AN ABSTRACT OF THE THESIS presented by Bridgette S. Davidson for the Degree of Master of Science in Biology, March 19, 1992.

Title: Variation in the secondary metabolites of the sponge *Dysidea* cf. *avara.*

Approved: Valerie J. Paul
Valerie J. Paul, Chairperson, Thesis Committee

Dysidea cf. *avara,* a common sponge on reefs within Apra Harbor on Guam, exhibits significant spatial variation in secondary metabolite concentrations. One of the secondary metabolites in the sponge, avarol, deterred feeding by reef fish at natural concentrations. I hypothesized that secondary metabolite concentrations in sponges are plastic and that biological and physical environmental factors affect production of the secondary metabolites and tested this hypothesis by transplantation between sites. To examine the factors influencing site-to-site variation in secondary metabolite concentrations in *Dysidea* cf. *avara* I tested the effects of 1) artificial grazing using scissors, and 2) light and UV radiation on secondary metabolite concentrations. Variation in secondary metabolites was evaluated by two methods: 1) chemical analysis by quantitative HPLC and then 2) extracts of experimental sponges were compared with appropriate controls in field assays designed to quantify the effects on potential predators. Transplantation of sponges caused their secondary metabolite concentrations to become similar to control sponges, even though differences were significant between the two transplant study sites. Feeding assays of extracts from transplant experiment sponges showed no conclusive results. Artificial grazing and light manipulations did not significantly alter concentration's of secondary metabolites or reef fish's preference for sponge extracts in feeding assays. Thus, some factor other than light or grazing

appears to be responsible for variation in secondary metabolite production in these sponges. A possibility is that observed variation in secondary metabolite concentrations is due to variation in food availability and/or metals at the different sites.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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VARIATION IN THE SECONDARY METABOLITES OF THE SPONGE *DYSIDEA* CF. *AVARA*

BY

BRIDGETTE S. DAVIDSON

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INTRODUCTION

Many plants and animals produce secondary metabolites that are toxic or act as feeding deterrents (Rosenthal and Janzen 1979; Van Alstyne and Paul 1988; Rosenthal and Berenbaum 1991). Intraspecific variation in concentrations and types of secondary metabolites has been observed in many terrestrial plants and marine organisms (Rosenthal and Janzen 1979; Paul and Van Alstyne 1988; Green *et al.* 1992; Lindquist *et al.* 1992; Paul 1992). Variation can be within individuals, temporal or spatial. This study is primary concerned with spatial variation in secondary metabolites. Factors influencing chemical variation are not fully understood and are of interest to ecologists, natural products chemists and pharmacologists.

Spatial variation in secondary metabolites has been observed in several marine algae and invertebrates. *Halimeda* was found to have higher levels of secondary metabolites in areas of higher rates of herbivory (reef slope), than in areas of lower rates of herbivory (reef flats) (Paul and Van Alstyne 1988). *Udotea cyathiformis* and *Rhipocephalus phoenix* individuals from reefs contained about two times the level of secondary metabolites as individual's from seagrass beds (Paul and Fenical 1986). *Halimeda goreauii* individuals from shallow reefs contained greater level of secondary metabolites as individual's from deep reef sites (Paul and Fenical 1986). A red alga, a soft coral and ascidians were also noted as having spatial variation in secondary metabolites (Phillips and Tower 1982; Green *et al.* 1992; Lindquist *et al.* 1992).

Spatial variation in secondary metabolite concentrations may be caused by differences in grazing pressure which can influence secondary metabolite production. When secondary metabolite production increases due to herbivory, this enhanced level of defense is termed an inducible defense. Inducible defenses due to herbivory have been studied in two marine algae (Van Alstyne 1988; Renaud *et al.* 1990). When

grazing was simulated by clipping the alga *Fucus distichus*, an increase in the concentration of polyphenolics was found relative to algae that had not been clipped (Van Alstyne 1988). Manual clipping and grazing by sea urchins of the brown alga, *Padina gymnospora*, produced plants that significantly deterred grazing by sea urchins relative to unmanipulated *Padina* (Renaud *et al.* 1990). Renaud *et al.* inferred that this deterrence was probably due to changes in algal chemistry, but the putative chemical change was never analyzed.

Only a few studies have been conducted with marine organisms that involved manipulating physical environmental factors to see how these affect secondary metabolite production. Light induced a change in secondary metabolite levels in the sponge *Rhopaloeides odorabile* (Thompson *et al.* 1987). Both depth and light were manipulated by transplanting and shading the sponges. The diterpene content was a function of available light, with exposed sponges at 5 m depth having significantly greater quantities of diterpenes than sponges that were shaded at this depth. Light probably did not effect phototrophic symbionts because they were not found in the sponge matrix, and the secondary metabolites did not absorb UV-b light and therefore probably did not protect the sponge from UV radiation. Thompson *et al.* (1987) concluded that the most likely explanation for the changes in concentration was to deter surface fouling by algal growth, which is most intense under highly illuminated conditions. Carbon, nitrogen and phenols were measured over time in three populations of the brown alga, *Fucus vesiculosus* (Ilvessalo and Tuomi 1989). A significant negative correlation was found between phenolic content and the ratio of nitrogen to carbon; therefore, phenolic compounds were being produced when there was excess carbon in the brown algae and may have varied as a function of nitrogen availability in the environment.

This study focuses on the marine sponge *Dysidea* cf. *avara*. Marine sponges have been less studied than seaweeds by chemical ecologists even though they contain many bioactive secondary metabolites. Patterns of chemical variation have not been well documented in most marine invertebrates including sponges. *Dysidea* cf. *avara*'s secondary metabolites (avarol and avarone) are of pharmacological interest for *in vivo* and *in vitro* antileukemic activity, inhibition of HIV-1 replication and antimutagenic effects (Loya and Hizi 1990; Belisario *et al.* 1991; 1992). If ecological factors affecting production of the secondary metabolites in *Dysidea* spp. were known, culture of *Dysidea* spp. and possibly other sponges could better maximize yields of secondary metabolites of interest.

I hypothesized that secondary metabolite concentrations in *Dysidea* cf. *avara* are plastic and that biological and physical environmental factors affecting their production.

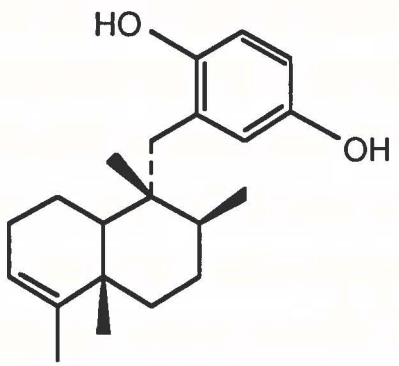
I examined spatial variation by addressing four questions: 1) What are the patterns of site-to-site variation in secondary metabolite concentrations? 2) Is the observed spatial variation the result of environmental (as opposed to genetic) controls? 3) Does artificial grazing have an effect on secondary metabolite concentrations? and 4) Does light (including UV radiation) affect the secondary metabolite concentrations in the sponge? Two approaches were taken to document the effects of various manipulations on the chemistry of *Dysidea cf. avara* First, the changes in secondary metabolite concentrations were quantified by high performance liquid chromatography (HPLC). Second, extracts were compared with appropriate controls in field feeding assays designed to quantify the effects of extracts on potential predators (reef fish). In addition, I also performed field feeding assays to determine if the secondary metabolites of *Dysidea* cf. *avara* deter feeding by potential predators.

BIOLOGY AND CHEMISTRY OF *DYSIDEA*

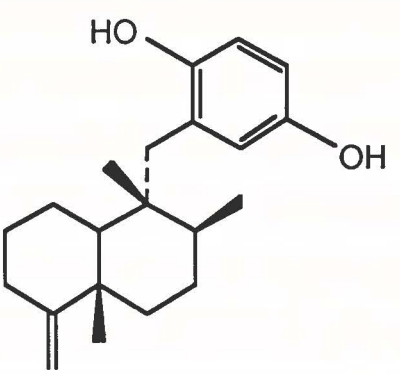
Dysidea sponges are in the class Demospongia, order Dictyoceratida and family Dysideidae. The species used in this study has been identified as *Dysidea* sp., similar to *Dysidea avara* (Pat Bergquist pers. comm.). *Dysidea* spp. have an association with bacteria and cyanobacteria (blue-green algae). Both are mainly extracellular and appear to compete with each other for space. These associations are mutualistic symbioses, where the sponge receives food by consuming the bacteria and cyanobacteria as needed and the bacteria and cyanobacteria are provided with a suitable place to live (Bergquist 1978). When the cyanobacteria are consumed by phagocytoses their glycogen reserves can be transferred to the sponge (Sara 1971).

Dysidea cf. *avara* occurs at depths of 3 to 25 meters on Guam. The sponge is pink to blue in color with many raised points on the surface. The sponge grows attached to algae and hard substrata such as rubble, coral and ship wrecks. The sponge lacks the defense of spicules.

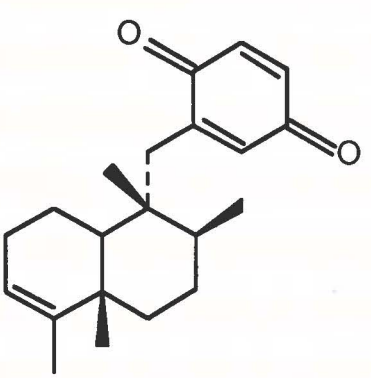
Dysidea cf. *avara* contains the secondary metabolites avarol, isoavarol, avarone and isoavarone (Fig. 1). The secondary metabolites avarol and avarone were first described by Minale *et al.* (1974). Isoavarol was described by Shubin, L. K. *et al.* (1990). Isoavarone has not been previously described in the literature.



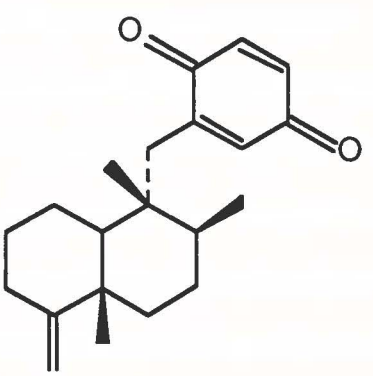
AVAROL



ISOAVAROL



AVARONE



ISOAVARONE

Figure 1: Molecular structures of avarol, isoavarol, avarone and isoavarone.

MATERIALS AND METHODS

Chemical Analysis

Sponges were individually extracted exhaustively (usually three times was adequate) in a 1:1 solution of dichloromethane:methanol. The extracts were then filtered and separated into organic and water layers, and the solids dried at 60^{o}C for 3 days and weighed. The organic solvent was then evaporated in a rotary evaporator, and the remaining extract residue weighed. Crude extract yields were calculated as mass of extract per dry mass of the sponge.

When performing the chemical analysis, isoavarol coeluted in most solvent schemes with avarol. These compounds were analyzed together as a mixture (approximately 1:1 avarol and isoavarol). When referring to avarol in this paper I actually mean the mixture of avarol and isoavarol. Similarly avarone coeluted with isoavarone as an approximately 1:1 mixture, and I will refer to the mixture as avarone. Identification of all metabolites used as standards for high-performance liquid chromatography (HPLC) and in the feeding assays was confirmed by proton nuclear magnetic resonance (NMR) spectroscopy.

The secondary metabolite concentrations were quantified by a Beckman high-performance liquid chromatography (HPLC) instrument with integrator. The HPLC consisted of a 20 µl injection loop, Beckman model 110B solvent pump with an Alltech 25 cm X 4.6 mm Spherisorb 5 micron silica column. To measure avarol contents, samples were diluted to 2.5 mg/ 100 µl, with an injection of 20 µl in a solvent mixture of 25% ethyl acetate/ 75% hexane. To measure avarone contents, samples were diluted to 10 mg/ 100 µl, with an injection of 20 µl in a solvent mixture of 8% ethyl acetate/ 92% hexane. Peak areas were measured by automatic electronic integration. Peaks of interest were identified by comparison with a series of dilutions of the pure compounds

used to obtain standard curves. Calculations were then done using the standard to determine the quantities of avarol and avarone in each sample. Concentrations of each metabolite are expressed as percent dry mass of the sponge.

Pure compounds were obtained from a mass extraction of the sponge in a 1:1 solution of dichloromethane:methanol. The organic layer was then separated using silica gel vacuum-flash column chromatography. Solvent scheme used was: 100% hexane, 10% ethyl acetate/ 90% hexane, 15% ethyl acetate/ 85% hexane, 25% ethyl acetate/ 75% hexane, 50% ethyl acetate/ 50% hexane, and then 100% ethyl acetate. Avarone was usually obtained from the 15% ethyl acetate/ 85% hexane fraction and avarol from the 50% ethyl acetate/ 50% hexane fraction. Compounds were further purified from the flash column fractions by the use of a Waters HPLC instrument with an Alltech 250 mm X 10 mm Spherisorb 10 μm silica column.

Feeding Assays

The extracts being tested were individually incorporated into an artificial diet consisting of 2.5 g carrageenan (Sigma #C-1013), 4 g of paraffin wax, 70 ml water, and 50 g of squid homogenate (250 ml water: 500 g squid). The carrageenan, paraffin wax and water were mixed and heated in a microwave oven for 75 seconds. The squid homogenate was then added and the mixture heated for 15 seconds in the microwave. The extract was then dissolved in dichloromethane and mixed into the artificial food. The control consisted of the above diet, but only the solvent dichloromethane was added. The food was then poured into stainless steel potato slicers that were composed of a 7 X 7 grid of 1 cm X 1 cm squares that were ~ 1 cm high. Prior to the addition of the diet, black plastic O-rings (3/8 in OD, 1/4 in ID) were placed in each cell in the potato slicer. After the food had gelled, individual cubes were removed and placed on 50 cm long pieces of 3 strand, 1/4 in yellow polypropylene rope by passing a safety

pin, that is attached to the rope, through the o-ring. Each rope held four food cubes. Safety pins were attached equidistantly along the top 30-40 cm of the rope.

Feeding assays were done at Western Shoals reef in Apra Harbor at a depth of 4-5 m. The ropes were set out on the reef in pairs (control and treated), by attaching them to the bottom. Fish observed feeding from the ropes included *Abudefduf vaigiensis, Abudefduf sexfasciatus, Naso vlamingii, Acanthurus thompsoni, Cheilinus fasciatus* and *Thalassoma lutescens.* Ropes were collected and scored after three or more food cubes were completely eaten from the pair. Scoring consisted of recording the number of cubes eaten by reef fish on the control and treated rope for each pair. Differences between the number of control and treated food cubes consumed were compared with a Wilcoxon signed rank test for paired comparisons (2-tailed test). All statistical analysis were performed by the program Statistix version 3.5 by Analytical Software.

Avarol and avarone were isolated from a mass extraction of *Dysidea* cf. *avara*, as explained in the chemical analysis section above. Avarol and avarone were individually tested in the feeding assays at concentrations of 0.2%, 0.5%,1%, 2%, 3%, 4%, and 5% dry mass of the artificial diet. A separate feeding assay comparing avarol at 2.3% with avarol at 2% plus avarone at 0.3% was conducted to look at the combined effect of these metabolites. These ratios were tested because they fell in the middle range of the naturally occurring concentrations for the sponge.

Whole sponge extracts from the following experiments were tested in feeding assays to determine if the changes in the whole extracts affected feeding by reef fishes. Each experiment pooled individual control extracts and then separately pooled treated extracts to obtain enough extract to test in the field. Feeding assays were conducted using 7% extract per dry mass of the artificial food, which was the average % yield of extracts per dry mass of sponges.

Sampling of Different Sites

Individual sponges were collected over a four month period (5/7/91-9/19/91) from different sites in Apra Harbor, Guam: Gab Gab II (18 meters) 5/7/91, Sponge Mound (21 meters) 7/23/91, from two different depths at Western Shoals (3 meters) 7/9/91 and (4 meters) 5/16/91, and American Tanker (8 meters) 9/19/91 and (25 meters) 7/5/91. All sponges from one reef and one depth were collected on the same day. A maximum of ten sponges were collected from any one site due to limitations of dive time and density of sponges. The sponges were brought back to the lab and analyzed as described below for secondary metabolite concentration. Additionally, a new deep reef site (25 meters) at American Tanker plus the two previous American Tanker sites were sampled on the same date (1/25/93) so a statistical comparison could be made of the secondary metabolite concentrations among the different sites. Data were arcsine square-root transformed because concentrations were expressed as percentages and a one-way ANOVA was performed on the American Tanker sites.

Sponge extracts from American Tanker shallow reef (9/19/91) versus deep ship wreck (7/5/91) collections were tested to determine if there was a significant difference in fish preference for whole extracts between these two sites.

Transplant Experiment

Ten sponges each were transplanted between two depths (shallow 7 meters and deep 25 meters) near the American tanker ship wreck. Sponges that were attached to small rubble were selected to prevent damage in the move, and the tags were attached to the rubble to further minimize damage to the sponges. Control sponges were handled the same amount of time and under the same conditions as the treated sponges and were placed back at the depth from which they were collected, ten shallow and ten deep. All

sponges were collected after one month and analyzed as described above in chemical analysis. Data were arcsine square root transformed and analyzed with a two-way ANOVA (study sites and original collection sites) for each compound.

This experiment was conducted twice. The first one was out for two months, but the deep site did not survive Typhoon Omar. The second attempt was collected before Typhoon Brian, limiting the experiment to one month.

Sponge extracts from the second transplant experiment were tested: extracts of deep sponges transplanted to shallow versus shallow control sponges, extracts of shallow sponges transplanted to deep versus deep control sponges, extracts of control deep versus control shallow, and extracts of shallow sponges transplanted to deep versus deep sponges transplanted to shallow to determine if there was a significant difference in reef fish preferences for any of the treated or control extracts.

Artificial Grazing Experiment

American Tanker deep site (on the ship wreck) in Apra Harbor was used as the experimental site because of the abundance of sponges in the area. The sponges were tagged with marked cable ties. The ties were attached at the base of the sponges so that recreational divers would not notice them. Thirty control sponges and thirty treated sponges were selected haphazardly and tagged. Treated sponges were damaged by clipping them at approximately 1.5 cm intervals on their top surface to a depth of approximately 1.5 cm with scissors. No tissue was actually removed from the sponges.

Ten controls and ten treated sponges were collected at intervals of one week, two weeks and three weeks after clipping. Collected sponges were placed in individual zip lock bags and brought to the lab for immediate extraction. The secondary metabolite contents were analyzed by HPLC as described above. Differences in secondary

metabolite contents between control and treated (damaged) sponges at each time interval were analyzed by t-tests after proportional data were arcsine square root transformed.

The above artificial grazing experiment was repeated, except that the sponges were collected after 24 hrs.

The above transformed data for all four time periods were analyzed with a two-way ANOVA (time and treatment) for each compound.

Sponge extracts from the artificial grazing experiment were tested, treated versus control extracts. The time intervals were tested separately; therefore four feeding assays were performed: sponges that were collected at 24 hr, 1 week, 2 weeks, and 3 weeks intervals.

Light Experiment

A shallow reef (7m depth) at the American Tanker contains the sponge *Dysidea* cf. *avara* in abundance. Light reaching the sponges was manipulated by placing a 1' x 1' x 1/8" Plexiglas® platform over each individual sponge. Three types of Plexiglas® were purchased for the platforms. Sponge sizes were about 5 cm high by a 5 cm diameter. The Plexiglas® platforms were suspended about 10 cm over the sponges by fastening, with electrical ties, the corners to rebarb stakes anchored to the reef. The platforms allowed free water movement under them. Shade was provided for ten sponges by placing a non-translucent platform, made of 1/8" regular Plexiglas® painted black, over each individual sponge. Ten sponges had a Plexiglas® II UVT platform over each sponge, which allowed passage of the ultraviolet including all UV-visible radiation above 275 nm transmitted (included all UV-A and UV-B radiation) and the visible light spectrum. Ten sponges had a Plexiglas® UF-3 platform that blocked out UV light and short-wavelength violet light below 450 nm transmitted, but allowed passage of the visible light spectrum. A small amount of scattered light probably

reflected in from the sides of the platform but was not measured. After 2.5 months, all sponges were collected and analyzed as described above in chemical analysis. Data were arcsine square root transformed and analyzed with a one-way ANOVA for each compound.

Sponge extracts from light experiments were tested, total light block versus controls and UV block versus controls, to determine if there was a significant difference in preference for any of the treated or control extracts by reef fish.

RESULTS

Feeding Assays of Pure Compounds

In the feeding assays avarol significantly deterred feeding by fish at 1%, 2%, 3%, 4% and 5% concentrations (Fig. 2). Additional testing of lower concentrations showed avarol was still deterrent at 0.5%, but at 0.2% avarol was attractant (Fig. 3). Therefore, avarol did deter fish from feeding at a natural concentration of 2% and 3%.

Avarone significantly deterred feeding by fish at 3% and 5% concentrations (Fig. 4). At 4% concentration avarone was almost attractant at P=0.084. Additional feeding assays conducted at 0.2% 0.5% and 1% showed no effect on feeding by reef fish (Fig. 5). Avarone did not deter fish from feeding at a natural concentration of 0.2%.

Fish showed no feeding preference for a combination of avarol (2%) and avarone (0.3%) compared to just avarol (2.3%) (Fig. 6).

Sampling of Different Sites

Dysidea cf. *avara* shows variation in secondary metabolite concentrations between different sites in Apra Harbor, Guam (Fig. 7 and 8). No obvious trends were noticed. The two American Tanker sites showed the greatest difference in avarol concentrations with the mean concentration from the deep (25m) site about twice the mean concentration of the shallow (8m) site. The avarone concentrations in the sponges varied the greatest from Gab Gab II to Western Shoals. Gab Gab II data was from a small sample of two sponges due to a lack of *Dysidea* cf. *avara* at that site. Western Shoals sponges showed variation in secondary metabolite concentrations at about the same depth, 3 and 4 meter sites, on different dates. The American Tanker sites were

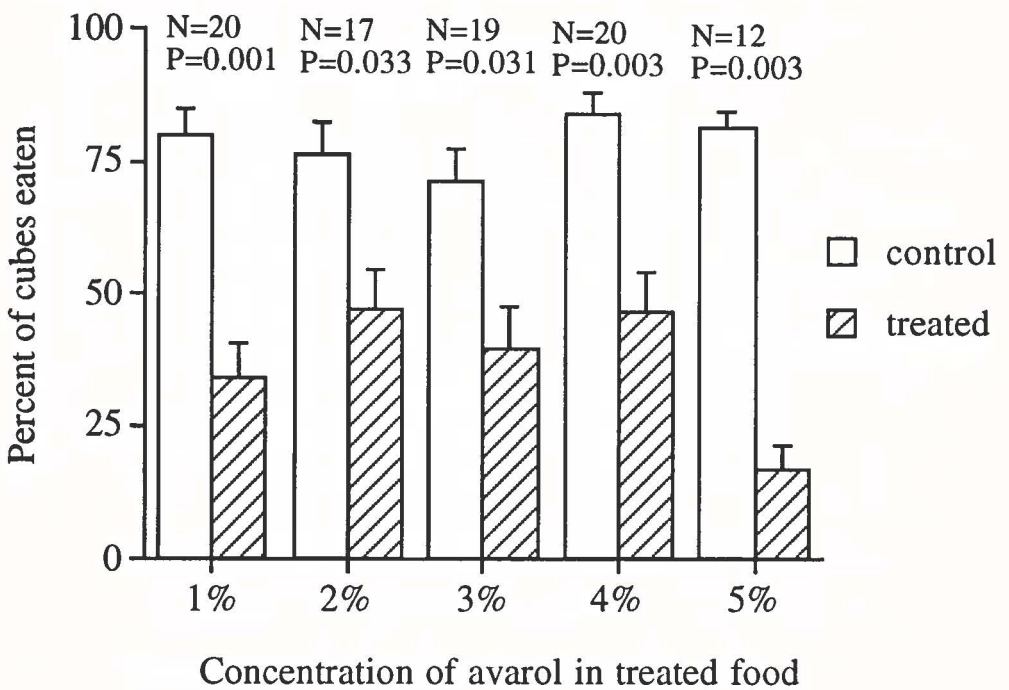


Figure 2: Effect of different concentrations of avarol on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

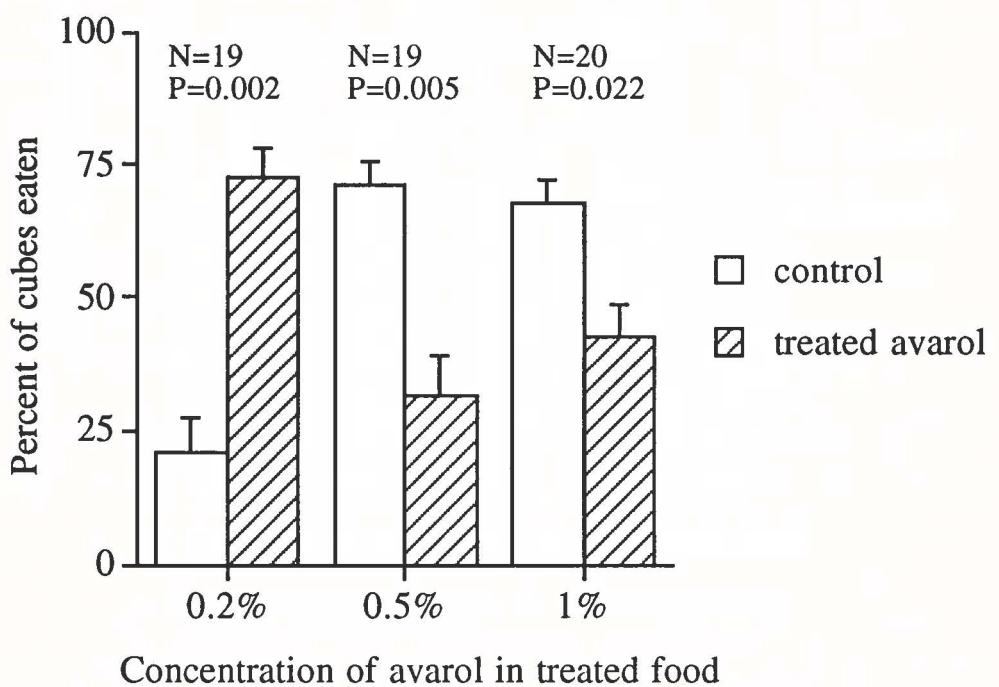


Figure 3: Effect of different concentrations of avarol on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

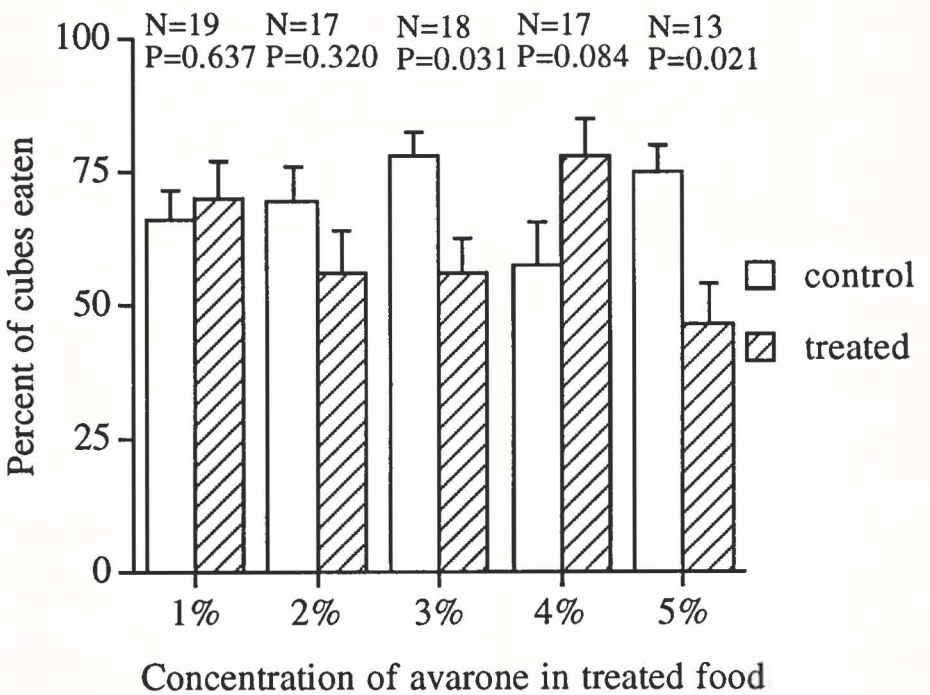


Figure 4: Effect of different concentrations of avarone on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

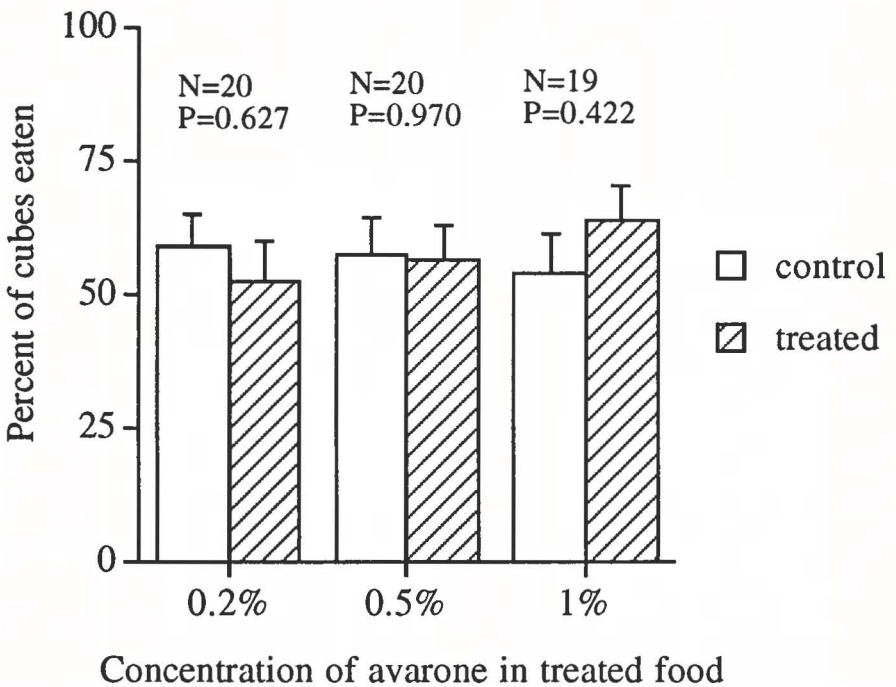


Figure 5: Effect of different concentrations of avarone on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

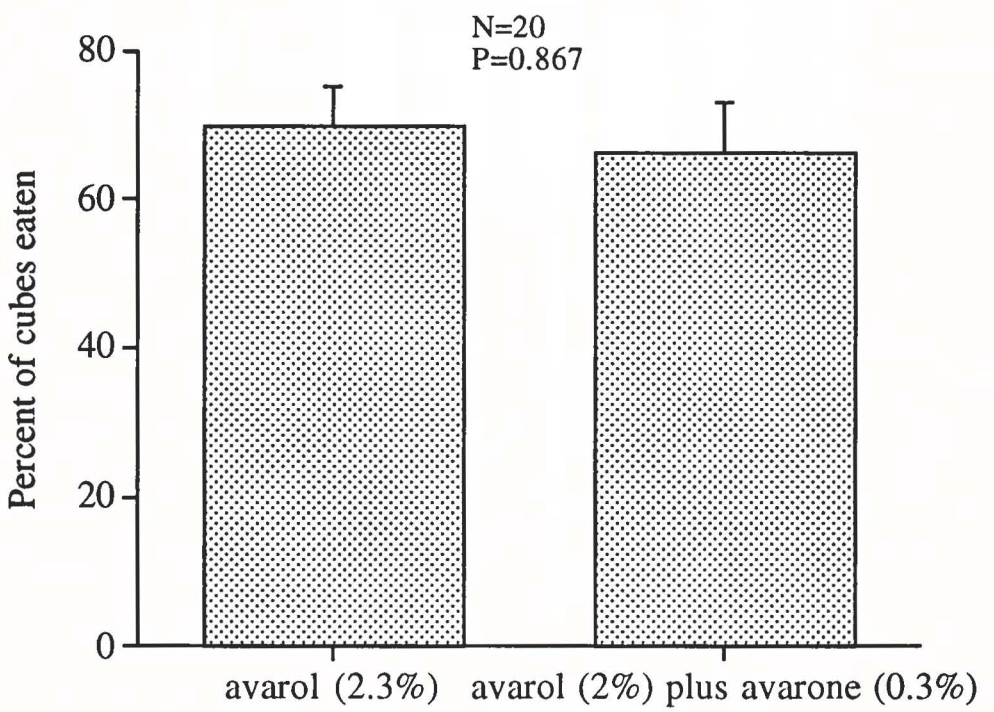


Figure 6: Effect of avarone versus avarol and avarone on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

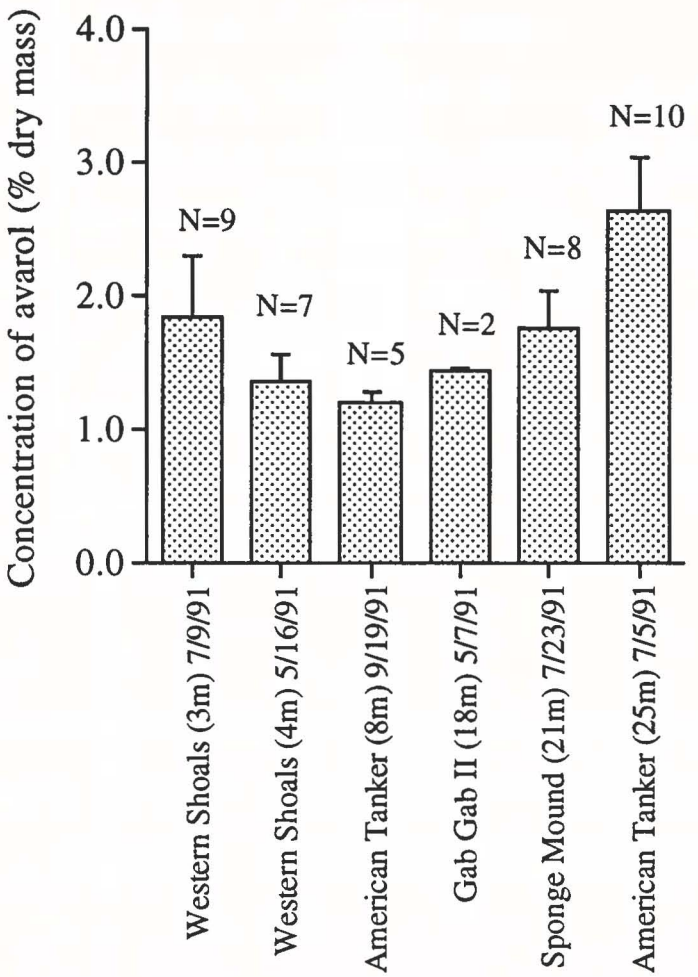


Figure 7: Concentration of avarol (% dry mass) in sponges from different locations in Apra Harbor, Guam. Values shown are mean + one standard error. N=number of individual sponges sampled from the collection site.

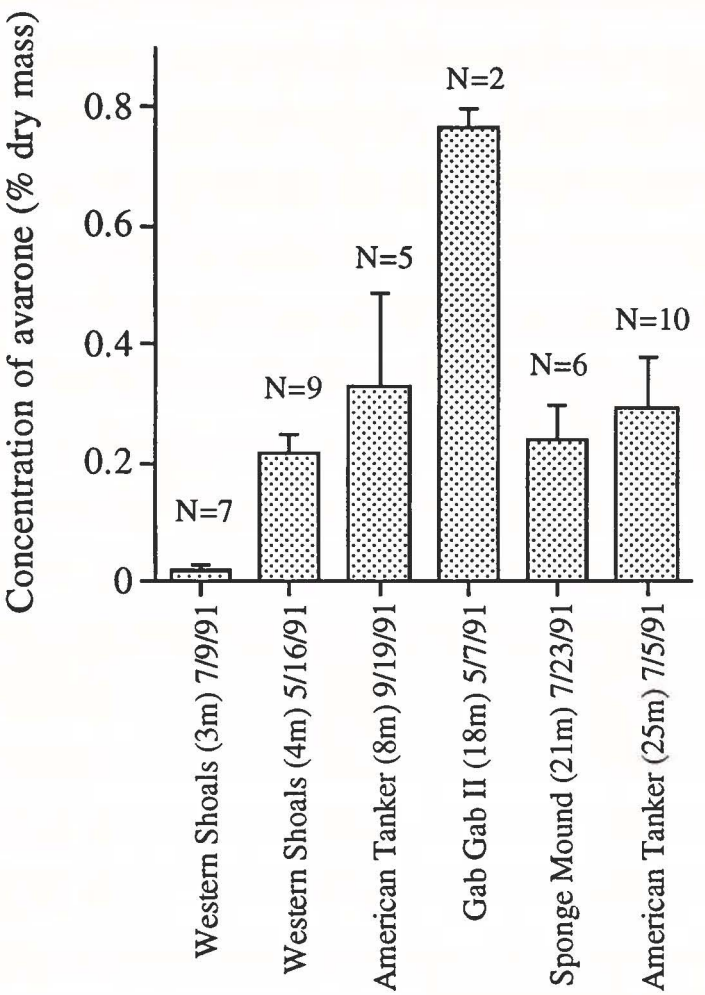


Figure 8: Concentration of avarone (% dry mass) in sponges from different locations in Apra Harbor, Guam. Values shown are mean + one standard error. N=number of individual sponges sampled from the collection site.

chosen for further studies because of the great variation between the sites in avarol concentrations and the abundance of the sponge.

Dysidea cf. *avara* contains the secondary metabolite avarol at a mean of 1.843% dry mass with a standard error of 0.168 for 41 samples and avarone at approximately 0.245% dry mass with a standard error of 0.040 for 39 samples.

Sampling of the three American Tanker sites showed avarol concentration to be highest on the deep tanker, lowest on the deep reef, and at intermediate concentrations on the shallow reef. The deep tanker site had significantly higher concentrations of avarol than the other two sites (Fig. 9) (Table 1). Avarone concentrations varied among these sites (Fig. 10) but not significantly (Table 2).

In feeding assays reef fishes showed no preference for sponge extracts from the deep tanker or shallow reef at American Tanker (Fig. 11), although fishes tended to eat more of the artificial food with the extract from the shallow sponges.

Transplant Experiment

Both transplant experiments indicated rapid environmental control of secondary metabolite concentrations. The surviving shallow depth of the first transplant experiment had similar avarol (Fig. 12) and avarone (Fig. 13) levels in the control and treated sponges after two months in the field.

The second transplant experiment (one month in the field) showed that avarol concentrations (Fig. 14) were significantly different between deep and shallow study sites, but no difference was attributable to site of origin of the sponges (Table 3). Chemical analysis of avarone concentrations (Fig. 15) also showed a significant difference between deep and shallow sites, but again no difference was attributed to site of origin of the sponges (Table 4).

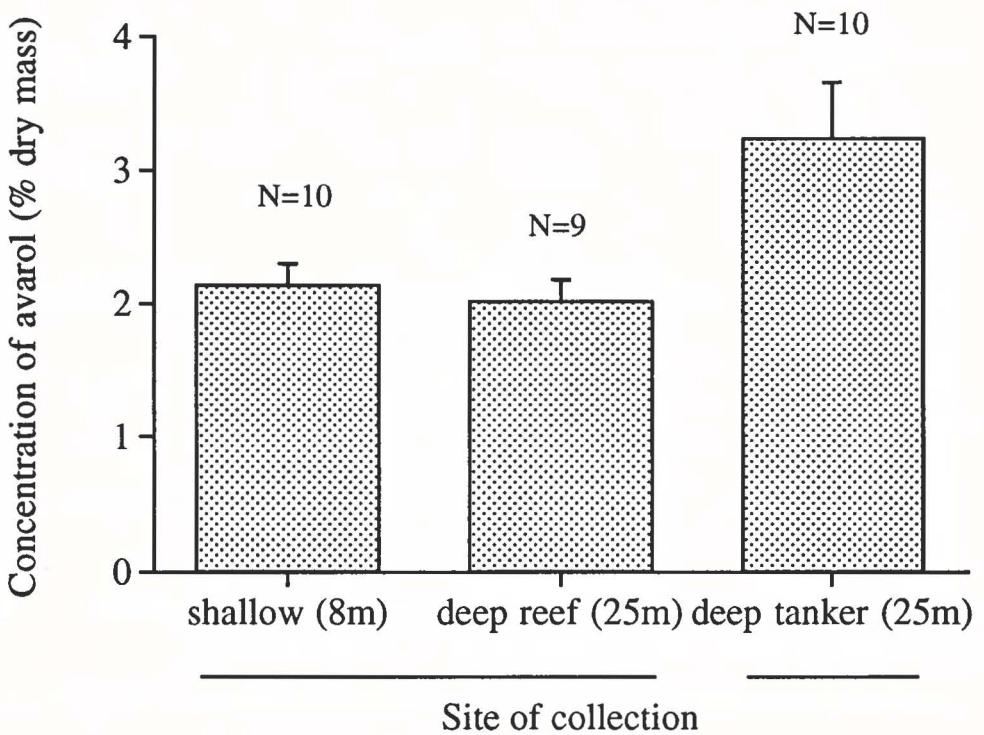


Figure 9: Concentration of avarol (% dry mass) in sponges from different locations at American Tanker in Apra Harbor, Guam. Values shown are mean + one standard error. N=number of individual sponges sampled from the collection site. Bars represent homogeneous groups (Tukey test).

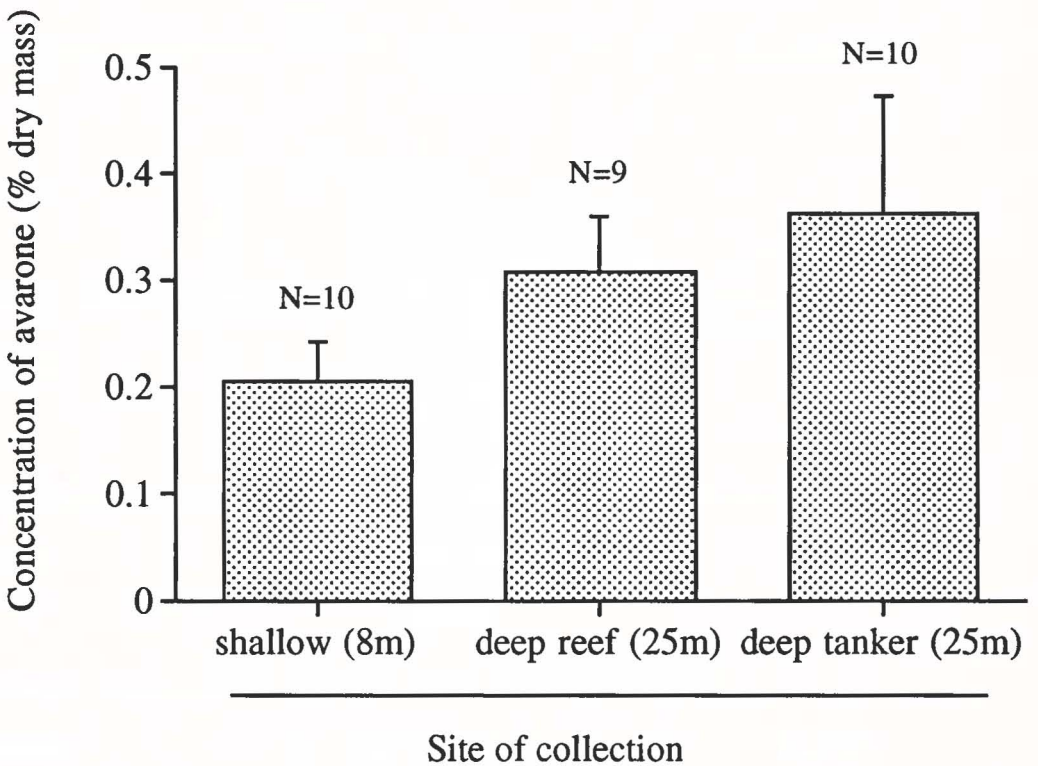


Figure 10: Concentration of avarone (% dry mass) in sponges from different locations at American Tanker in Apra Harbor, Guam. Values shown are mean + one standard error. N=number of individual sponges sampled from the collection site. Bars represent homogeneous groups (Tukey test).

Table 1: Effect of sampling site at American Tanker on sponge concentrations of avarol.

Source	DF	Sum of Squares	F	P
Between	2	0.007292	5.29	0.0118
Within	26	0.01791		

Table 2: Effect of sampling site at American Tanker on sponge concentrations of avarone.

Source	DF	Sum of Squares	F	P
Between	2	0.0007895	1.00	0.3799
Within	26	0.01021		

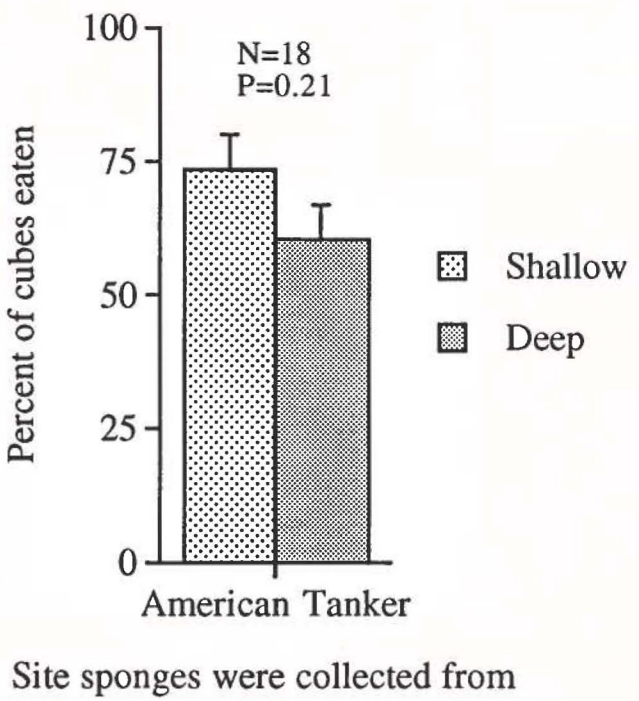
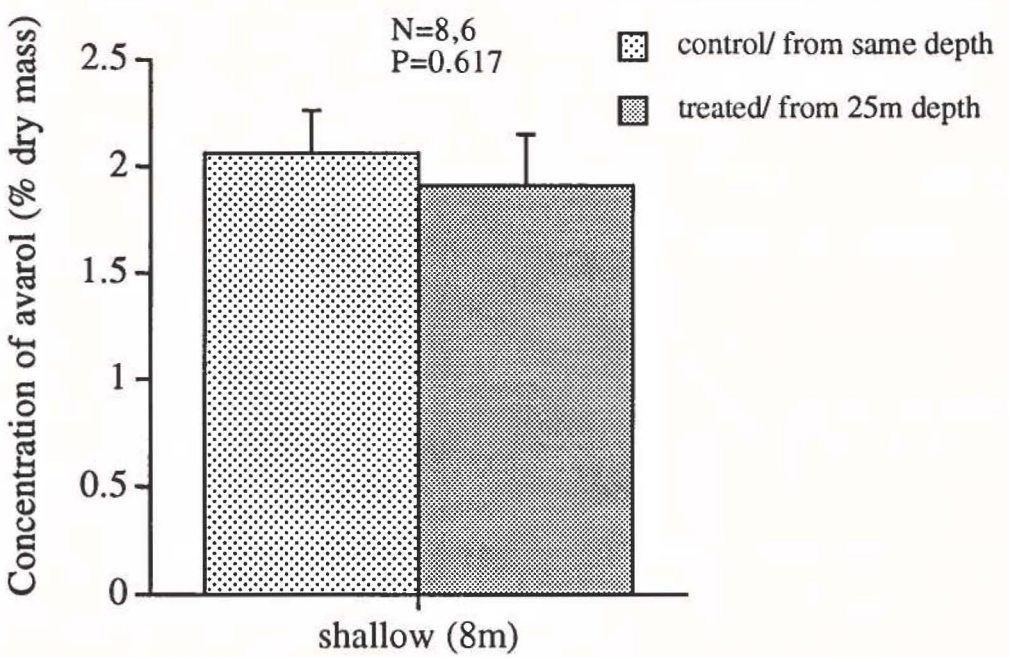


Figure 11: Effect of American Tanker pooled deep (tanker) extract versus shallow (reef) extracts on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two tailed Wilcoxon Sign Rank Test.



Site of experiment at American Tanker

Figure 12: Analysis of avarol concentrations in the transplant experiment. Values shown are mean + one standard error. N=number of replicate sponges placed on the reef. P=significant level using a t-test.

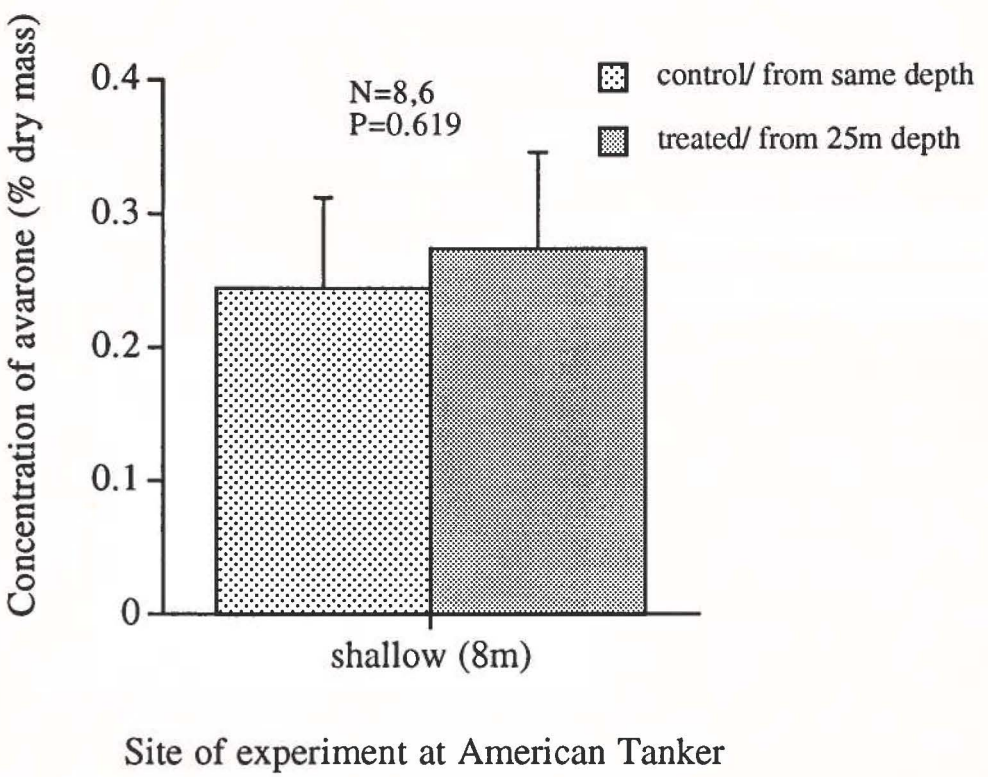


Figure 13: Analysis of avarone concentrations in the transplant experiment. Values shown are mean + one standard error. N=number of replicate sponges placed on the reef. P=significant level using a t-test.

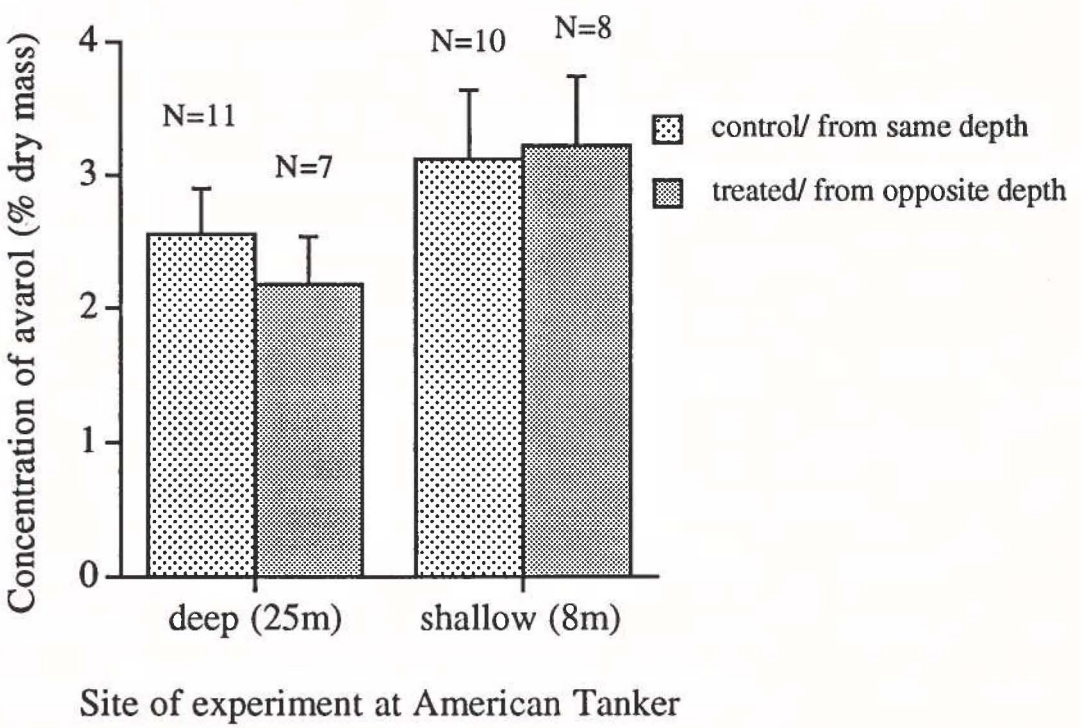


Figure 14: Analysis of avarol concentrations in the transplant experiment. Values shown are mean + one standard error. N=number of replicate sponges placed on the reef.

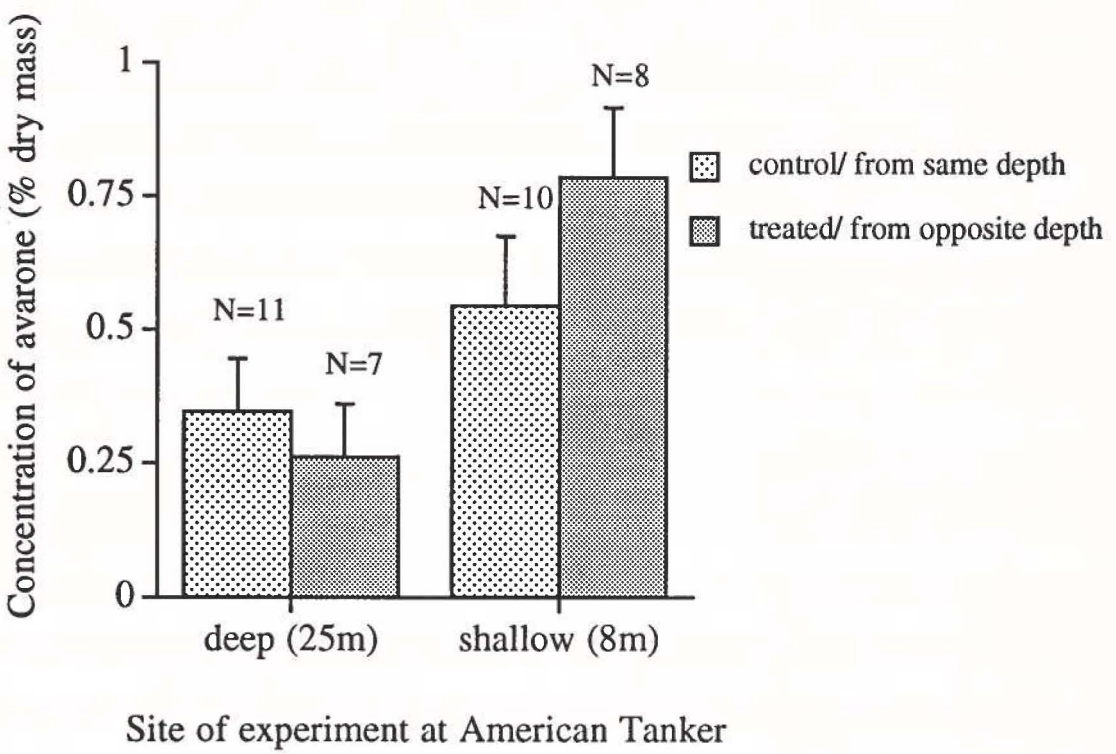


Figure 15: Analysis of avarone concentrations in the transplant experiment. Values shown are mean + one standard error. N=number of replicate sponges placed on the reef.

Table 3: Effect of experimental site and effect of where the sponge was originally collected, on concentrations of avarol.

Source	DF	Sum of Squares	F	P
Experiment Site	1	0.0059837	5.16	0.0299
Original Site	1	0.00050370	0.43	0.5144
Interaction	1	0.00008525	0.07	0.7880
Error	32	0.037081		

Table 4: Effect of experimental site and effect of where the sponge was originally collected, on concentrations of avarone.

Source	DF	Sum of Squares	F	P
Experiment Site	1	0.0079157	11.84	0.0016
Original Site	1	0.00086742	1.30	0.2637
Interaction	1	0.00033759	0.50	0.4825
Error	32	0.021400		

Feeding assays resulted in extracts of sponges transplanted from deep to shallow incorporated in the artificial food were consumed significantly less than extract from control shallow sponges. Extracts of sponges transplanted from shallow to deep incorporated in the artificial food were not consumed differently than extracts from control deep sponges(Fig. 16). Extracts of transplanted deep control sponges incorporated in the artificial food were not consumed differently than extracts from shallow control sponges. Extracts of sponges transplanted from shallow to deep incorporated in the artificial food were not consumed differently than extract from sponges transplanted from deep to shallow (Fig. 17).

Artificial Grazing Experiment

Chemical analysis of the avarol concentrations in the artificially grazed sponges that were allowed to recover 24 hours, 1 week, 2 weeks and 3 weeks after grazing demonstrated that, compared with controls, damaged sponges tended to have less avarol, although these differences were not significant (Fig. 18). The two-way ANOVA for avarol concentrations resulted in no significant effect for time or treatment (Table 5).

Chemical analysis of the avarone concentrations in the artificially grazed sponges that were allowed to recover 24 hours, 1 week, 2 weeks and 3 weeks were not significantly different than avarone concentrations of control sponges, (Fig. 19). The three week samples (both control and treated) had much higher levels of avarone (Fig. 19). The two-way ANOVA for avarone resulted in a significant difference for time, but not for treatment (Table 6).

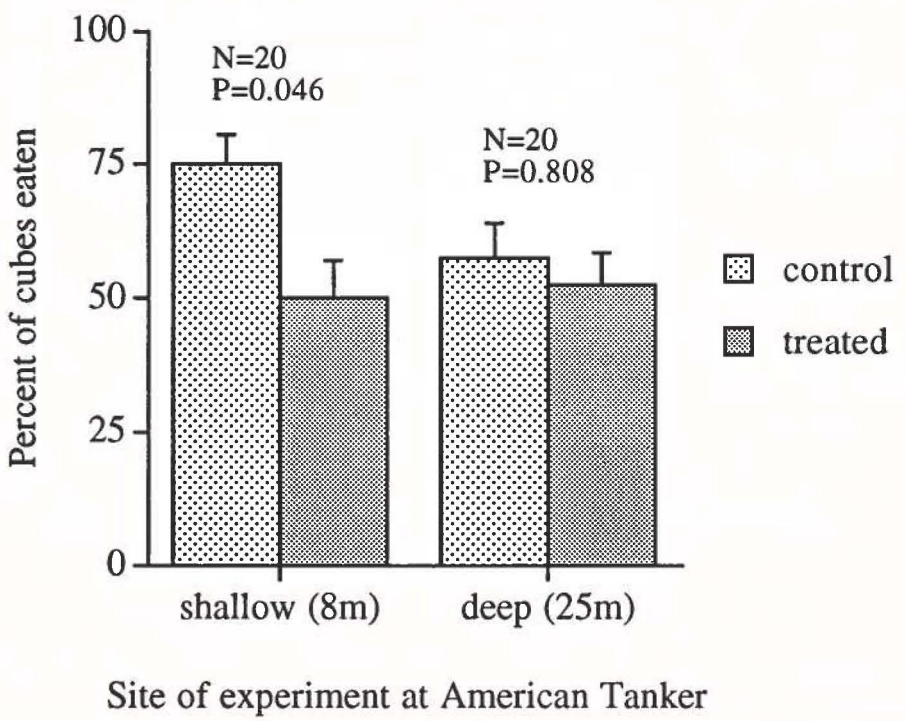


Figure 16: Effect of pooled transplant control extract versus treated extracts on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

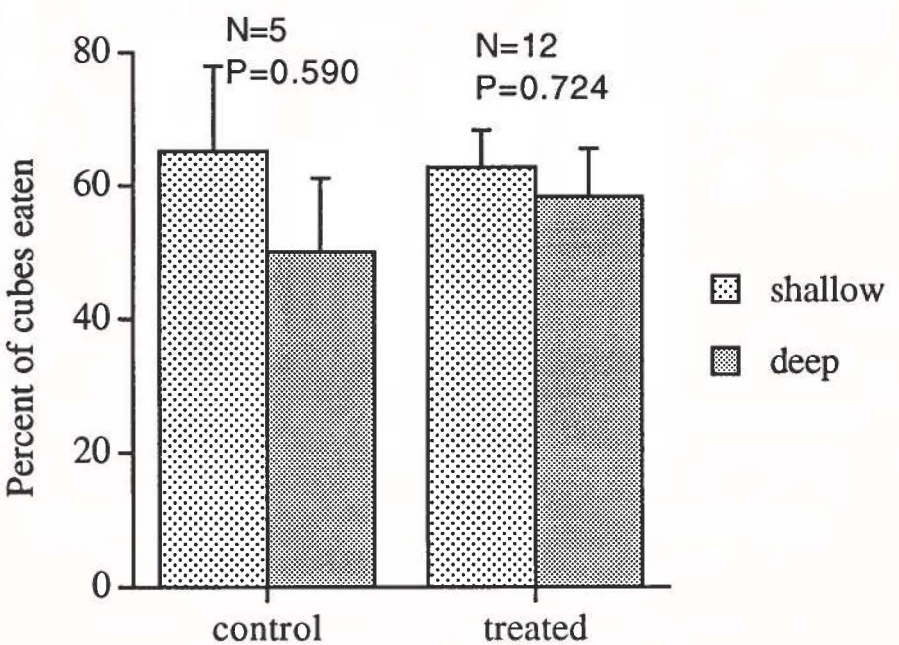


Figure 17: Effect of pooled transplant control extract versus treated extracts on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

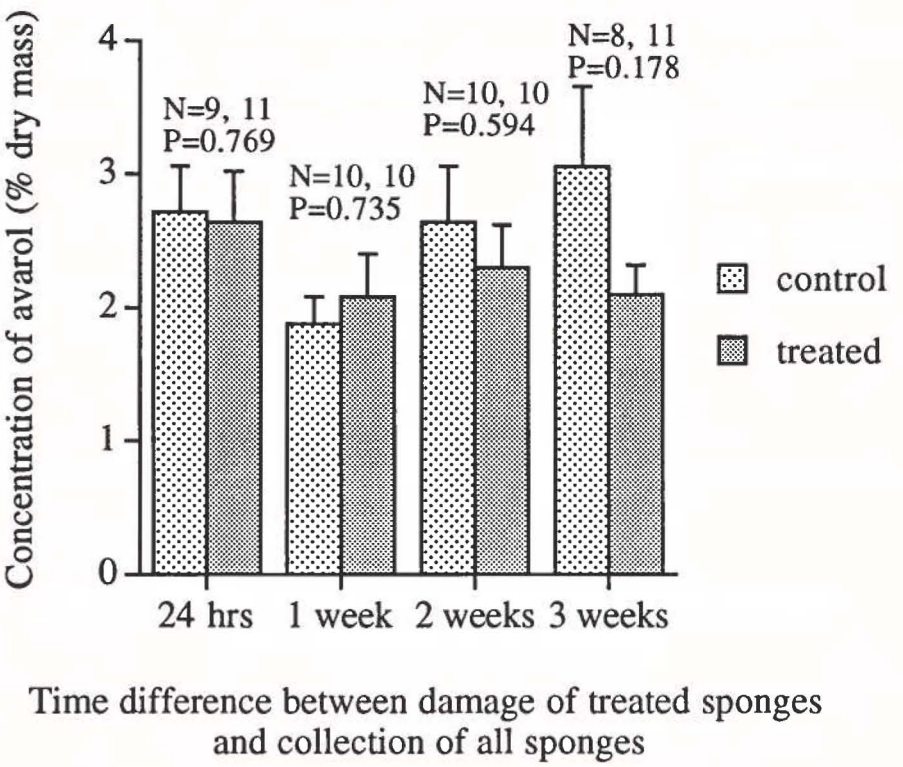


Figure 18: Analysis of avarol concentrations in the artificial grazing experiment after 24 hours, 1 week, 2 weeks, and 3 weeks of recovery. Values shown are mean + one standard error. N=number of replicate sponges (control, treated) and P= significance level of a two-tailed t-test. The 24 hr experiment was done at a different time than the 1, 2, and 3 week interval experiment.

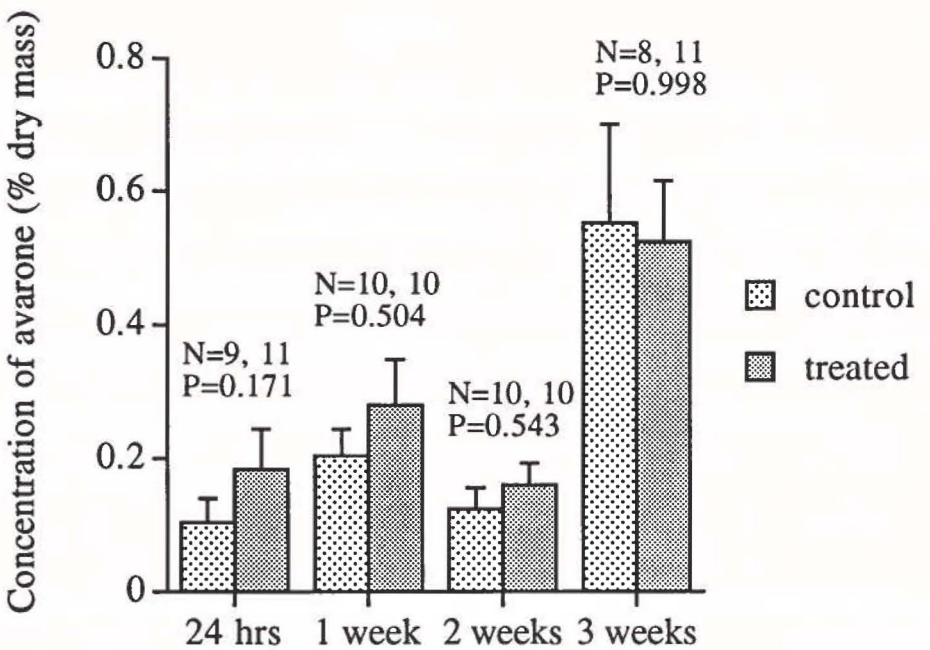


Figure 19: Analysis of avarone concentrations in the artificial grazing experiment after 24 hours, 1 week, 2 weeks, and 3 weeks of recovery. Values shown are mean + one standard error. N=number of replicate sponges (control, treated) and P= significance level of a two-tailed t-test. The 24 hr experiment was done at a different time than the 1, 2, and 3 week interval experiment.

Table 5: Effect of time of collection of sponges after treatment and effect of treatment (control or clipped) on concentrations of avarol.

Source	DF	Sum of Squares	F	P
Time	3	0.029838	1.11	0.3525
Treatment	1	0.0019020	0.21	0.6468
Interaction	3	0.023943	0.89	0.4536
Error	71	0.63788		

Table 6: Effect of time of collection of sponges after treatment and effect of treatment (control or clipped) on concentrations of avarone.

Source	DF	Sum of Squares	F	P
Time	3	0.018631	15.47	0.0000
Treatment	1	0.00067326	1.68	0.1994
Interaction	3	0.00034982	0.29	0.8333
Error	71	0.028495		

Feeding assays resulted in extracts of artificially grazed sponges, collected 24 hrs, 1 week, 2 weeks and 3 weeks after treatment, incorporated in the artificial food were not consumed differently than artificial food with extracts from control sponges (Fig. 20).

Light Experiment

Chemical analysis of secondary metabolite concentrations in sponges from the light experiment showed that both avarol (Fig. 21) and avarone (Fig. 22) concentrations did not significantly differ among treatments (Table 7 and 8).

Feeding assays resulted in extracts of sponges incorporated into artificial food that had light manipulations were not consumed any differently than artificial food with extract from control sponges (Fig. 23).

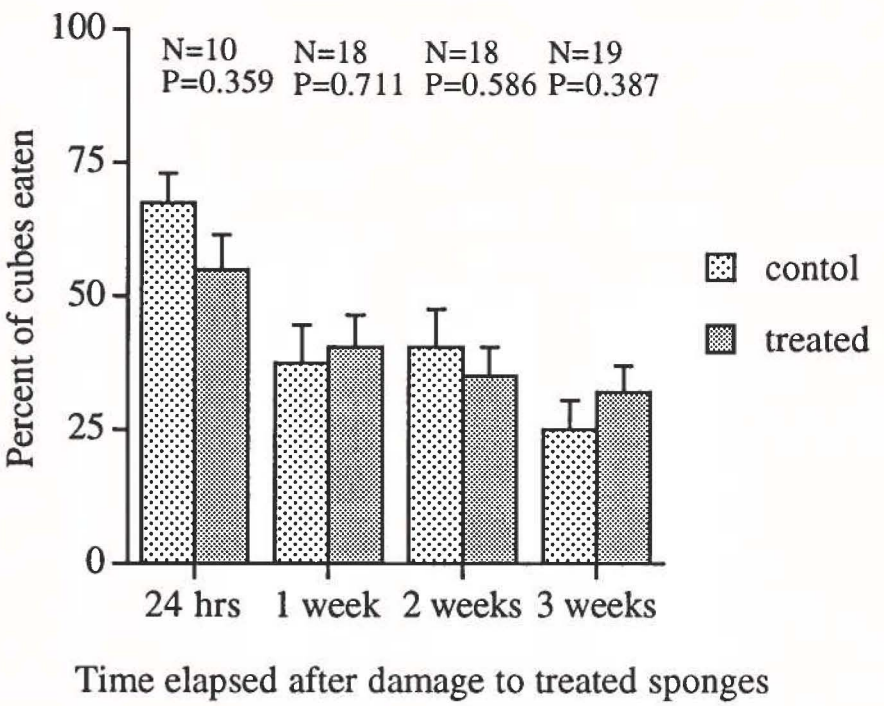


Figure 20: Effect of pooled artificially grazed control extract versus treated extracts on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

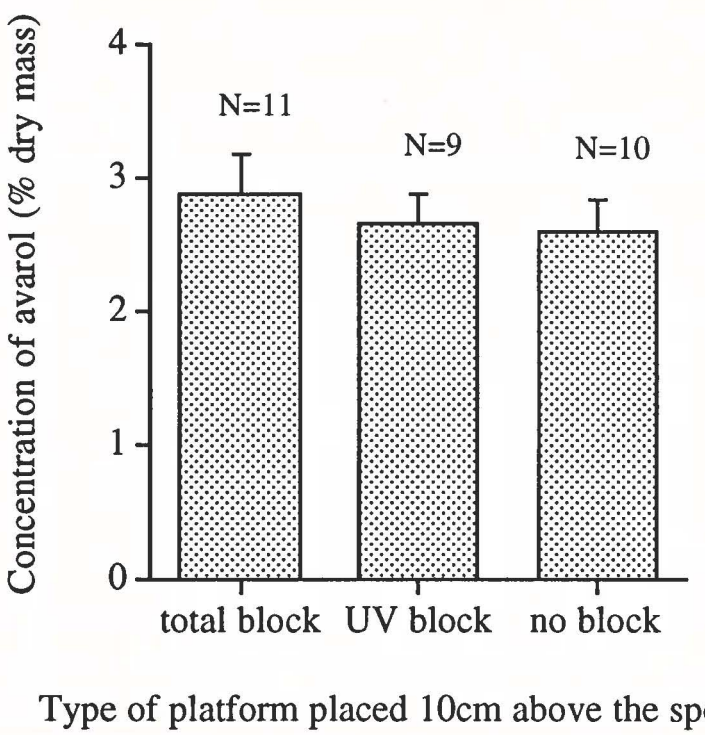


Figure 21: Analysis of avarol concentrations in the light manipulation experiment. Values shown are mean + one standard error. N=number of replicate sponges.

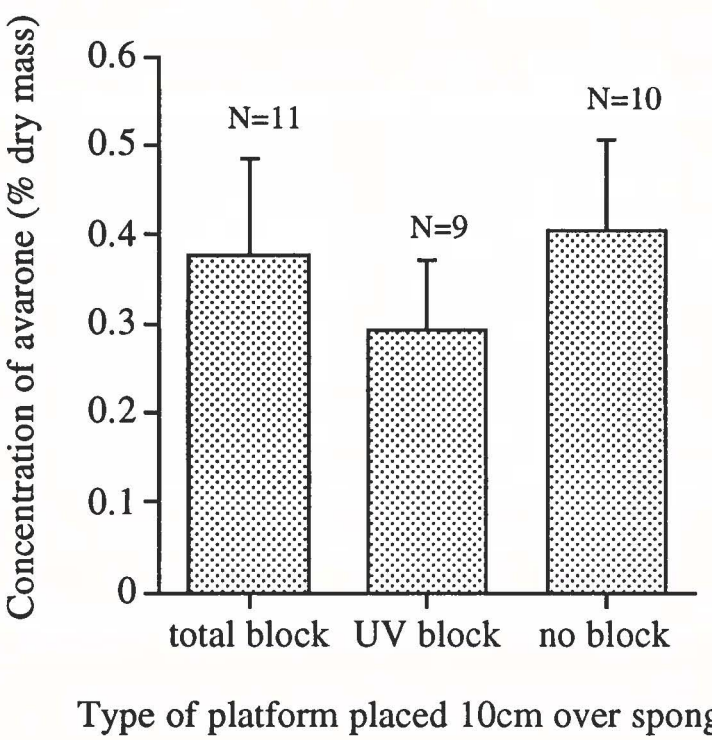


Figure 22: Analysis of avarone concentrations in the light manipulation experiment. Values shown are mean + one standard error. N=number of replicate sponges.

Table 7: Effect of light treatments on sponge's concentrations of avarol.

Source	DF	Sum of Squares	F	P
Between	2	0.0003197	0.25	0.7774
Within	27	0.01698		

Table 8: Effect of light treatments on sponge's concentrations of avarone.

Source	DF	Sum of Squares	F	P
Between	2	0.0004975	0.43	0.6523
Within	27	0.01547		

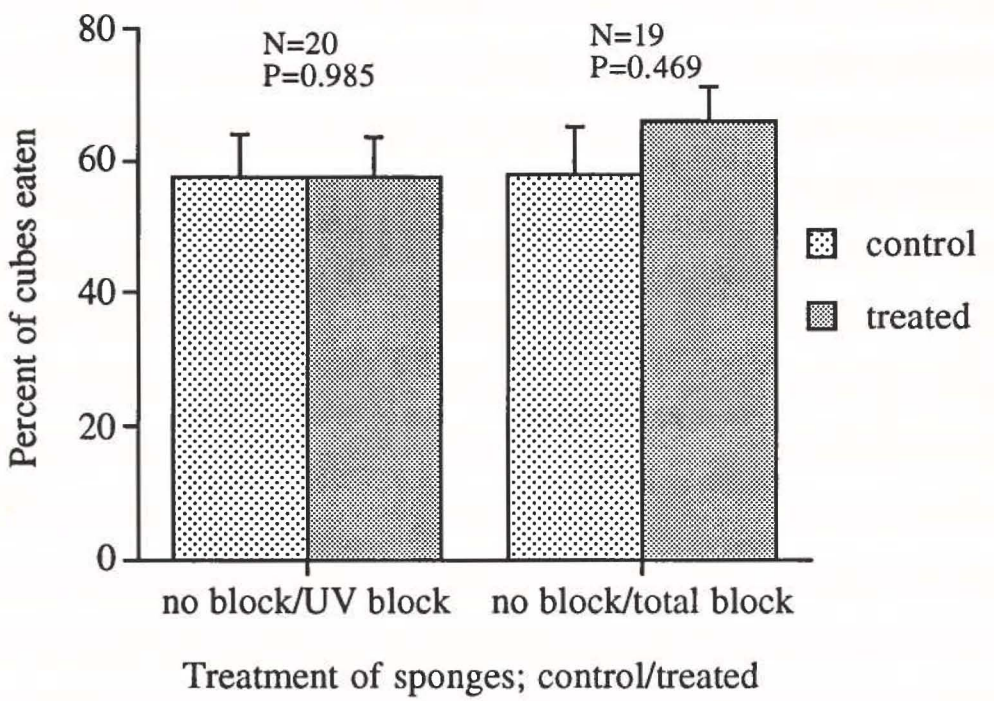


Figure 23: Effect of pooled light control extracts versus two treated extracts on feeding by reef fish. Values shown are mean + one standard error. N=number of replicate pairs placed on the reef, and P= significance level using a two-tailed Wilcoxon Sign Rank Test.

DISCUSSION

I found site to site variation in secondary metabolite concentrations. I was able to mimic the variation with transplants, but couldn't mimic this variation with manipulations of the sponge. Below I will first discuss the results of the feeding assays with the pure metabolites and then address each of these points in turn.

Testing of avarol demonstrated that it is a very effective deterrent towards fish predation at natural levels of 2% and 3% and even as low as 0.5% of dry mass (Fig. 2 and 3). These results suggest that the sponge is chemically defended against predatory fishes. The reef fish *Zanclus cornutus* consumes sponges as part of their diet (Myers 1989) and I have observed this fish eating other sponges. I have also observed nibbled areas on other sponges but never on *Dysidea* cf. *avara*. *Dysidea* cf. *avara* lacks the mechanical defense of spicules and may be relying on chemical defense. At a very low concentration of 0.2% of the dry mass of the artificial food, avarol was significantly attractant (Fig. 3). Similar effects have been noted in grasshopper feeding assays where compounds that acted as a feeding deterrent at natural concentrations acted as a significant phagostimulater at lower concentrations (Bernays, 1991).

Avarone did not act as a feeding deterrent at natural concentrations of 0.2% and 0.5% (Fig. 5), only at higher levels of 3%, and then it had varied results with it being almost attractant at 4%, P=0.08 (Fig. 4). Avarone may be involved in another aspect of the sponge's ecology, such as deterring predation by invertebrates, or controlling anti-fouling, or it may be a metabolic precursor or postcursor to avarol.

A combined effect of avarol plus avarone showed the same effectiveness as equal levels of avarol (Fig. 6). There appeared to be no observable synergistic effect of the combined metabolites and the combination did not detract from the deterrent effect of avarol.

Individual *Dysidea* sponges collected from different sites varied in the concentration of secondary metabolites they contained (Fig 7 and 8). However, this variation was not clearly related to depth or date. When the American Tanker sites were examined on the same day (1/25/93) to confirm a spatial variation, the shallow reef site had lower levels of avarol then deep ship wreck site, as was the case when previously sampled (shallow 9/19/91 and deep 7/5/91) (Fig. 7). The difference in avarol concentrations was significant when the American Tanker sites (1/25/93) were analyzed (Fig. 9).

Transplanted sponges changed their concentrations of secondary metabolites to levels similar to indigenous sponges at the transplanted site. This experiment showed that much of the observed variation in secondary metabolite concentrations is environmental and not genetic. This plasticity was demonstrated in both the surviving half of the first transplant experiment (shallow reef site) (Fig. 12 and 13) and at both sites for the second transplant experiment (Table 3 and 4). However, whole extract testing in fish feeding assays of the transplanted sponges did not correlate completely with the results from the chemical analysis. Although concentrations of avarol in shallow and deep sponges differed significantly, this did not have a significant effect on fish feeding(Fig. 17), possibly because of small sample sizes due to small quantities of extracts available. The only significant preference exhibited by fish was between shallow controls and treated (transplanted from deep site) sponges (Fig. 16). A possible explanation for this significance is that levels of avarol in the extracts were at the high end of the range and may have caused the fish to be more sensitive to the small variation present between the shallow control and treated sponges.

There was no significant effect on secondary metabolite concentrations in either the chemical analysis or feeding assays when testing effects of artificial grazing on sponges that had recovery times of 24 hrs, 1 week, 2 weeks and 3 weeks (Fig. 18, 19

and 20). The difference in avarol content between control and treated sponges became greater with time after damage to treated sponges, and the difference did not support an inducible defense (Fig 18). The treated sponges may have been stressed due to the damage and diverted energy from secondary metabolite production. The greatest difference in avarone content was at the 24 hr sampling and declined through the next three sample periods (Fig. 19). Artificial grazing on terrestrial plants has shown more frequently that mechanical damage does not effectively simulate herbivory (Baldwin 1990). Artificial grazing by scissors, the only type of artificial grazing tested, may not be simulating the tearing action or saliva deposited by an actual predator, but in both cases tissue is damaged.

Light (including UV radiation) had no significant effect on secondary metabolites in either the chemical analysis or feeding assays (Table 7 and 8) (Fig. 23). Four hypotheses would lead to the expectation of different levels of secondary metabolite concentrations depending on light levels: 1) secondary metabolite concentrations increase in illuminated areas because light stimulates the growth of photosynthetic symbionts which provided more primary metabolites (glycogen) to the sponges 2) secondary metabolite concentrations decrease in sponges exposed to UV radiation because the sponges are stressed by UV radiation 3) secondary metabolite concentrations increase in sponges exposed to UV radiation because secondary metabolites protect the sponges against UV radiation and 4) secondary metabolite concentrations increase in illuminated areas to prevent fouling by algae, as seen in the Thompson *et al.* (1987) study on a sponge from the Great Barrier Reef. My results showed that these hypotheses do not explain the spatial variation in secondary metabolite concentrations in *Dysidea* cf. *avara.*

Two major theories have been developed to explain intraspecific chemical variation in terrestrial plants: optimal defense theory and carbon-nutrient balance theory. These theories can also be applied to chemical variation in marine organisms.

Optimal defense theory is based on the assumption that there are costs to defenses and an organism would save energy which could be used for growth and reproduction if they produced the defense only where and when needed (Coley 1986; Fagerstrom 1989; Baldwin *et al.* 1990; Harvell 1990; Paul 1992). The optimal defense theory has been well documented in terrestrial plants (Rosenthal and Janzen 1979; Stienberg 1984; Schultz 1988; Spencer 1988) and some work has been conducted for marine algae (Van Alstyne 1988; Tugwell and Branch 1989; Renaud *et al.* 1990; Meyer and Paul 1992) and invertebrates (Harvell and Fenical 1989; Walls *et al.* 1991).

The carbon-nutrient balance theory is based on resource availability within a habitat and the effect of nutrient limitation on plant growth. It predicts that if nitrogen is limiting for protein synthesis relative to carbon, then extra carbon that is not being used for growth can be used to make carbon-based secondary metabolites. The quantity of secondary metabolite production by the plant will vary with the amount of extra carbon available (Bryant *et al.* 1983). This theory has been successful in predicting changes in secondary metabolite concentrations due to manipulated changes in the environment of several terrestrial plants (Bryant *et al.* 1983; Bryant *et al.* 1987; Tugwell and Branch 1989). For example Bryant *et al.* (1987) manipulated terrestrial trees by fertilization and shading. Both treatments produced a decline in the concentration of secondary metabolites, presumably by increasing the ratio of nitrogen to carbon, thereby reducing the amount of carbon available for secondary metabolites.

It is possible that the variation in secondary metabolite concentrations in *Dysidea* could be explained by the carbon-nutrient balance hypothesis. The observed spatial variation could be due to variation in food availability from the surrounding

environment, not from light affecting symbiotic cyanobacteria. Variation in food over time could account for the variation at a single site over time as observed in the grazing experiment where the concentration of avarone varied significantly among collection times (Fig. 19). Further studies should try to examine a relationship between abundance of secondary metabolites and food. Further studies should also include metals because the American Tanker ship wreck site showed the highest concentrations of avarol. Concentrations also varied at the other sites in the harbor, therefore I do not believe the variation among sites is due completely to the abundance of metal.

I conclude that variation in secondary metabolite concentrations from site to site is not an effect of grazing or of physical damage or of light including UV radiation. There is some other environmental factor affecting secondary metabolite concentrations in *Dysidea* cf. *avara*. Further studies need to examine the effects of food availability and metals on secondary metabolite concentrations in sponges. The sampling of the three American Tanker sites show that the sponges on the ship wreck had significantly higher concentrations of avarol. This could be due to the presence of the metal or because the ship wreck is at the edge of a reef in Apra harbor. These sponges may be receiving more water flow over them, supplying more food. The transplant experiment where secondary metabolites of deep sponges (that were placed on the reef) were significantly lower than the shallow reef sponges, could have been due to the tanker blocking some flow of the water to the deep study site. Nutrient availability could also account for variations over time at the same site which was observed in the grazing experiment with avarone.

LITERATURE CITED

Baldwin, I. T. 1990. Herbivory simulation in ecological research. *Trends in Ecology and Evolution* 5:91-93.

Baldwin, I. T., C. L. Sims and S.E. Kean 1990. The reproductive consequences associated with inducible alkaloidal responses in wild tobacco. *Ecology* 71:252-262.

Belisario, M. A., Ma. Maturo, R. Pecce, S. De Rosa and G. R.D. Villani 1992. Effect of avarol and avarone on in vitro-induced microsomal lipid peroxidation. *Toxicology* 72:221-233.

Belisario, M. A., R. Pecce, A. R. Arena, A. De Giulio, G. Strazzullo and S. De Rosa 1991. Effect of avarol, avarone and nine of their natural and synthetic derivatives on microsomal drug-metabolizing enzymes. *Toxicology Letters* 57:183-193.

Bergquist, P. R. 1978. *Sponges*. Berkeley: University of California Press.

Bernays, E.A. 1991. Relationship between deterrence and toxicity of plant secondary compounds for the grasshopper *Schistocerca americana. Journal of Chemical Ecology* 17:2519-2526.

Bryant, J.P., F.S. Chapin and D.R. Klein 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357-368.

Bryant, J.P., F.S. Chapin, P.B. Reichardt and T.P. Clausen 1987. Response of winter chemical defense in Alaska paper birch and green alder to manipulation of plant carbon/nutrient balance. *Oecologia* 72:510-514.

Coley, P. D. 1986. Costs and benefits of defense by tannins in a neotropical tree. *Oecologia* 70:238-241.

Fagerstrom, T. 1989. Anti-herbivory chemical defense in plants: a note on the concept of cost. *The American Naturalist* 133:281-287.

Green, D., Y. Kashman and Y. Benayahu 1992. Secondary metabolites of the yellow and gray morphs of the soft coral *Parerythropodium fulvum fulvum*: comparative aspects. *Journal of Natural Products* 55:1186-1196.

Harvell, C. D. and W. Fenical 1989. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.): Intracolony localization of defenses. *Limnology and Oceanography* 34:382-389.

Harvell, C. D. 1990. The ecology and evolution of inducible defenses. *The Quarterly Review of Biology* 65:323-340.

Ilvessalo, H. and J. Tuomi 1989. Nutrient availability and accumulation of phenolic compounds in the browm alga *Fucus vesiculosus*. *Marine Biology* 101:115-119.

Lindquist, N., M. E. Hay and W. Fenical 1992. Defense of ascidians and their conspicuous larvae: adult vs. larval chemical ecology. *Ecological Monographs* 62:547-568.

Loya, S. and A. Hizi 1990. The inhibition of the human immunodeficiency virus type 1 reverse transcriptase by avarol and avarone derivatives. *Federation of European Biochemical Societies Letters* 269:131-134.

Meyer, K.D. and Valerie J. Paul 1992. Intraplant variation in secondary metabolite concentration in three species of *Caulerpa* (Chlorophyta: Caulerpales) and its effects on herbivorous fishes. Marine Ecology Progress Series 82:249-257.

Meyers, R. F. 1989. *Micronesian Reef Fishes.* Guam, Coral Graphics p 251.

Minale, L., R. Riccio and G. Sodano 1974. Avarol, a novel sequiterpenoid hydroquinone with a rearranged skeleton from the sponge *Dysidea avara. Tetrahedron Letters* 38:3401-3404.

Paul, J.V. 1992. *Ecological Roles of Marine Natural Products.* London: Comstock Publishing Associates.

Paul, J.V. and William Fenical 1986. Chemical defense in tropical green algae, order Caulerpales. *Marine Ecology - Progress Series* 34:157-169.

Paul, V.J. and K. L. Van Alstyne 1988. Chemical defense and chemical variation in some tropical Pacific species of *Halimeda* (Halimedaceae; Chlorophyta). *Coral Reefs* 6:263-269.

Renaud, P. E., M. E. Hay and T. M. Schmitt 1990. Interactions of plant stress and herbivory: intraspecific variation in the susceptibility of a palatable versus an unpalatable seaweed to sea urchin grazing. *Oecologia* 82:217-226.

Phillips, D.W. and G.H.N. Towers 1982. Chemical ecology of red algal bromophenols. Including temporal, interpopulational and within-thallus measurements of lanosol levels in *Rhodomela larix* (Turner) C. Agardh. *Journal of Experimental Marine Biology and Ecology* 58:285-293.

Rosenthal, G. A. and M. R. Berenbaum 1991. *Herbivores: Their Interactions with Secondary Plant Metabolites., second edition* . San Diego: Academic Press.

Rosenthal, G. A. and D. H. Janzen 1979. *Herbivores: Their Interactions with Secondary Plant Metabolites.* New York: Academic Press.

Sara, M. 1971. Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variablilis* (Demospongiae). *Marine Biology* 11:214-221.

Schultz, J. C. 1988. Plant responses induced by herbivores. *Trends in Ecology and Evolution* 3:45-49.

Shubina, L.K., S.N. Fedorov, V.A. Stonik, A.S. Dmitrenok and V.V. Isakov 1990. Avarol and isoavarol from the Pacific sponge *Dysidea* sp. *Khim Prir Soedin* 3:353-361.

Spencer, K. C. 1988. *Chemical mediation of coevolution.* New York: Academic Press Inc. pp. 345-348.

Stienberg, P.D. 1984. Algal chemical defence against herbivores: allocation of phenolic compounds in the kelp *Alaria marginata. Science* 223:405-407.

Thompson, J. E., P. T. Murphy, P. R. Bergquist and E. A. Evans 1987. Environmentally induced variation in diterpene composition of the marine sponge *Rhopaloeides odorabile. Biochemical Systematics and Ecology* 15:595-606.

Tugwell, S. and G. M. Branch 1989. Differential polyphenolic distribution amoung tissues in the kelps *Ecklonia maxima, Laminaria pallida* and *Macrocystis angustifolia* in relation to plant-defence theory. *Journal of Experimental Marine Biology and Ecology* 129:219-230.

Van Alstyne, K. L. 1988. Herbivore grazing increases polyphenolic defenses in the brown alga *Fucus distichus. Ecology* 69:655-663.

Van Alstyne, K. L. and V. J. Paul 1988. The role of secondary metabolites in marine ecological interactions. *Proceedings of the 6th International Coral Reef Symposium, Australia* 1:175-186.

Walls, J. T., A. J. Blackman and D. A. Ritz 1991. Distribution of amathamide alkaloids within single colonies of the bryozoan *Amathia wilsoni. Journal of Chemical Ecology* 17:1871-1881.