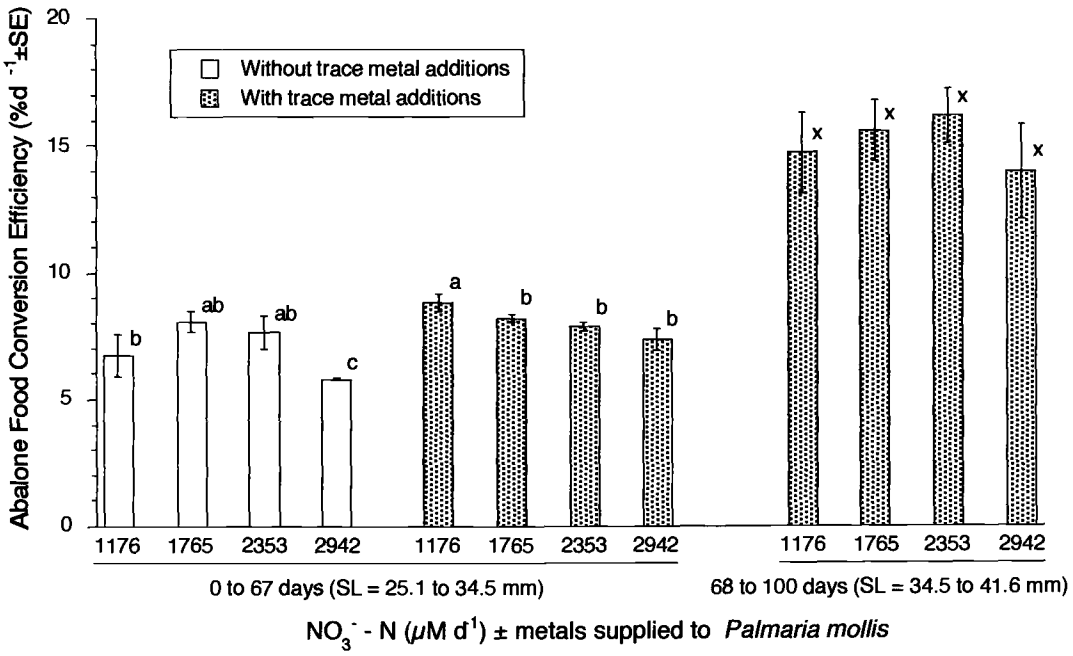


Figure 4.5: Shell length increase of *Haliotis rufescens* (μm SL d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.3-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, c, d, and x indicate significant differences within each sampling period (ANCOVA, SNK; $P = 0.04$).

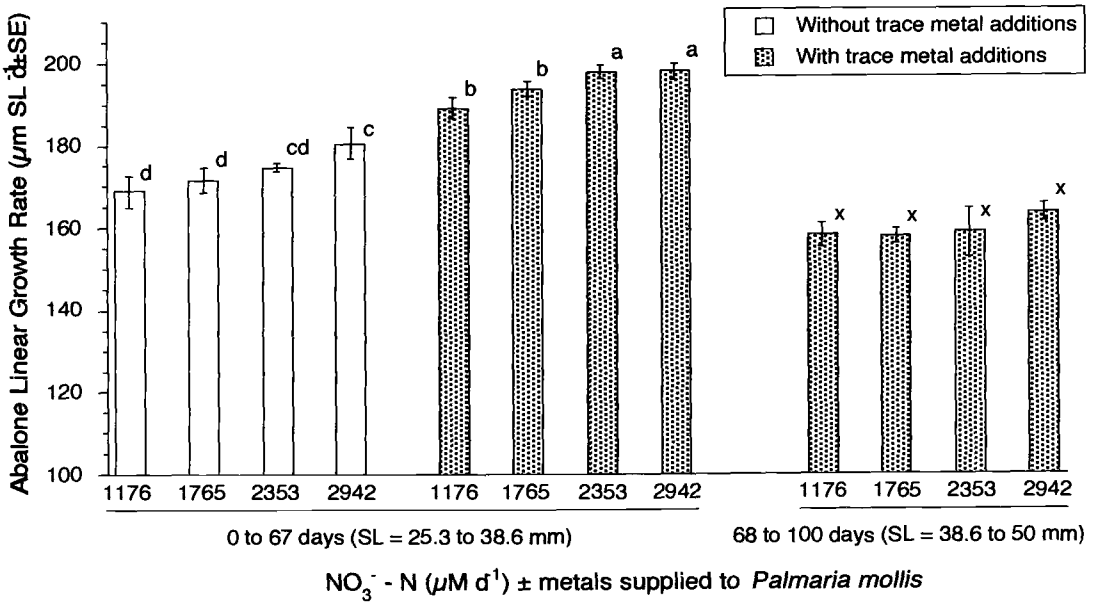


Figure 4.6: Specific growth rate of *Haliotis rufescens* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.3-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, c, and x indicate significant differences within each sampling period (ANCOVA, SNK; P = 0.047).

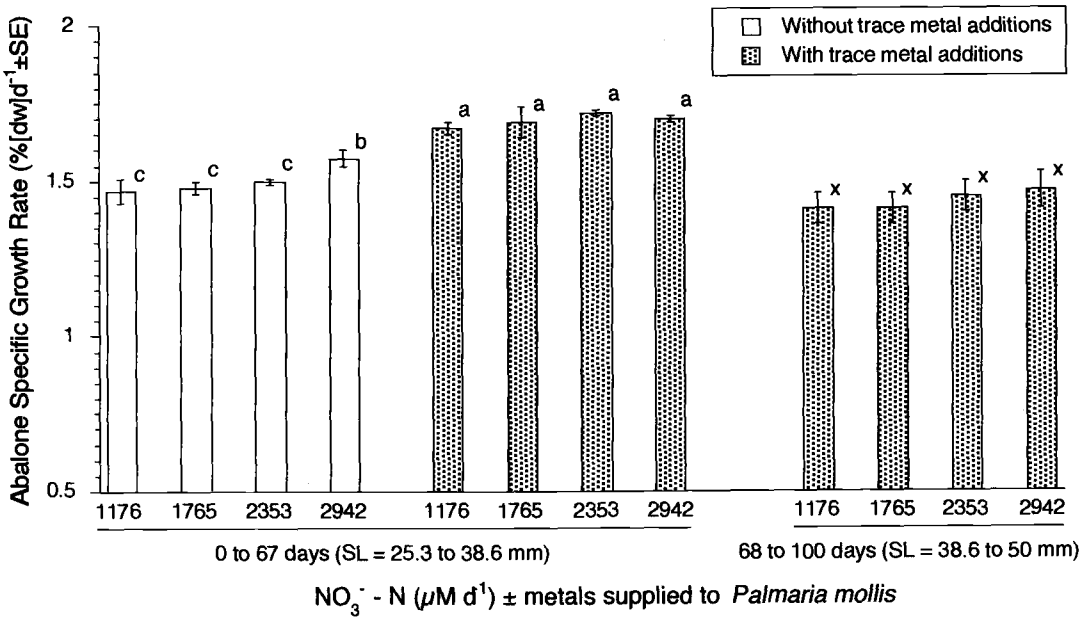
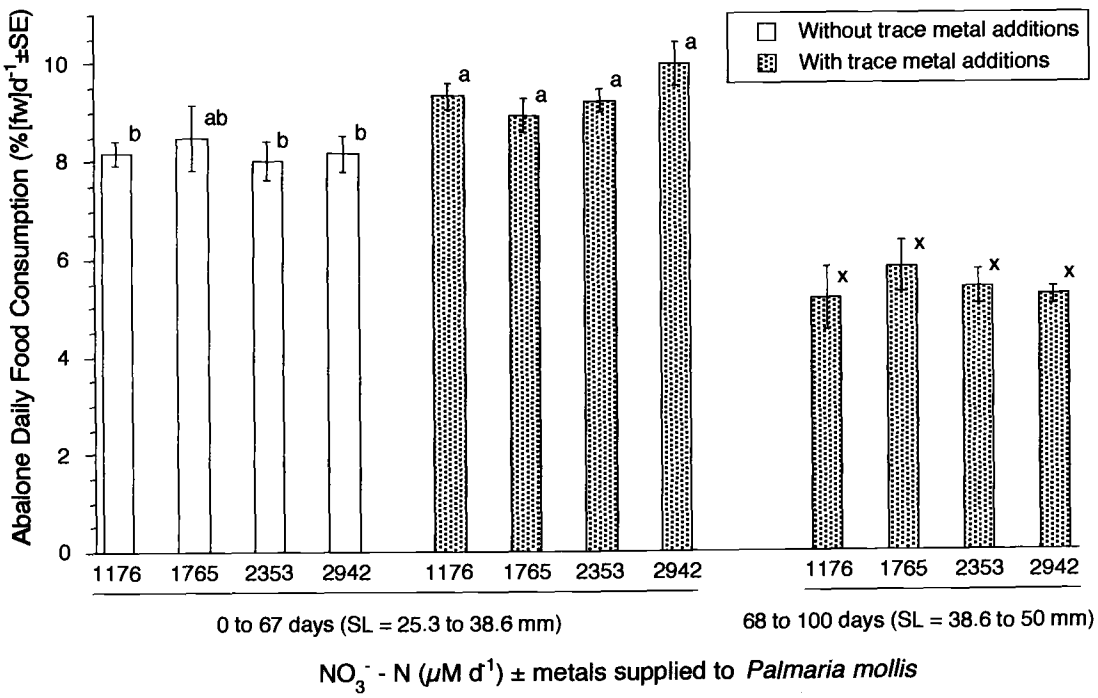


Figure 4.7: Daily food consumption of *Haliotis rufescens* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.3-mm abalone per vessel. Water temperature was 18±1° C. Letters a, b, and x indicate significant differences within each sampling period (ANCOVA, SNK; P = 0.021).



Feed conversion efficiencies (FCE's) during the first 67 days among *P. mollis* treatments ranged from 11.4±0.7% (1176 μM NO_3^- - N d^{-1}) to 13.04±0.5% (2353 μM NO_3^- - N and 0.75f + Zn metals d^{-1}) with no significant difference (ANCOVA; P = 0.867) among treatments (Fig. 4.8). For the remaining treatments tested from day 68 to 100 of the experiment, FCE's for *H. rufescens* ranged from 13.65±0.58% (1765 μM NO_3^- - N and 0.75f + Zn metals d^{-1}) to 17.22±0.98% (2942 μM NO_3^- - N and 0.75f + Zn metals d^{-1}). FCE's were significantly (ANCOVA, SNK; P = 0.034) correlated with NO_3^- - N load supplied to *P. mollis* from day 68 to 100 (Fig. 4.8).

The shell color of both *H. discus hannai* and *H. rufescens* reflected consumption of red pigments found in *P. mollis*. For all of the *P. mollis* diets tested, the shell color of *H. discus hannai* was typically dark green with red to brown mottling, while that of *H. rufescens* was rose to burgundy red in color. Prior to the present study, *H. rufescens* had been reared on a diet of *Macrocystis sp.* and *Nereocystis luetkeana* which had produced a green shell color, making the transition to the *P. mollis* diet apparent.

On day 27 of the experiment it was discovered that gonads of approximately one in every three *H. rufescens* were significantly developed. This was particularly pronounced in treatments where *P. mollis* was supplied with trace metals and was observed in both males and females (average size ≈ 30 mm), although most (> 75%) of the ripening abalone observed were male. By day 100 of the experiment approximately 48% of the *H. rufescens* (average

size ≈ 46 mm) and 20% of *H. discus hannai* (average size ≈ 37 mm) had strong gonad development (4, 0-4).

Biochemical composition of P. mollis

The effects of NO_3^- load and addition of trace metals to *P. mollis* on its protein, carbohydrate, and lipid content are shown in Table 4.3. Over the range of nitrate supplied, protein content averaged between 19.2 to 31.3%. A paired t-test showed that protein (as determined from tissue N) was significantly higher for *P. mollis* supplied with trace metals compared to cultures without trace metal additions (paired t-test; $P = 0.0063$). When *P. mollis* tissue cultured under the different nitrate levels were pooled into those with and without trace metal additions there was no significant difference in carbohydrate (33.4 to 39.3%) or total lipid (2.41 to 2.74%) (ANOVA; $P = 0.637$, $P = 0.481$, $P = 0.713$ respectively) (Table 4.3).

Figure 4.8: Food conversion efficiency of *Haliotis rufescens* (% d^{-1} ± SE) fed on diets of *Palmaria mollis* cultured with additions of PO_4^-, different nitrate loads and either with or without additions of 0.75 *f* + Zn trace metals. Triplicate culture vessels for each treatment were initially stocked with eight, 25.3-mm abalone per vessel. Water temperature was 18±1° C. Letters a, x, and y indicate significant differences within each sampling period (ANCOVA, SNK; $P = 0.034$).

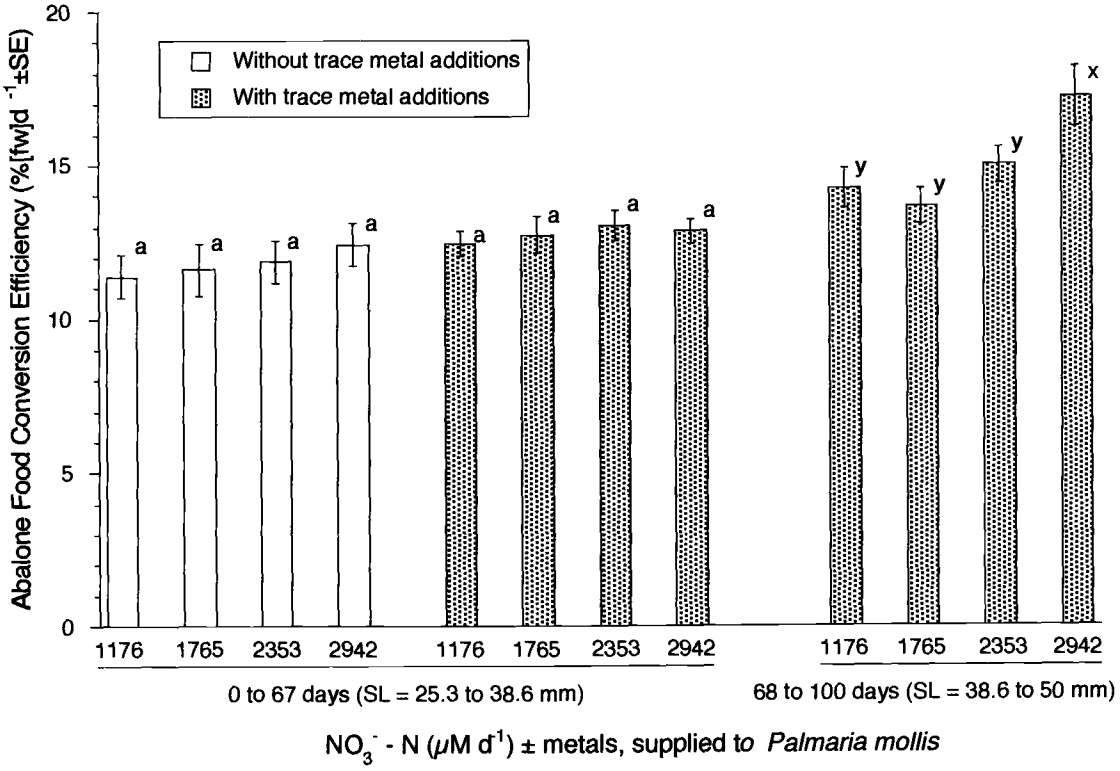


Table 4.3: Dry weight protein (± SE), carbohydrate (± SE), and total lipid (± SE) of *Palmaria mollis* as a function of nitrate load d^{-1} under a moderately high light (PFD = 51.64 mol photons m^{-2} d^{-1}). Sodium nitrate range values (100 – 250 mg l^{-1}) represent pooled tissue samples for the effect of trace metals on proximate analysis. Nutrients were delivered once every three to four days. Values represent means ± SE. ND = not determined.

Abalone Diets: *Palmaria mollis* nutrient regime d^{-1}					
$NaNO_3$ (mg l^{-1})	NO_3^- (μM)	0.75*f* + Zn	Protein (%dw±SE)	Carbohydrate (%dw±SE)	Total lipid (%dw±SE)
100	1176	no	19.2±1.6	ND	ND
150	1765	no	25.5±3.0	ND	ND
200	2353	no	31.3±2.2	ND	ND
250	2942	no	29.4±1.8	ND	ND
100	1176	yes	25.1±2.4	ND	ND
150	1765	yes	30.8±4.8	ND	ND
200	2353	yes	31.2±2.9	ND	ND
250	2942	yes	30.4±1.5	ND	ND
100-250	1176-2942	no	26.1±2.8	39.3±3.6	2.74±0.4
100-250	1765-2942	yes	30.3±4.1	33.4±5.2	2.41±0.2

DISCUSSION

Bioassay experiment

Abalone seed were used in bioassays to determine the direct effects on abalone survival and growth of nutrients used in *P. mollis* culture. Such bioassays are important in order to design successful co-culture sytems or when cultures of the two species are arranged in series (cf., Day and Fleming, 1992; Fleming 1995a and b).

Results showed the additions of PO_4^-, different NO_3^- loads, and 0.75 *f* + Zn trace metals added to culture water had no significant affect on survival, linear growth or SGR for *H. discus hannai* (Table 4.2). On the contrary, abalone growth rates tended to increase in treatments where nutrients were added, perhaps due to abalone grazing on enhanced diatom growth occurring on exposed surfaces in the presence of added nutrients. Such enhanced diatom growth could be expected in co-cultures of *P. mollis* and abalone, provided the *P. mollis* does not out-compete diatoms (Austin et al., 1990; Demetropoulos, *pers. obs.*; George Trevelyan, *pers. comm.*).

Of the trace metals used in the *f* medium (cf., Guillard and Ryther, 1962), Cu is probably the most toxic for abalone. Hahn (1989b) reported the Cu LD_{50} (96 hr) for *H. rufescens* adults was 65 ppb. During this experiment the maximum concentration (added once every 4 days) was 15 ppb. While this is probably well below what might be considered toxic in the short-term, there may be long-term sub-lethal effects of Cu on abalone growth and disease

resistance. Therefore, caution should be taken when using the *f* medium for co-culture of abalone.

Feed experiment

While many studies have been conducted on macroalgal dietary preferences of abalone (Sakai, 1962; Kikuchi et al., 1967; Uki et al., 1986; Hahn, 1989b; Gao et al., 1990; Levin, 1991; Mercer et al, 1993; Rosen et al., 2000) there is little published on the indirect effects of nutrients on the nutritional value of seaweeds for abalone. We tested a range of nutrient-supplemented *P. mollis* diets to determine how different nutrient regimes would affect growth and algal utilization by two abalone species, *H. discus hannai* and *H. rufescens*. As first reported by Rosen et al. (2000), results suggested culture conditions of *P. mollis* can have a significant affect on growth, DFC, and FCE of both *H. discus hannai* and *H. rufescens*.

Initial increases in linear growth and SGR for the first 67 days of the experiments showed growth was a function of both nitrate load and addition of trace metals to *P. mollis* cultures. During this first 67 day period, linear growth of *H. discus hannai* (SL = 29.4 to 39.4 mm) was highest when fed *P. mollis* cultured with 83.3 μM (as NaH_2PO_4), a nitrate load of 1176 to 2353 μM NO_3^- and 0.75*f* + Zn trace metals d^{-1} while that of *H. rufescens* (SL = 32.2 to 44.0 mm) was best when fed *P. mollis* fertilized with 83.3 μM PO_4^- d^{-1} (as NaH_2PO_4), a nitrate load of 2353 to 2942 μM NO_3^- - N and 0.75*f* and Zn trace metals d^{-1}. Both

species showed significant increases (ANCOVA, SNK; $P < 0.001$) in SGR when fed on *P. mollis* fertilized with 0.75*f* + Zn trace metals d^{-1}, with *H. discus hannai* showing a further significant improvement in FCE. Over the course of the entire experiment (100 days), both *H. discus hannai* and *H. rufescens* grew best when fed on *P. mollis* fertilized with 1176 to 2353 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1}. These values compare well with optimal nutrient loads for growth of *P. mollis* itself (Demetropoulos and Langdon, *this volume*).

Linear growth and SGR's for *H. discus hannai* maintained at 18±1° C and supplied with the most nutritive *P. mollis* diets were high compared to those reported for abalone fed on other artificial and natural diets. Fleming et al. (1996) reviewed growth rates of various abalone species ranging in shell length from 25 to 50 mm, and fed on a variety of artificial diets (at 18 to 22° C). They showed linear growth rates of 50 to 138 μm SL d^{-1} for a variety of species including *H. discus hannai* as compared to growth rates of 94.8 to 140.5 μm SL d^{-1} for the same sized animals in this study. In spite of a sub-optimal cultivation temperature (18±1° C vs. an optimal temperature of 22.5° C [Hahn, 1989c]) the linear growth rate of 139 μm SL d^{-1} for abalone fed on the most nutritive *P. mollis* diet was comparable to the highest values reported by Uki et al. (1986) for *H. discus hannai* which were fed on a good algal diet of *Eisenia bicyclis* at a temperature of 21±1° C.

Typical growth rates of *H. rufescens* in commercial production have ranged from 33 to 50 μm SL d^{-1} (Trevelyan et al., 1998) when animals were fed on *Macrocystis*

pyrifera at temperatures generally ranging between 12 to 16° C. Evans and Langdon, (2000) showed growth could be significantly improved to 123 μm SL d^{-1} when *H. rufescens* were fed on diets of *P. mollis* supplied with moderate concentrations of nitrate and phosphate. Our growth rates for similar sized animals were as high as 198 μm SL d^{-1} (first 67 days) and 161 μm SL d^{-1} between day 68 and 100, with an average of 171 μm SL d^{-1} for the entire 100-day experiment. However, this difference was likely due to both cultivation at a higher temperatures -- 18±1° C in this study compared with culture temperatures ranging from 12 to 16° C reported by Evans and Langdon (2000) as well as the addition of 0.75*f* + Zn trace metals to the *P. mollis* cultures.

Food conversion efficiencies in this study compared favorably with those reported by Mercer et al. (1993) who found an FCR of 15% for *H. discus hannai* fed *P. palmata*. From day 0 to 67 *H. discus hannai* showed a negative and significant decrease in FCE with increasing nitrate load delivered to *P. mollis* cultures (Fig. 4.4). The low FCE's for *H. discus hannai* fed on a diet of *P. mollis* supplied with between 1765 and 2942 μM d^{-1} NO_3^- d^{-1} (both with and without 0.75*f* + Zn trace metals) suggested that abalone were not able to convert the higher NO_3^- load diets into tissue with greater efficiency. While it is difficult to say exactly why this was the case, increased *P. mollis* toughness may have played a role (Demetropoulos, *unpublished data*). This is consistent with the observation that FCE's showed an opposite (but not significant) trend once the abalone got larger and presumably were better able to utilize tougher algal

tissue (Fig. 4.4). Since the best abalone growth over the entire experiment occurred at a nitrate load of between 1176 and 2353 μM NO_3^- - N and 0.75*f* + Zn metals d^{-1} (Figs. 4.1 and 4.2) it is probably best to supply this range of nitrate loads (with trace metals) to *P. mollis* for maximum *H. discus hannai* growth over a size range from 25 to 35 mm SL.

Both FCE's and DFC's of *H. rufescens* were not significantly affected by nitrate additions to *P. mollis*. For *H. rufescens*, FCE's over the entire experiment were typically greater than those of Rosen et al., (2000) who fertilized *P. mollis* with 2353 μM NO_3^- - N d^{-1}, 416 μM PO_4^- d^{-1} (as NaH_2PO_4) and provided no trace metals. Our results indicate an FCE for *H. rufescens* of between 13.7 to 17.2% when abalone were fed on a diet where *P. mollis* was fertilized with between 1176 and 2942 μM NO_3^- - N d^{-1} (with 0.75*f* + Zn metals) (Fig. 4.8). However, since the best abalone growth occurred when *P. mollis* was fertilized with 2353 to 2942 μM NO_3 - N d^{-1} and 0.75*f* + Zn trace metals (Figs. 4.5 and 4.6; day 0 to 67), it is probably best to apply these nitrate loads to *P. mollis* cultures for maximum growth (size range 25.1 to 47.9 mm SL) and FCR's of *H. rufescens*.

The present work with *P. mollis* and *H. rufescens* also suggests an important relationship between diet and shell color. The tenet that red abalone generally prefer brown seaweed (Tenore, 1976; Hahn, 1989c) is likely more a function of the historical relative abundance of kelp in the natural environment rather than optimal nutrition and/or autecological feeding behavior. Based on comparative shell color alone (*P. mollis* yielding a red

shell and *Macrocystis sp.* and/or *Nereocystis luetkeana* yielding a green shell), *H. rufescenes* forages on far more than brown algae in its natural environment and the same is probably true of flat, pink, and white abalone (Demetropoulos, *pers. obs.*).

The minimum size at sexual maturity for *H. rufescens* in this experiment was reached at approximately 30 mm, with male *H. rufescens* observed more commonly. Hahn (1989b) reported the minimum size for *H. rufescens* was 39.5 mm (females) and 50 mm (males). Thus, the use of *P. mollis* also appeared to enhance the onset of gonad development in *H. rufescens*.

Biochemical analysis

The protein composition of *P. mollis* in this study (19.2 to 31.3%) was high compared to reported values for *P. palmata*: 8.13 to 23.69% (Morgan and Simpson, 1981b) and 15.93 to 18.38% (Mercer et al., 1993). However, Morgan, et al. (1980) showed wild-harvested *P. palmata* typically consisted of 8-35% crude protein, 38-74% carbohydrate and 0.2-3.8% total lipid, with variation in these constituents attributed mainly to season.

Mercer et al. (1993) suggested that a diet composed of > 15% protein, 20–30% carbohydrate, and 3-5% lipid was necessary for optimal growth of abalone. Further, Mai et al. (1995b) found the optimum dietary protein content for *H. discus hannai* was closer to between 25 and 35%. Like *P. palmata*, our study suggests *P. mollis* provided a balanced diet for abalone, with a protein content of 30.4

to 31.2%, carbohydrate of 33.4 to 39.3% and total lipid of 2.41 to 2.74% when *P. mollis* was fertilized with daily average concentrations of 83.3 μM (as NaH_2PO_4), a nitrate load of 1176 to 2353 μM NO_3^- (as $NaNO_3$) and 0.75*f* + Zn trace metals.

The fact that addition of trace metals to *P. mollis* cultures significantly increased protein (paired t-test; $P = 0.0063$ and Table 3) was somewhat surprising. At this point, it is difficult to determine if the increase in protein itself produced significantly higher abalone growth rates for both species. Increased growth of abalone fed on *P. mollis* fertilized with trace metals may be in the direct use of the metals by the abalone or increased levels of other biochemical constituents of *P. mollis* (or its epiphytes) such as vitamins or specific fatty acids.

However, in addition to nutritional composition, dietary preference of abalone is determined many factors including; morphology, texture, odor, and the presence of unpalatable chemicals (McShane et al., 1994; Fleming, 1995a; Marsden and Williams, 1996; Rivero and Viana, 1996; Stepto and Cook, 1996). Provided with culture conditions described above (cf., Demetropoulos and Langdon, *this volume*), the thallus morphology of all *P. mollis* diets was similar to that described as "A" by Rosen et al. (2000). In their study, this morphology produced the highest growth rate, suggesting *P. mollis* morphology may significantly influence feeding preference by *H. rufescens*. This is consistent with the findings McShane et al. (1994) who suggested food toughness is a primary factor in determining abalone feeding preference. Indeed,

P. mollis cultured under very low light (< 10 mol photons m^{-2} d^{-1} [PAR], stocking density 10 g [damp wt.] l^{-1}) can become quite tough and typically results in poorer growth of *H. discus hannai* and *H. rufescens* as compared to *P. mollis* grown under higher light which is generally softer and provides better growth for these species (Demetropoulos, *unpublished data*).

In summary, the present work shows ambient concentrations of nutrients supplied to *P. mollis* to maintain optimal growth under high light and low seawater exchange (1 vol d^{-1}) were not directly deleterious to abalone growth (viz., *H. discus hannai*). Further, the work showed growth, FCE, and DFC of *H. discus hannai* and *H. rufescens* could be increased though indirect manipulation of nutrient loads provided to *P. mollis*.

Increased SL and SGR of *H. discus hannai* indicated that *P. mollis* cultured under low NO_3^- load was more nutritious than *P. mollis* cultured under high NO_3^- load. For *H. rufescens*, increases in SL and SGR indicated *P. mollis* cultured under high NO_3^- load was more nutritious than *P. mollis* cultured under low NO_3^- load. Perhaps most importantly, both *H. discus hannai* and *H. rufescens* growth were significantly increased when *P. mollis* was provided with trace metals (Fe, Mn, Zn, etc.). Biochemical composition of the *P. mollis* diets suggested this latter result was not attributable to the presence of more or less protein, carbohydrate, or lipid.

The trends in abalone growth and FCE of this study suggested nutrient concentrations (on a per day basis) which should be applied to *P. mollis* cultures for optimal abalone feed were approximately 83 μM – P, 1765 to 2353 μM

NO_3^- - N and 0.75f + Zn trace metals for *H. discus hannai* and 83 μM – P, 2353 to 2942 μM NO_3^- - N and 0.75f + Zn trace metals for *H. rufescens,* under natural light conditions ranging from 20 to 55 mol photons m^{-2} d^{-1}. Typically, in order to optimize growth of the algae itself, these nutrients should be delivered at night to *P. mollis* cultures every three to five days as described by Demetropoulos and Langdon (*this volume*).

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CONCLUSIONS

The goal of this research was to optimize culture conditions of *P. mollis* as a biofilter and food to be used in low exchange or no exchange recirculating seawater systems for abalone culture. While bacteria-dominated recirculating marine systems are currently in use, problems associated with maintaining such systems have led to exploration of alternatives such as macroalgae biofilters. However, macroalgae biofilters require optimization of parameters such as nutrients, inorganic carbon, light, density, salinity, and temperature in order to grow properly and provide the highest nutrition to co-cultured species – in this case abalone.

The present work shows that under moderate to high PFD's (17-53 mol photons m^{-2} d^{-1}) and reduced water volume exchanges (1 vol d^{-1}), growth of *P. mollis* can be significantly enhanced by supplying a revised version of the *f* medium developed by Guillard and Ryther (1962), during the dark cycle every 5 to 7 days. Maximum growth rates of *P. mollis* occurred at NO_3^- concentrations of 2942 μM – N d^{-1}, and while the use of NO_3^- and NH_4^+ as a combined source of nitrogen showed no significant difference over NO_3^- alone in the short-term (several weeks), NH_4^+ as a primary N source appeared to be somewhat toxic on a long-term basis (7 weeks or greater). Surprisingly, phosphorus in excess of 83.3 μM (delivered as 10 mg l^{-1} NaH_2PO_4) depressed specific growth rates of *P. mollis*. Finally, the addition of trace metals (Fe, Mn, Zn, and Cu) significantly increased growth rates over cultures supplied with just nitrate and phosphate alone.

The addition of inorganic carbon in the form of both CO_2 and HCO_3^- increased *P. mollis* yields by approximately 53% compared with a treatment that received no inorganic carbon. However, a question still remains as to whether the addition of CO_2 and HCO_3^- are economically the best methods of supplying inorganic carbon as compared to simply increasing flush water to 15 vol d^{-1} or greater during periods of peak photosynthesis. The economics will vary depending on the cost of supplying water to cultures compared to the cost of CO_2 and HCO_3^-.

Under the highest photon flux density supplied on a specific light density basis, *P. mollis* produced exceptional growth rates of up to 18% d^{-1}. This result indicates that with proper density, DIC, macro/micro nutrients, and water motion, *P. mollis* can grow well for sustained periods of time (months) under PFD's which are considerably higher than PFD's typical of temperate regions.

The combined effect of optimizing *P. mollis* culture conditions resulted in an SGR that averaged 12 to 18% d^{-1} with a protein content between 27 to 33% (dw). This makes Pacific dulse one of the highest protein-producing organisms in the world.

The ambient nutrient concentrations (N, P, and trace metals) delivered to *P. mollis* had no observable negative effect on abalone growth. Furthermore, nutrient concentrations for the best *P. mollis* growth had a significant positive effect on abalone growth, with the greatest effect occurring when trace metals (Fe, Zn, Mn, Cu, Mo, and Co) were added to *P. mollis* cultures. Based

on the current work, the best nutrient conditions for *P. mollis* growth also resulted in high abalone growth rates.

With the completion of this phase of the research, we have begun to harmonize the biology of the two co-cultured organisms and address the economic considerations of light energy (PAR), nutrients, and inorganic carbon in terms of mass culture yields and growth of two valuable abalone species, *H. rufescens* and *H. discus hannai*.

Future work should develop a nutrient delivery system linked to the average daily PAR absorbed by a "standing stock" of *P. mollis*. In this way nutrient additions will be driven by photosynthetic demand and nutrient availability. Maximum production should parallel or slightly lag behind the amount of solar radiation being delivered (cf, McLachlan and Bird, 1986; McLachlan et al., 1986). Ultimately, seasonal production of both *P. mollis* and abalone in culture systems should be predictable as part of a computer model. The model could be based on PAR delivered to cultures and correlated with both ambient nutrient inputs and *P. mollis* internal nutrient concentrations (i.e., N, P, and trace metals).

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APPENDICES

APPENDIX 1

Composition of the *f* medium (Guillard and Ryther, 1962) used as the basis for nutrient additions in *Palmaria mollis* culture. Values are provided in terms concentration (mg l^{-1} and μM) by element.

Compound	mg l^{-1} d^{-1}	μM d^{-1} (element)
$NaNO_3$	150	1765 – N
NaH_2PO_4	10	83.3 – P
Fe EDTA (13% Fe)	10	23.6 – Fe
$MnCl_2 \cdot 4H_2O$	0.36	1.82 – Mn
$ZnSO_4 \cdot 7H_2O$	0.044	0.15 – Zn
$CuSO_4 \cdot 5H_2O$	0.02	0.08 – Cu
$CoCl_2 \cdot 6H_2O$	0.02	0.08 – Co
$Na_2MoO_4 \cdot 2H_2O$	0.012	0.06 – Mo

APPENDIX 2

Summary of two ANOVA tests for specific growth rate of *Palmaria mollis* as a function of nitrate additions (as $NaNO_3$) under artificial low light (PFD = 23.68 mol photons m^{-2} d^{-1}) and high light (PFD = 51.64 mol photons m^{-2} d^{-1}).

1.) Experiment was conducted at HMSC under artificial low light (PFD = 23.68 mol photons m^{-2} d^{-1}), opaque 3.8 l pitchers (depth, 28 cm) receiving 1 vol d^{-1} of seawater.

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Nitrate additions	310.21	6	51.70	126.79	< 0.0001
Residual	5.71	14	0.408		

2.) Experiment was conducted at HMSC under artificial high light (PFD = 51.64 mol photons m^{-2} d^{-1}), opaque 3.8 l pitchers (depth, 28 cm) receiving 1 vol d^{-1} of seawater.

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Nitrate additions	463.50	6	77.25	397.88	< 0.0001
Residual	2.72	14	0.194		

APPENDIX 3

ANOVA test for specific growth rate of *Palmaria mollis* as a function of either constant seawater exchange (7.5 and 15 vol d^{-1}) or variable seawater exchange (7.5 vol d^{-1}) or CO_2 sparaging to control pH at constant seawater exchange (7.5 d^{-1}). Experiment was conducted at NELH, Hawaii under natural high light (PFD = 47.1 mol photons m^{-2} d^{-1}).

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Seawater Exchange	10.531	5	2.106	22.40	< 0.0001
Block	0.090	2	0.045	0.048	0.625
Residual	2.633	28	0.094		

APPENDIX 4

ANOVA tests for specific growth rate of *Palmaria mollis* as a function of temperature and light. Experiments were conducted under high natural light at NELH, Hawaii, (PFD = 55 mol photons m^{-2} d^{-1} or 0.016 mol photons g^{-1} [fresh wt.] d^{-1}) and at HMSC, Oregon, under artificial light (PFD ranging from 13 to 29 mol photons m^{-2} d^{-1} or 0.010 to 0.021 mol photons g^{-1} [fresh wt.] d^{-1}, respectively).

1.) One-way ANOVA test: Experiment conducted at NELH under natural light, translucent 125 l tanks (depth, 65 cm) receiving 60 vol d^{-1} of seawater.

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Temperature	12.268	2	6.134	3.346	0.0629
Residual	27.495	15	1.833		

2.) Two-way ANOVA test: Experiment conducted at HMSC under high artificial light (13 mol photons m^{-2} d^{-1}) and low artificial light (13 mol photons m^{-2} d^{-1}) in opaque 3.8 l pitchers (depth, 28 cm) receiving 1 vol d^{-1} of seawater.

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Temperature	971.49	5	194.30	301.91	< 0.0001
Light	94.39	1	94.39	146.66	< 0.0001
Temperature X Light	78.75	5	15.75	24.47	< 0.0001
Residual	15.45	24	0.64		

APPENDIX 5

ANCOVA test for specific growth rate (%[dw] d^{-1} ± SE) of *Haliotis discus hannai* (SL ≈ 24mm to 34mm) after 61 days of exposure to various nutrient regimens differing in nitrate load and the addition of 0.75 *f* + Zn (d^{-1}) trace metals. Initial abalone body weight was used as the covariate.

Source of variation	Sum of Squares	d.f.	Mean Square	F-Value	P-Value
Nutrient Regimne	0.119	6	0.020	1.988	0.195
Initial Abalone Wt.	0.001	1	0.001	0.098	0.764
Interaction (Nutrient Regime X Initial Abalone Wt.)	0.119	6	0.020	1.998	0.193
Residual	0.070	7	0.010		