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Intraspecific variation in the red alga *Portieria hornemannii*: monoterpene concentrations are not influenced by nitrogen or phosphorus enrichment

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Abstract The red alga *Portieria hornemannii* (Lyngbye) Silva was selected to test the effects of enhanced nutrient availability on the production of carbon-based secondary metabolites, because of its notable site-to-site variation in monoterpene production. On Guam, the major secondary metabolite produced by this alga is ochtodene, a cyclic monoterpene. Quantitative high-performance liquid-chromatography analysis of the extracts of *P. hornemannii* collected from six sites on Guam showed that both ochtodene and triglyceride concentrations differed significantly among sites. Internal nitrogen and phosphorus content of the algae did not correlate with the observed variation in chemistry. Experimental enhancement of N-alone, P-alone or N + P in the field for 5 wk failed to induce a significant change in ochtodene concentrations in the alga, while triglyceride concentrations increased significantly in the N + P treatment. Ochtodene and triglyceride concentrations did not change among similar treatments in shaded (18 d) and unshaded (11 d) fertilization experiments conducted in the laboratory. Variation in ochtodene concentrations in *P. hornemannii* cannot be attributed to N and P availability; however, the decrease in ochtodene and triglyceride concentrations during the shaded laboratory experiment suggests that light may be a factor influencing monoterpene biosynthesis. The difference in ochtodene concentration between the initial and final sets of field controls collected for the unshaded laboratory experiment suggests that temporal variation might also contribute to differences observed among the algae at the different sites.

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Introduction

Intraspecific chemical variation in secondary metabolite production has been noted for a variety of plants in marine and terrestrial environments (Hay and Steinberg 1992). Recent studies focus mostly on hypotheses that attribute this variation to phenotypic responses (Tuomi 1992). One of these hypotheses, the carbon/nutrient-balance hypothesis, suggests that the allocation of resources to the production of secondary metabolites in a plant depends primarily on the resource regime available (Bryant et al. 1983, 1987a,b; Baldwin and Ohnmeiss 1994; Baldwin et al. 1994). The hypothesis postulates that the concentration of secondary metabolites in a plant will vary quantitatively with resource availability, i.e. carbon and nitrogen (Bryant et al. 1983; Price et al. 1989). For plants that produce carbon-based secondary metabolites (such as terpenes or polyphenolics), conditions that increase the C:N ratio (e.g. high light, low nutrients) should result in an increase in the concentration of secondary metabolites and a decrease in growth rate, while conditions that decrease the C:N ratio (e.g. shade, high nutrients) should result in a decrease in growth rate accompanied by a decrease in the concentration of secondary metabolites. For plants that produce nitrogen-based secondary metabolites (such as alkaloids), conditions that increase the C:N ratio should result in a decrease in the concentration of secondary metabolites and the growth rate, while conditions that decrease the C:N ratio should result in a decrease in growth rate and an accumulation of secondary metabolites (Bryant et al. 1983).

Resource enhancement and depletion experiments with carbon, nitrogen and combined nitrogen and phosphorus treatments conducted with plants and brown algae that synthesize carbon-based compounds such as polyphenolics and tannins achieve results well within the predictions of the carbon/nutrient-balance hypothesis (Coley et al. 1985; Bryant et al. 1987a; Yates and Peckol 1993; Arnold et al. 1995; Hartley et al. 1995).

However, the response of monoterpene biosynthetic pathways in terrestrial plants to changes in nutrient availability has varied considerably, often exhibiting changes outside the predictions of the carbon/nutrient-balance hypothesis (Mihaliak and Lincoln 1985; Muzika et al. 1989; Lerdau et al. 1994; Hartley et al. 1995). Fertilization experiments conducted with plants that produce monoterpenes did not always result in quantitative changes of secondary metabolites; instead, in some cases qualitative changes were observed in monoterpene biosynthesis (Mihaliak and Lincoln 1985; Mihaliak et al. 1987; Lerdau et al. 1994; Hartley et al. 1995).

Tropical marine waters around coral reefs can be considered to be nutrient-limited (Parsons et al. 1984b; Lapointe 1987; Lapointe et al. 1993; Littler et al. 1993) or to be a closed system where excess nitrogen and phosphate are not readily available for uptake by macroalgae (Atkinson 1989). Noting the nutrient regime in this environment, it is not surprising that the majority of secondary metabolites produced by marine algae are carbon-based. This trend is reflected in the algae so far investigated in studies of marine natural products (Faulkner 1995 and literature cited therein). Only cyanobacteria and a few macrophytic algal species produce nitrogen-containing compounds in detectable quantities (Ireland et al. 1989).

The branching red alga *Portieria hornemannii* (Lyngbye) Silva [order Gigartinales, family Rhizophyllidaceae; formerly *Chondrococcus hornemannii* and *Desmia hornemannii* (Silva et al. 1987)] was selected to test the effects of enhanced nutrient availability on the production of carbon-based secondary metabolites, because it exhibits notable site-to-site variation in secondary metabolite production (Paul et al. 1987; Coll and Wright 1989; Wright et al. 1990; Fuller et al. 1992) and has been shown to produce halomon, a potent biomedical agent targeted for cancer research (Fuller et al. 1992, 1994). The acyclic monoterpene, halomon [6(R)-bromo-3(S)-(bromomethyl)-7-methyl-2,3,7-trichloro-1-octene] was first isolated as a pure compound from a sample of *P. hornemannii* collected in Chanaryan, Batan Island, Philippines, in April 1986 (Fuller et al. 1992). This compound has exhibited unique selective antitumor activity in the National Cancer Institute's human tumor and disease-oriented in vitro screen (Fuller et al. 1992). To date, halomon has only been isolated from two other collections of *P. hornemannii*, one from Hawaii in 1975 (Burreson et al. 1975b) and one again from Chanaryan in April 1992 (Fuller et al. 1994). Other collections from Chanaryan and a variety of locations in the Pacific have not yielded this compound in detectable amounts; instead, an array of related halogenated acyclic and cyclic monoterpenes have been reported (Fuller et al. 1992, 1994). Different sets of major secondary metabolites have been isolated from samples collected from sites separated by as few as 10 km (Burreson et al. 1975a; Wright et al. 1990). This remarkable site-to-site chemical variation has been documented for collections at various sites in Hawaii, Guam, Australia, Japan and the Philippines (Burreson et al. 1975a; Paul et al. 1987; Coll and Wright 1989; Wright et al. 1990, 1991; Fuller et al. 1992).

Portieria hornemannii can be found in a variety of habitats on Guam: on reef flats (e.g. Anae Island) and on the reef slope from 3 m (e.g. Anae Island, Gun Beach) to 35 m (e.g. Iates Point). Preliminary studies of these populations of *P. hornemannii* suggested that ochtodene is the major secondary metabolite produced by this alga on Guam, with several acyclic monoterpenes as minor metabolites (Paul et al. 1987). Feeding assays on Guam have shown ochtodene to be an effective feeding-deterrent to herbivorous reef fishes (Paul et al. 1987, 1990, 1993). Site-to-site variation and the influence of nutrient availability on secondary metabolite production were not previously examined in populations of *P. hornemannii* on Guam. In this study, the following questions were addressed: (1) Is there site-to-site variation in secondary metabolite production in *P. hornemannii* on Guam?; (2) can this variation be correlated with internal nitrogen and phosphorus stores in the seaweed?; (3) is it possible to influence monoterpene production in *P. hornemannii* by altering the resources available to individual thalli?

Materials and methods

Collection sites and sampling methods

Portieria hornemannii (Lyngbye) Silva was collected from six sites on Guam (Fig. 1): three sites on the leeward side, Double Reef (13°36′N; 144°50′E), Anae Island (13°23′N; 144°38′E) and Gun Beach (13°31′N; 144°48′E) and three sites on the windward side of the island, Janum (13°33′N; 144°56′E), Pago Bay (13°25′N; 144°48′E), and Tagachang (13°24′N; 144°47′E). Double Reef and Janum are the northernmost sites and have an input of nutrient-rich fresh water from the Guam aquifer (Matson 1991). Anae Island and Tagachang, the southernmost sites, are located near more residential development than the northernmost sites and receive some runoff during the rainy season. Anae Island is ≃450 m offshore, with a small fringing reef on its eastern side form which *P. hornemannii* was collected. Gun Beach and Pago Bay are sites with commercial or residential development directly above or on the beach that contribute considerable runoff to the reefs during the rainy season. Nutrient-rich, fresh aquifer-water also leaks onto the reef at Gun Beach (Matson 1991). *P. hornemannii* was collected from the northern end of Gun Beach. Fresh water from the Pago River empties into Pago Bay at the southern end of the bay. Samples were collected at the northern end of Pago Bay.

Portieria hornemannii grows as small individual thalli, 3 to 8 cm tall and 5 to 10 cm in diameter. They occur 0.5 to 3 m apart attached to reef rock in areas with heavy current. Whole thalli for the bulk extractions were collected haphazardly over the reef. Bulk extractions of combined thalli were carried out to obtain milligram quantities of pure compounds for structural identification and to use for calibration curves for quantitative analyses. Whole thalli collected to observe individual variation were sampled haphazardly at least 0.5 m apart, and were extracted and analyzed separately.

Internal nutrient analysis

Prior to analysis, *Portieria hornemannii* samples for the site-to-site variation study and fertilization experiments were cleaned of epiphytes, rinsed with seawater, and blotted dry. One frond, weighing

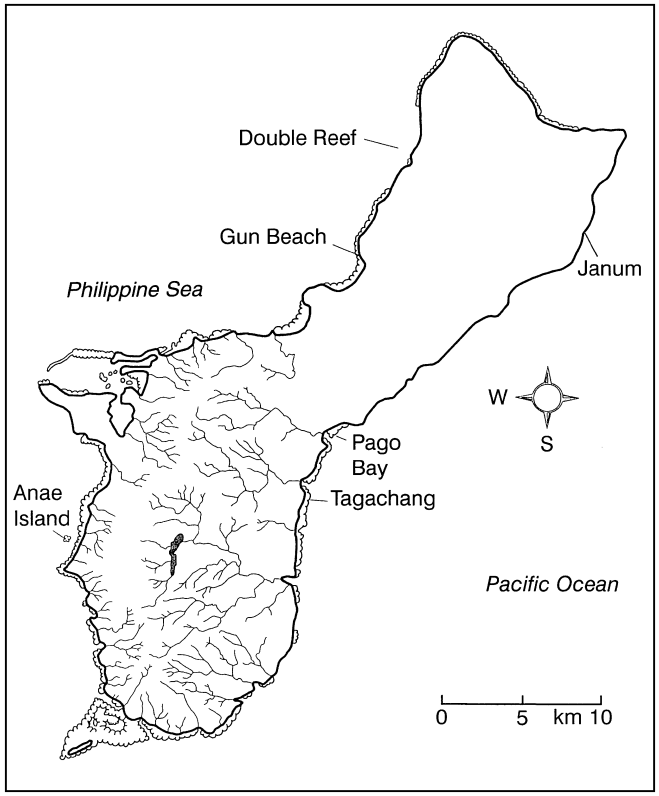

Fig. 1 *Portieria hornemannii*. Collection and study sites on Guam. Samples collected from Double Reef (3 to 10 m), Anae Island (0.3 to 10 m), Gun Beach (0.3 to 8 m), Janum (0.3 to 8 m), Pago Bay (1.5 to 10 m) and Tagachang (5 to 10 m) for analysis of site-to-site variation. In situ fertilization-experiment was conducted at north end of Gun Beach; algae for laboratory fertilization-experiments were collected from Anae Island

3 to 30 mg (dry mass), was cut from each individual thallus with forceps, oven-dried at 62 °C for 24 h, and stored in a tightly closed vial. These samples were submitted to the College of Agriculture and Life Sciences at the University of Guam for analyses of total Kjeldahl nitrogen (TKN) (TKN in soil/plant, Quickchem method 13-107-06-2-D) and total Kjeldahl phosphorus (TKP) (total phosphorus in Kjeldahl digests, Quickchem method 13-115-01-1-B) on an automated ion analyzer (Lachat Instruments, Milwaukee, Wisconsin).

Data for TKN and TKP were collected as the percent yield of nitrogen and phosphorus from dry mass of the alga. Percentages were arcsine-square-root or log-transformed before statistical analysis. Bartlett's test of equal variance was used to test for homogeneity of variances. Transformed data were compared by one-way analysis of variance (ANOVA) when the assumption of homogeneity of variances was met. Tukey's honestly significant-difference (HSD) pairwise comparison of means test was used to determine which means were different ($p < 0.05$). When the data did not meet the requirements for an ANOVA, means were compared by a non-parametric Kruskal–Wallis test (Sokal and Rohlf 1981). Statistix 4.0 (Analytical Software, St. Paul, Minnesota, USA) was used for all statistical analyses.

Chemical analysis

The remainder of the cleaned algal tissue from each sample that was not analyzed for internal nutrients was ground in a Virtis Hi-Speed "23" homogenizer in a small amount of methanol (MeOH) and immersed in 1:1 dichloromethane (DCM)/MeOH. Bulk samples and samples of individual thalli were extracted exhaustively in 1:1 DCM:MeOH over a 72 h period. The remaining algal material was dried in an oven at 62 °C. The extracts were partitioned between hexanes and water to remove salts and water-soluble components, then the solvents were evaporated to yield a crude extract.

The crude extract of the bulk collections was partitioned by normal-phase silica-gel vacuum-flash chromatography in a 5 to 20% hexanes/ethyl acetate gradient. Qualitative separation was achieved by preparative normal-phase high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 501 HPLC pump and a R401 differential refractometer. The column was an Alltech Econosil 10U silica column (25 cm × 10 mm). Samples were injected on the column through a 2 ml loop. The compounds were separated in pure hexanes or 5% ethyl acetate/hexanes solvent systems. The chemical structure for ochtodene was confirmed by comparison with previously reported chemical shifts for ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (McConnell and Fenical 1978; Paul et al. 1987). Similar techniques were used to investigate minor compounds.

Crude extracts of individual thalli were partitioned through a Florisil (4 to 6 mg) column with 20% ethyl acetate/hexanes to remove chlorophyll pigments and polar materials, before quantitative analysis on an analytical HPLC instrument with an integrator in 100% hexanes. This system consisted of a Beckman Model 110B solvent pump, Model 156 refractive index detector and Model 427 integrator. The analytical column was an Alltech Spherisorb S-5-W Silica 5U column (25 cm × 4.6 mm). One mg (10 µl) samples were injected onto the column through a 20 µl loop. Standard curves were obtained for ochtodene and triglycerides.

Organic extract yields were calculated from total dry mass of extract plus extracted algal material. The mass of pure compound per injection was calculated from the standard curve and converted to percent yield of pure compound from the total dry mass. The dry mass of individual thalli, organic extract and pure compound yields were statistically analyzed by one-way ANOVA as previously described.

Site-to-site variation

One bulk sample and 14 to 17 individual samples were collected per site between 25 and 31 August 1994. For each site, five individuals that were analyzed for secondary chemistry were also analyzed for TKN and TKP as previously described in subsection "Internal nutrient analysis". The mean yields of TKN and TKP were compared by a Kruskal–Wallis test for differences among sites. Chemical data were log-transformed, and means were compared by one-way ANOVA for differences among sites.

In situ field nutrient-enrichment experiment

A field nutrient-enrichment experiment was conducted at Gun Beach for 5 wk, from 20 May to 27 June 1994. Toroidal seep-through bags of fertilizer were attached by cable ties to aluminum screens (30 cm × 30 cm, center hole 12 cm × 12 cm) fastened by masonry nails to the reef around the base of individual *Portieria hornemannii* thalli at depths of 3 to 5 m. The bags were attached 10 to 15 cm away from the center hole, so that the algae were not disturbed. Commercially available agricultural fertilizers were used for nitrogen [Par Ex ID with isobutylidenediurea (IBDU), 34-0-0, time release], phosphorus [triple super phosphate (TSP), 0-45-0] and combined nitrogen and phosphorus (34:45:0 urea:phosphate:potassium) enhancement. IBDU, which contains nitrogen in the form of urea, was chosen for the experiment because it was the only form of nitrogen available in a slow-release formula. The bags on the control screens were filled with dried sand from Gun Beach. A total of 96 screens were attached to the reef, 24 of each treatment and 24 control plates. Water samples (300 ml each) were collected in empty, acid-washed nalgene bottles within 20 cm of the base of 10 of each treament and 10 control plates periodically over 9 d. Phosphate concentrations were determined by reaction with a

molybdic acid-ascorbic acid-trivalent antimony solution, followed by spectrophotometric analysis at 880 nm as described by Parsons et al. (1984a). Urea concentrations were not measured. Divers removed depleted treatment bags and replaced them with full bags every 7 to 9 d. Algae were not disturbed during the replacement of bags. Control bags were not changed.

After 5 wk, approximately half of the N-alone (n = 11), P-alone (n = 9), N + P (n = 8) and control (n = 8) individuals were recovered from the reef for analysis of internal nutrients and secondary metabolites according to the procedures previously described in earlier subsections. The remaining individuals were not present at the end of the experiment.

Shaded laboratory fertilization-experiment

An enhanced nutrient experiment was conducted at the University of Guam Marine Laboratory in shaded flow-through tanks from 25 August to 13 September 1994. Thirty *Portieria hornemannii* individuals, attached to rocks collected from Anae Island by hammer and chisel on 25 August, were transported in coolers containing seawater to the laboratory, where 10 individuals (initial field controls) were promptly prepared for analysis of internal nutrients and secondary metabolites as previously described in subsection "Chemical analysis". Twenty individuals, still attached to the rocks, were weighed and then placed in separate, covered, flow-through tanks arranged in two rows of ten. Three treatments (enriched nitrate, enriched phosphate, and combined) and control tanks were assigned with a random-numbers table. Seawater was drawn from Pago Bay, and was UV-treated and filtered before reaching the tanks to limit the introduction of epiphytes.

"Pulsed" nutrient enrichment according to the methods of Lapointe (1987) and Lapointe and O'Connell (1989) was used for the fertilization of individual *Portieria hornemannii* for 18 d. Each evening, the seawater flow was stopped, and nutrients were added to the treatment tanks at approximately six times the ambient concentrations of seawater in Pago Bay (Matson 1991), nitrate (50 μM) in the form of $NaNO_3$ and phosphate (10 μM) in the form of NaH_2PO_4. Seawater flow was restored each day to prevent the water in the tanks from getting too warm. Periodically during the experiment, nutrient concentrations were measured in each tank in the evening after adding the treatments and in the morning before restoring seawater flow to monitor nutrient depletion overnight. At the end of the experiment, nutrient concentrations were measured in empty, clean tanks overnight to make certain that the added nutrients were not absorbed from the seawater onto the walls of the tanks during the experiment. Nitrate concentrations were determined by nitrate reduction through shaking with cadmium followed by spectrophotometric analysis at 543 nm, according to the methods of Jones (1984). Phosphate concentrations were determined as previously described. At the end of the experiment, the individuals of *P. hornemannii* were removed from the tanks and weighed, still attached to the rocks. The growth of *P. hornemannii* during the experiment was estimated by calculating the change in wet mass (final minus initial). Seaweed was prepared for internal nutrient and chemical analyses as described in earlier subsections.

Data were analyzed for significant differences among the control and three treatments. Nutrient depletion data were compared by a Kruskal–Wallis test. Growth data were log-transformed and compared by one-way ANOVA. Internal nutrient, percent yield of the extracts, and major secondary metabolite data were arcsine-square-root transformed and compared by one-way ANOVA.

Unshaded laboratory fertilizaton-experiment

Quantum flux density (μM quanta $m^{-2}s^{-1}$) was measured with a non-submersible model LI-190 SA quantum sensor (LI-COR 1000 Datalogger; LI-COR, Lincoln, Nebraska) every 2 h for 10 h (08:00 to 18:00 hrs) in two empty covered and two empty uncovered tanks on a sunny day (21 February 1995) to estimate the difference in light in shaded versus unshaded tanks.

A second laboratory fertilization-experiment was conducted from 13 to 24 February 1995, with the same procedures in previous subsection, except that the 20 treatment tanks were unshaded. The algae began to bleach in the tanks, so the duration of the experiment was only 11 not 18 d. A second set of field controls (n = 10) was collected on 28 February 1995, at the end of the experiment.

TKN data were compared by one-way ANOVA while TKP data were compared by a Kruskal–Wallis test. Percent yields of the extracts and major secondary metabolites were arcsine-square-root transformed, and compared by one-way ANOVA.

Results

Chemical analysis

Two major peaks were observed in the analytical HPLC traces of all *Portieria hornemannii* extracts analyzed. The ^{1}H NMR of the first major peak (Peak 1, retention time = 1.38 to 1.45 min) suggested that this compound was a triglyceride composed primarily of saturated fatty acids (evidenced by no olefinic protons in the 5.0 to 5.5 ppm range). The second major peak (retention time = 3.30 to 7.14 min) was a mixture of mostly ochtodene and a structurally related minor compound, "isoochtodene". ^{1}H NMR analysis of the extracts from the site-to-site variation study indicate that "isoochtodene" comprised 5 to 20% of the ochtodene and "isoochtodene" mixture in *P. hornemannii* individuals collected from all sites. "Isoochtodene" concentrations were highest in the algae collected from Anae Island. NMR analysis of the extracts from the control and treated algae from the in situ fertilization experiments at Gun Beach showed that the extracts were primarily triglyceride and ochtodene with <5% "isoochtodene". The separation of "isoochtodene" from ochtodene by HPLC has not been possible to date, and pure, "isoochtodene" has not been available for characterization by structural determination. Although the second peak is a mixture, it will henceforth be referred to as ochtodene, the major component of the extracts. Both ochtodene and triglyceride concentrations were analyzed quantitatively for site-to-site variation and changes due to enhanced fertilization in field and laboratory experiments.

Site-to-site variation

Portieria hornemannii showed significant site-to-site variation in mean dry mass per individual (Fig. 2a), extract (Fig. 2b), ochtodene (Fig. 2c) and triglyceride (Fig. 2d) yields. The mean dry mass per individual of the samples from Double Reef was approximately twice that of the samples from the other five sites (Fig. 2a). Samples from Anae Island and Pago Bay exhibited a significantly greater extract yield than samples from Double Reef. The samples from Anae Island had the highest mean yield of ochtodene, 1.11 ± 0.2% of the dry mass (Fig. 2c). Ochtodene yields in the Pago Bay and Gun Beach extracts were 0.63 ± 0.1% and 0.48 ± 0.03%

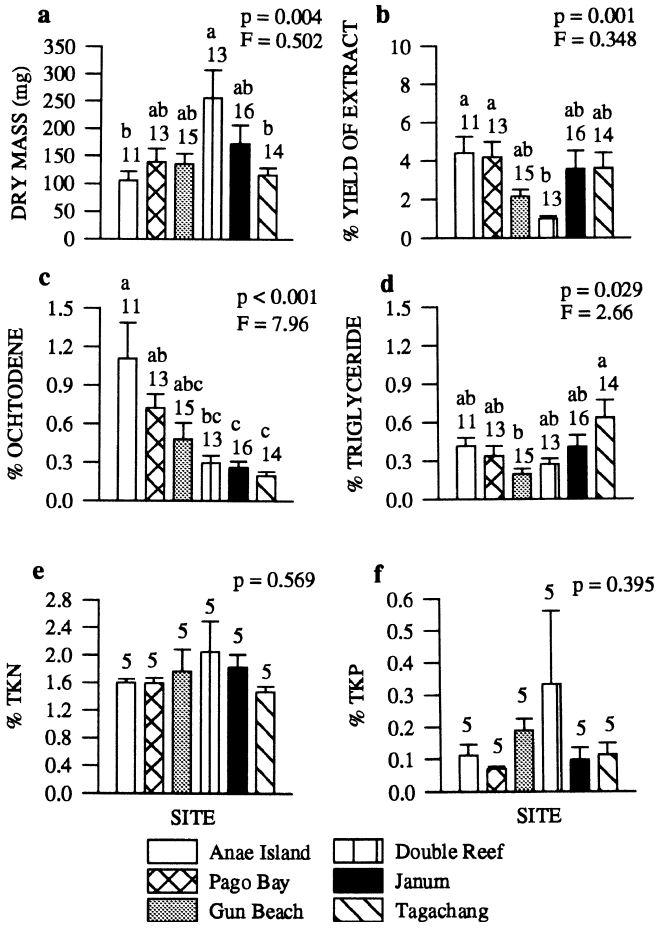

Fig. 2 *Portieria hornemannii*. Mean dry mass (**a**), extract (**b**), ochtodene (**c**), triglyceride (**d**), total Kjeldahl nitrogen, TKN (**e**) and total Kjeldahl phosphorus, TKP (**f**) yields (+ 1 SE) for samples collected between 25 and 31 August 1994. Dry mass, TKN and TKP were analyzed by Kruskal–Wallis test. Data for yields were arcsine-square-root transformed and analyzed by one-way ANOVA followed by Tukey's HSD [*Identical letters above bars* indicate similar means (p >0.05); *number above bars* number of samples analyzed per site]

of the dry mass. The samples from Double Reef, Janum, and Tagachang exhibited the lowest yields, 0.30 ± 0.05%, 0.26 ± 0.05% and 0.20 ± 0.03% of dry mass, respectively. Tagachang samples, which had the lowest yield of ochtodene, exhibited the highest yield of triglyceride, 0.64 ± 0.1% of dry mass. Samples from Double Reef had a significantly lower yield of triglyceride than samples from Tagachang.

There was no significant difference observed in the internal nitrogen (Fig. 2e) or phosphorus (Fig. 2f) stores among sites. The mean value for internal nitrogen for all the samples analyzed was (≃1.6% of the dry mass. The mean value for internal phosphorus for all the samples was 0.15% of dry mass. There was no correlation observed between the yields of ochtodene and internal nitrogen or phosphorus (r^2 = 0.001, p = 0.321; r^2 = –0.029, p = 0.585), or of triglyceride and internal nitrogen and phosphorus (r^2 = 0.021, p = 0.502; r^2 = 0.015, p = 0.431).

In situ fertilization-experiment

Mean phosphate concentrations around the P-alone and N + P treated-algae ranged from 5.6 to 25.0 μ*M* when the bags were first laid on the reef, and dropped to ≃0.5 μ*M* after 5d. The concentrations around the control and N-alone-treated algae ranged from 0.4 to 2.5 μ*M* for the duration of the experiment.

Mean dry mass per individual (Fig. 3a) and ochtodene (Fig. 3c) concentrations did not differ significantly among treatments. Organic extract (Fig. 3b) and triglyceride (Fig. 3d) yields for the N + P-treated algae were significantly higher than for the control and other two treatments. The mean extract yield of the N + P-treated algae was 14.53 ± 2.2% of the dry mass, twice that observed for the control, N-alone and P-alone treatments.

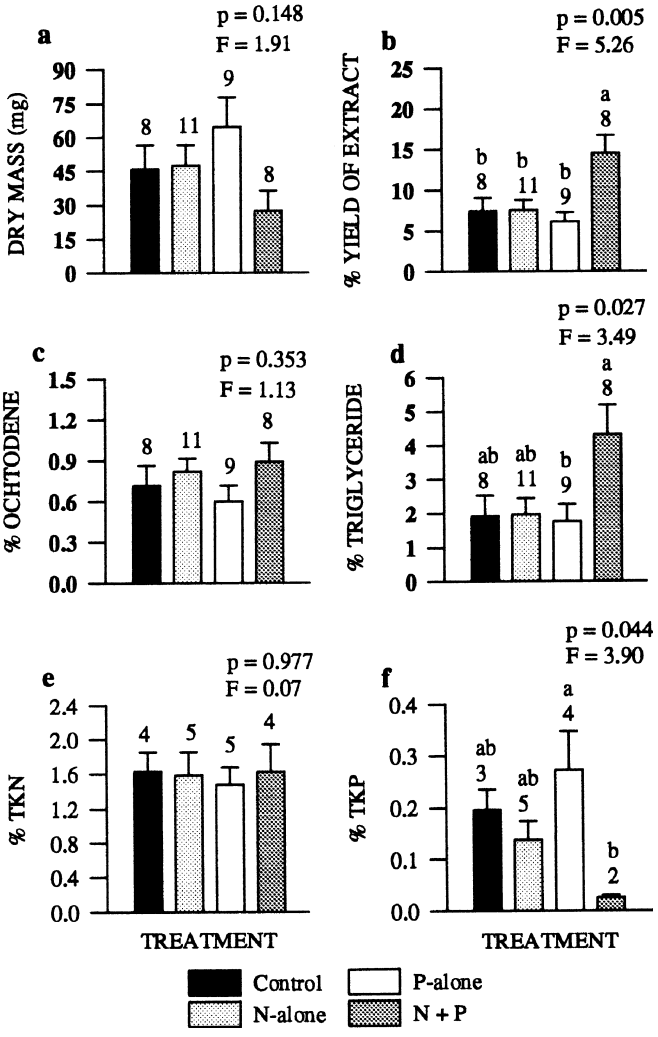

Fig. 3 *Portieria hornemannii*. In situ fertilization-experiment. Mean dry mass (**a**), extract (**b**), ochtodene (**c**), triglyceride (**d**), TKN (**e**) and TKP (**f**) yields (+ 1 SE) for control, N-alone, P-alone and N + P treatment. Data were log-transformed and analyzed by one-way ANOVA followed by Tukey's HSD [*Identical letters above bars* indicate similar means (p >0.05); *numbers above bars* number of samples analyzed]

The extract yields for the site-to-site variation portion of this study were also considerably lower, ranging from 1 to 4% of dry mass (Fig. 2b). The mean yield of triglyceride was 4.32 ± 0.9%, double that of the control, N-alone and P-alone treatments. The mean yields of triglyceride for all *Portieria hornemanii* used in the field experiment were considerably higher compared with the algae extracted from Gun Beach for the site-to-site variation study, which had a mean yield of 0.27 ± 0.04% (Fig. 2d).

Because of the small size of *Portieria hornemannii*, two or three samples of the same treatment were pooled in some cases for internal nutrient analysis to increase the number of replicates analyzed. At least three individual samples were analyzed per treatment; however, the number of samples per treatment may be less because the phosphorus concentrations of some individual thalli were below the detectable limits of the ion analyzer. No significant difference was observed in internal nitrogen content among treatments (Fig. 3e). The average for all treatments was ≃1.6% of the dry mass, as previously seen for the site-to-site internal nitrogen levels (Fig. 2e). Significant differences were observed in internal phosphorus content (Fig. 3f). The individuals fertilized with N + P were significantly lower in phosphorus content. The internal phosphorus concentration of two of the four N + P samples analyzed were below the detection level of the instrument. No correlation was observed between the yields of ochtodene and internal nitrogen or phosphorus ($r^2 = 0.008$, $p = 0.31$; $r^2 = 0.016$, $p = 0.389$), or triglyceride and internal nitrogen or phosphorus ($r^2 = 0.055$, $p = 0.609$; $r^2 = 0.078$, $p = 0.815$).

Shaded laboratory fertilization-experiment

The light intensity on a sunny day available to empty, shaded tanks in the laboratory during daylight hours (08:00 to 18:00 hrs) ranged from 60 to 456 μ*M* (Fig. 4). NO_3^- and $H_2PO_3^-$ were depleted from the enhanced nutrient-treated tanks during the evening "pulse" (Fig. 5), whereas the nutrients in scrubbed empty tanks without *Portieria hornemannii* were not depleted.

No significant difference was observed in the change in wet mass of *Portieria hornemannii* among treatments in the shaded experiment in August to September 1994 ($F = 0.25$, $p = 0.863$). The algae in all treatments showed an increase in wet mass, with high variability within treatments. Mean dry mass (Fig. 6a), extract yield (Fig. 6b), ochtodene (Fig. 6c) and triglyceride (Fig. 6d) yields differed significantly between the initial field control and the control and treated algae kept in the tanks during the experiment. The algae collected for the field controls were smaller, but yielded significantly more extract. The ochtodene and triglyceride yields were 1.11 ± 0.3% and 0.41 ± 0.07%, respectively, almost four times greater than for the algae kept in the tanks. No significant difference was observed in mean algal size, extract yield, ochtodene and triglyceride concentrations among algae in the control and the various treatments. No significant difference was observed for internal nitrogen (Fig. 6e) and phosphorus (Fig. 6f) content among the field control, laboratory control and various fertilization treatments. The mean internal nitrogen concentration for all *P. hornemannii* analyzed was 1.6% of the dry mass. No correlation was observed between the yield of either ochtodene and internal nitrogen or internal phosphorus ($r^2 = 0.056$, $p = 0.827$; $r^2 = 0.033$, $p = 0.484$), or triglyceride and internal nitrogen or internal phosphorus content ($r^2 = 0.181$, $p = 0.034$; $r^2 = 0.044$, $p = 0.552$).

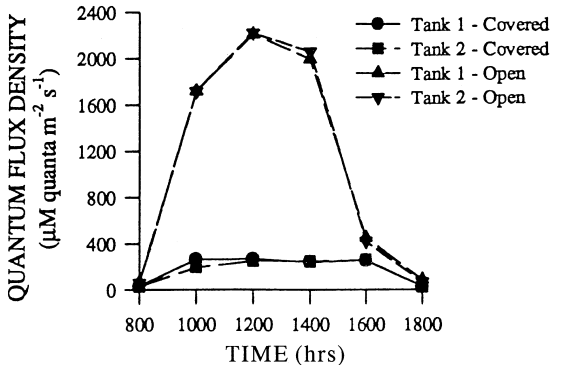

Fig. 4 *Portieria hornemannii.* Quantum flux density (μ*M* quanta m^{-2} s^{-1}) measured on 21 February 1995 in two empty covered and two uncovered tanks used to keep individual algae in shaded and unshaded fertilization-experiments

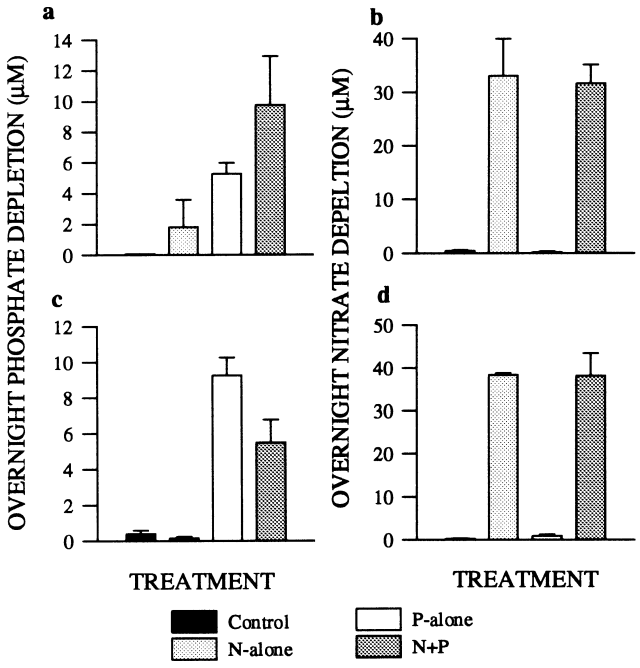

Fig. 5 *Portieria hornemannii.* Phosphate (**a,c**) and nitrate (**b,d**) depleted from control and treated laboratory tanks in 12 h on 9/10 June 1994 (**a,b**) and 15/16 June 1994 (**c,d**). Depletion data compared by Kruskal–Wallis test

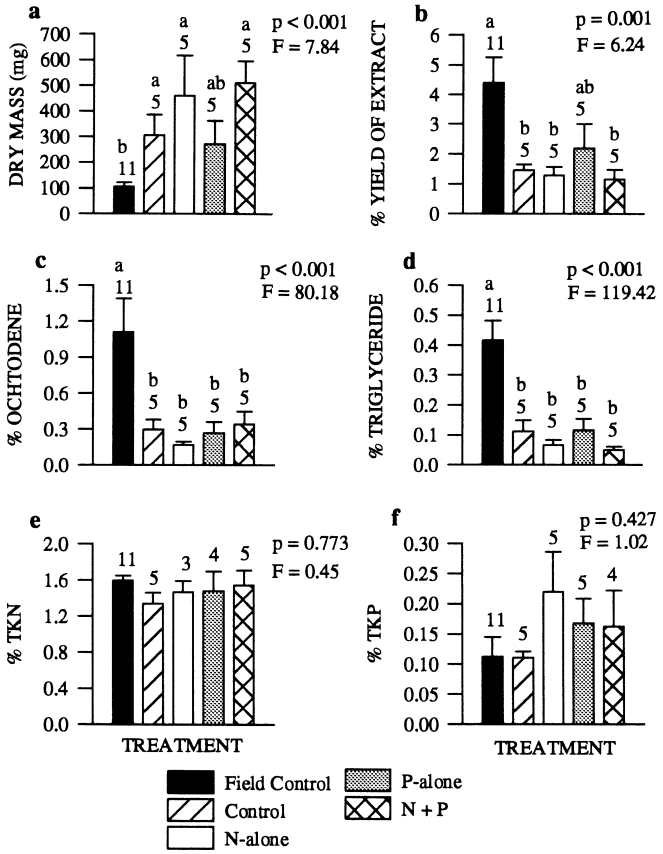

Fig. 6 *Portieria hornemannii*. Shaded laboratory fertilization-experiment. Mean dry mass (**a**), extract (**b**), ochtodene (**c**), triglyceride (**d**), TKN (**e**) and TKP (**f**) yields (+ 1 SE) for control, N-alone, P-alone and N + P treatment conducted during August to September 1994. Data for yields were log-transformed and analyzed by one-way ANOVA followed by Tukey's HSD [*Identical letters above bars* indicate similar means ($p > 0.05$); *number above bars* number of samples analyzed per treatment]

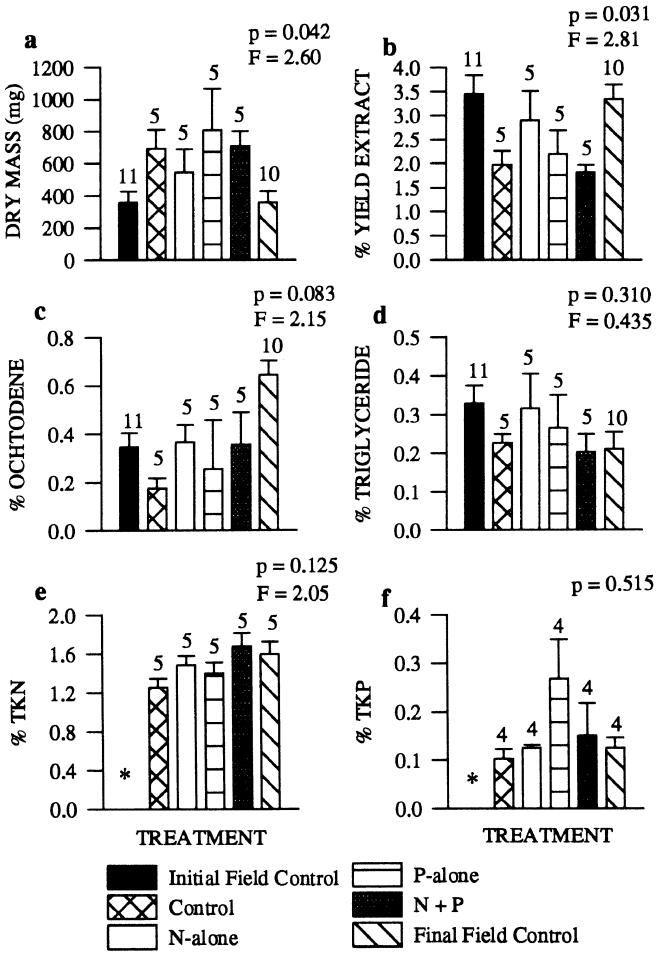

Fig. 7 *Portieria hornemannii*. Unshaded laboratory fertilization-experiment. Mean dry mass (**a**), extract (**b**), ochtodene (**c**), triglyceride (**d**), TKN (**e**) and TKP (**f**) yields (+ 1 SE) for control, N-alone, P-alone and N + P treatment conducted during February 1995. Data for dry mass, extract, ochtodene, triglyceride and TKN yields were log-transformed and analyzed by one-way ANOVA. Tukey's HSD comparison of means did not indicate significant differences among dry mass and extract-yield means. Data for TKP yields were compared by Kruskal–Wallis test [*Number above bars* number of samples analyzed; *samples collected for initial field controls were not analyzed for internal nutrients]

Unshaded laboratory fertilization-experiment

Light intensity available to the unshaded tanks ranged from 60 to 2250 μM, a five-fold increase relative to the shaded tanks (Fig. 4). The *Portieria hornemannii* in the unshaded experiment conducted in February 1995 did not exhibit significant differences in the change in wet mass among treatments ($F = 1.22$, $p = 0.337$). Mean dry mass (Fig. 7a) and extract yield (Fig. 7b) were significantly different among treatments; however, Tukey's HSD test for comparison of means for both algal dry mass and extract yield failed to show a difference in the means ($p > 0.05$). The *P. hornemannii* initial field controls from Anae Island were approximately one half the size of the individuals in the tanks at the end of the experiment, but similar in size to final field controls. The extract yields of the 9 and 28 February 1995 field controls and the N-alone treatment were high, 3.25 ± 0.42%, 3.48 ± 0.81% and 2.89 ± 0.62%, respectively. The yields of ochtodene (Fig. 7c) and triglyceride (Fig. 7d) did not differ significantly among the controls and treatments. A trend in temporal variability was detected in ochtodene production. The field controls collected on 28 February 1995 exhibited a mean ochtodene yield of 0.64 ± 0.09%, twice that of the field controls collected on 9 February 1995 and the control and treatment algae used for the experiment.

No significant differences were observed for the internal nutrient stores of nitrogen (Fig. 7e) and phosphorus (Fig. 7f) among the control and the treated algae. Mean values for the internal nitrogen stores ranged between 1.2 and 1.6%. While not significantly different, stores of phosphorus were observed in the phosphate-treated algae of 0.27 ± 0.08% compared with 0.1% seen for the other treatments. Field controls collected on 9 February 1995 from Anae Island were not analyzed for internal nutrient stores. No correlation was observed between the yields of either ochtodene and internal ni-

trogen or internal phosphorus ($r^2 = 0.132$, $p = 0.065$; $r^2 = 0.104$, $p = 0.120$), or triglyceride and internal nitrogen content ($r^2 = 0.014$, $p = 0.4$; $r^2 = 0.071$, $p = 0.988$).

Discussion

Studies of the carbon/nutrient-balance hypothesis in the marine environment have focused on the temperate brown alga *Fucus vesiculosus*. The polyphenolics produced by this alga were reported to accumulate under nitrogen deficiency, while carbon content did not seem to influence secondary metabolite production. From these results it was hypothesized that external resource availability could modify the accumulation of polyphenolic compounds (Ilvessalo and Tuomi 1989). More direct tests of the effects of nutrient availability with *F. vesiculosus* (Yates and Peckol 1993) and *Lobophora variegata* (Arnold et al. 1995) have shown that polyphenolic production is inversely proportional to nitrogen availability, suggesting that algae that produce polyphenolics should respond to changes in nutrient availability as predicted by the carbon/nutrient-balance hypothesis. In a recent study of the two brown seaweeds *Dictyota ciliolata* and *Sargassum filipendula*, that produce terpenes and pholorotannins as their major secondary metabolites, respectively, nutrient enhancement did not decrease the concentrations of carbon-based secondary metabolites as predicted by the carbon/nutrient-balance hypothesis (Cronin and Hay 1996). In a field nutrient-enhancement experiment with *D. ciliolata*, increased nutrients resulted in increased terpene concentrations, while nutrient enhancement of *S. filipendula* in the field did not change phlorotannin concentrations (Cronin and Hay 1996).

In this study with the red alga *Portieria hornemannii*, neither ochtodene nor triglyceride concentrations decreased as a result of the enhanced nitrogen and phosphorus regimes in the field, shaded or unshaded laboratory experiments. Triglyceride yields were significantly increased across all treatments in the field fertilization-experiment compared with those algae extracted for site-to-site variation and the laboratory experiments. This is in contrast to studies with the diatoms *Chaetoceros gracilis* and *Phaetodactylum tricornutum*, in which nutrient stress increased triglyceride concentration (Parrish and Wangersky 1987; Lombardi and Wangersky 1991). Another study, with seven species of microalgae, reported that not all species accumulated lipids under N-depletion (Reitan et al. 1994). In some cases, either no correlation was displayed or total lipid concentration decreased.

The response of monoterpene biosynthetic pathways in terrestrial plants to changes in nutrient availability varies considerably (Mihaliak and Lincoln 1985; Muzika et al. 1989; Lerdau et al. 1994; Hartley et al. 1995). In some cases, increased monoterpene concentrations have been correlated with decreased nitrogen availability, while other studies have found the opposite relationship, and others again saw no effect at all (Lerdau et al. 1994). A recent study with Sitka spruce (*Picea sitchensis*) seedlings, which produce polyphenolics, tannins and monoterpenes, showed that polyphenolic and tannin concentrations varied within the predictions of the carbon/nutrient balance hypothesis, while monoterpene concentrations did not vary during the time frame of the experiment (Hartley at el. 1995).

Physiological studies of terrestrial plants that produce monoterpenes have shown that monoterpenes are synthesized and stored in discrete cells (Falk et al. 1990; Brun et al. 1991; Lerdau et al. 1994). The variable response of monoterpene biosynthesis to nutrient stress has been suggested to depend more on how resource availability limits photosynthate supply, which controls the differentiation of storage cells in the plant, than on the availability of excess carbon for monoterpene biosynthesis (Lerdau et al. 1994). An increase in the production of storage cells and/or monoterpene concentrations resulting from an increase in nutrient availability may only occur if the algae are nutrient-limited at the time of fertilization (Mihaliak and Lincoln 1985).

The quantitative changes observed in triglyceride yields between the shaded and unshaded experiments suggest that light is a potential factor affecting the production of triglycerides in *Portieria hornemannii* (Figs. 6d, 7d). A difference was not observed in triglyceride concentration for the controls of the shaded and unshaded laboratory experiments, indicating that there is little temporal variation. Ochtodene yields did not differ in overall concentration between the shaded and unshaded experiments (Figs. 6c, 7c). However, in the laboratory fertilization-experiment conducted during August to September 1994 (Fig. 6c), there was a significant decrease in the yield of ochtodene in the algae kept $\simeq 3$ wk, in the shaded laboratory tanks across all treatments compared with the initial field controls, suggesting that light is also a potential factor affecting ochtodene biosynthesis. Light has been shown to be a factor in cell differentiation of storage structures and monoterpene production in terrestrial plants (Brun et al. 1991; Lerdau et al. 1994) and in terpene production in the brown alga *Dictyota ciliolata* (Cronin and Hay 1996).

We do not know what factors are responsible for the observed variation in ochtodene production among different sites on Guam. The results from the shaded laboratory experiment suggest that light may be a factor, but there is no evidence suggesting that the algae are more shaded at some sites than at others. The significant differences in ochtodene production in *Portieria hornemannii* may be due to genetic variability in populations among sites. Monoterpene production has been shown to be under strong genetic control in many species of terrestrial plants (Zavarin et al. 1990). Red algal spores are transported by currents to potential settling areas (Lobban and Harrison 1993); different genetic popula-

tions of *P. hornemannii* may thus arise at various sites on Guam, depending upon current patterns.

Monoterpenes have been shown to be induced by herbivory in terrestrial environments (Lerdau et al. 1994). The effect of herbivory on monoterpene production was not addressed in this study, but is a possible factor in the site-to-site variability of ochtodene production for *Portieria hornemannii* on Guam.

Alternatively, variation could relate to the age of individuals at different sites. Density of *Portieria hornemannii* varies considerably at different sites around Guam, suggesting that there is seasonal variation in settlement. Since it was not possible to control for age in the field samples, algae collected for the site-to-site variation study may have been at different stages in the life cycle as there were significant differences in the size of the individuals from some sites (Fig. 2a). Quantitative differences in the ochtodene concentrations observed between the *P. hornemannii* collected in August 1994 from Gun Beach (0.4% dry mass) (Fig. 2c) and the thalli used in the field experiment conducted in June 1994 (0.6 to 0.9% dry mass) (Fig. 3c) also suggest that the age of the algae may contribute to intraspecific variation. Similar differences were also observed for the 9 February 1995 (0.3% dry mass) and 29 February 1995 (0.6% dry mass) (Fig. 7c) field controls used in the unshaded laboratory experiment. Temporal variation in monoterpene biosynthesis has been seen in bulk extracts from collections from the Great Barrier Reef and the Philippines (Coll and Wright 1989; Fuller et al. 1992, 1994). Monoterpene concentrations have been shown to vary with age in *Mentha* ×piperita, depending upon the developmental stage of the storage structures (Brun et al. 1991). Further research in this area should focus upon investigations of temporal variation and the potential effects of herbivory on monoterpene production.

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