Factors mediating metamorphosis of *Leptastrea purpurea* (Cnidaria: scleractinia) planulae

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INTRODUCTION

Many marine invertebrates have a planktonic dispersal phase before settling into a benthic adult lifestyle. How the larvae find a suitable habitat on which to settle has been the subject of much research. It has become increasingly evident in recent years that active habitat selection by larvae plays an important role in the ultimate distribution of the adults of many species. The ability of larvae to detect habitat-specific cues has been recognized for a range of phyla that is almost as diverse as the cues themselves, and this ability evidently allows the larvae to maximize their own post-settlement survival. Water flow, light levels, surface texture and/or composition, lipophilic (suface-bound) or hydrophilic (water-soluble) chemical cues arising from conspecifics, prey, hosts, or surface-associated biofilms have all been implicated as natural sources of inducers. Sometimes it appears that an intricate, sequential combination of factors must be present for successful settlement (Crisp, 1974), and these requirements can vary appreciably even among similar species.

The terms settlement and metamorphosis describe both the behavioral and physiological transition from the mobile larvae to the benthic adult. Settlement has been described as a reversible, substrate exploratory behavior that culminates in attachment, whereas metamorphosis is a largely non-reversible physiological change (Pawlik, 1992). Oftentimes, however, the terms are used interchangeably. Because attachment without

metamorphosis was rarely seen by the 16-24 hour endpoint in the experiments of the scleractinian coral *Leptastrea purpurea* described here, only complete metamorphosis was used to monitor recruitment success. Metamorphosis in coral planulae can be characterized as an obvious oral-aboral flattening of the attached juvenile, with distinct septal mesenteries radiating from the center oral region, as described by Negri *et al.* (2001).

Scleractinian metamorphosis

Corals have two different reproductive strategies: either they broadcast their gametes into the water column during a synchronous spawning period, or brood their larvae and release them after they are fully competent. Brooding corals (which tend to have smaller adult colonies) have a ready food supply (lecithotrophy) and harbor zooxanthellae inherited from the parent colony, whereas broadcasting corals (which are generally larger as adults) have planktotrophic larvae that are more abundant, smaller, lack a food supply, and do not inherit zooxanthellae from the parent. While researchers (looking at fossil records) found that Indo-Pacific brooding and broadcasting genera have similar ranges (with the exception that many brooding species having narrower longitudinal ranges), brooders survive preferentially because lecithotrophic larvae have higher recruitment success than do planktotrophic larvae in lower-quality habitats (Edinger and Risk, 1995). Further, the amount of zooxanthellae that an individual lecithotrophic larva receives from the parent is haphazard (Gaither, 1999), and may – through energetic constraints - influence settlement capacity as well.

Crustose coralline algae as an inducer

For numerous invertebrate species across diverse phyla, red crustose coralline algae (CCA; *Rhodophyta, Corallinaceae*) have been implicated as the source of a natural inducer of settlement and metamorphosis. Within the photic zone, CCA are among the most abundant marine organisms that live on hard substrata, which are habitats also preferred by many invertebrates, including corals. In general, CCA are found in abundance in areas with moderate amounts of wave action and/or current and an absence of intense herbivory. In the tropics, for instance, zones of free-living CCA often have insufficient cover to provide refuge for herbivorous fishes from predators (Steneck, 1986). This reduction in herbivory in CCA-dominated habitats may increase recruitment success for settling invertebrates. The morphogenic signal associated with CCA appears specific to a small number of genera; many other encrusting red algae that look similar to the naked eye, (and account for the greatest coverage of surface area in the reef environment), do not induce the larvae to settle or metamorphose (*reference* Edinger & Risk, (?)). The growth forms of CCA that have been found to be the most effective are typically thin crusts on dead coral substrata, and are themselves frequently overgrown in encounters with other low-profile organisms such as other species of CCA, sponges, bryozoa, and fleshy algae.

Although some larvae, such as abalone (Morse& Morse, 1984) and vermetid gastropods (Kelly, 2004), are apparently dependent on the presence of CCA, attachment does not always occur directly on the algae. Contact with or proximity to CCA may be required for these larvae to induce settlement, but they often then swim or crawl to nearby CCA-free microhabitats before metamorphosis occurs. Therefore, continuous

contact with the inducer may not be required to complete metamorphosis after settlement. Rahmani and Ueharai (2001) found that aqueous extracts of CCA induced sea urchin (*Echinometra*) metamorphosis, and that the chemicals were gamma-aminobutyric acid (GABA)-mimetic in their interaction with larval receptors. Swanson *et al.* (2004) determined that the sea urchin *Holopneustes purpurascens* is induced to metamorphose by a water-soluble cue emanating from a red alga that is a primary substrate for new recruits. This settlement cue was identified as histamine, itself a neurotransmitter. Unlike previous research where a neurotransmitter was used to artificially mimic the function of a natural settlement cue, these studies show that are naturally produced by algae in the marine environment. However, this type of inducer is not relevant across all phyla; the possible occurrence of a water-soluble inducer that diffuses from CCA was excluded in several experiments with other marine invertebrate larvae in which supernatants removed after prolonged contact with morphogenically active CCA were by themselves not inducers (Morse, et al.1988, _____).

Microbial inducers

Rather than preferring CCA *per se*, marine invertebrate larvae may respond in some cases to the interaction between bacteria and the surface to which the larvae attach. The presence of a surface film of microorganisms promotes the settlement of many species, but inhibits the settlement of others (Pawlik, 1992). Marine biofilms have been reported to induce metamorphosis in crowns-of-thorn starfish larvae (*Acanthaster planci*), soft corals (*Anthozoa*), jellyfish (*Scyphozoa*), and *Hydrozoa.* While larvae may be responding to the chemical nature of the bacterial film, fouled surfaces also decrease

the "wettability" of the surface, perhaps making attachment easier. Webster *et al.* (2004) observed successful coral recruitment on biofilms on glass coated with CCA recruits as well as glass without any evidence of CCA, although this occurred only after allowing a biofilm to develop for longer than 2 weeks. On the biofilms where CCA were present, the larvae preferentially settled on top of the algal recruits; when CCA was absent from the biofilm the larvae settled on clean glass away from the biofilm. Negri *et al.* (1994) also found that coral larvae settle and metamorphose in response to biofilms that lack CCA. In order to survey normal levels of coral larval recruitment, Heyward *et al.* (2002) used terracotta tiles conditioned in seawater for 3 weeks to allow a biofilm to develop, and had a success rate averaging 0.27 recruits per tile after 6 weeks. Golbuu (1996) found that the brooding coral *Stylaraea punctata* responded to biofilm in preference to other substrates, including CCA.

A bacterium identified as *Pseudoalteromonas spp.* isolated from the CCA *Hydrolithon onkodes* was able to induce significant levels of metamorphosis, although sterile CCA substrate, extracts of CCA, and extracts from the skeleton of some coral species acted as inducers as well (Negri, *et al.* 2001). For the serpulid polychaete *Hydroides elegans*, however, Lau and Qian (2001) found two bacterial strains highly inducive to larval settlement, and also discovered that both formaldehyde and streptomycin treatments reduced larval settlements in a dose-dependent manner. The authors concluded that both treatments kill bacteria with little damage to the surface chemistry of bacterial walls, and thus the presence of the living bacterial population is a requirement for induction for *Hydroides elegans*. Although formaldehyde may actually damage cell walls when used in this treatment, the inducer for this organism is perhaps a

readily degradable metabolic product of the bacteria concentrated in the extracellular matrix of the cell. In any case, as is the case with CCA species themselves, many strains of bacteria do not induce larval settlement at all.

For some species, it may take an entire assemblage of bacterial types to induce a response, and the cue may ultimately be due to the ability of the bacteria to communicate among themselves. Known collectively as quorum sensing, a group of chemical signals (termed AHL's (*N*-acylhomoserine lactones)) have been shown to induce motile zoospores of the green seaweed *Enteromorpha* to settle (Joint et al, 2002). Upon reaching a threshold level, AHL's activate the LuxR family of transcriptional regulator proteins, forming a complex that turns on genes for additional AHL synthesis. Using mutant strains of *Vibrio anguillarum* (defective in AHL synthesis or expressing foreign AHL synthases from recombinant plasmids) or synthetic AHL compounds, Joint *et al.* (2002) found that wild-type biofilms induced high levels of zoospore settlement and that attachment could be similarly induced by synthetic AHL compounds of varying chain lengths (C6-C14), whereas the AHL-negative mutant failed to induce settlement. This study provides evidence that eukaryotic organisms may be able to exploit prokaryotic signaling, although this has not yet been demonstrated for any species of marine invertebrate larvae.

Morphogen characterization

Attempts have been made to further describe morphogenic inducers of various species of larvae, although most of the chemical cues that have been identified have not been conclusively characterized. Low levels of the compound within the environment and the quick dilution of water-soluble cues make complete characterization difficult. Larvae

of many species, however, respond to low-molecular-weight water-soluble cues. Morse *et al.* (1988) found that boiling living CCA significantly reduced their effectiveness as an inducer, but did not inhibit the metamorphosis induced by live CCA placed in the same container. This demonstrated that the reduced activity seen with the boiled samples alone was not due to the production of a toxic inhibitor of the larval response, but instead is a result of a change in the morphogenetic inducer itself. Upon fractionization through chromatography, however, the major portion of the morphogenetic activity was found in the water-insoluble fraction, while the water-soluble fraction was less inductive and appeared to be slightly toxic to coral planulae Also, the (antibiotic-treated) skeleton of the coral *Goniastrea sp.* and coral rubble were found to induce metamorphosis in some corals, suggesting that either multiple inducers for metamorphosis exist or specific inducers may originate from a variety of sources found in their natural habitat (Heyward, Negri 1999). The structure of the morphogen recognized by *Agaricia humilis* larvae is consistent with both a high-molecular mass polysaccharide molecule of a red algal cell wall and a cell wall produced by an associated bacterium. However, since decalcification of the CCA extract appeared in this case to enhance inducer activity, the algal cell wall is still considered the most likely source of morphogenic compounds for this species (Morse *et al.,* 1994).

This proposed study is an effort to determine the factors that induce settlement and metamorphosis of larval *Leptastrea purpurea,* a scleractinian coral. In preliminary laboratory studies I found that this species metamorphoses in the presence of crustose coralline algae encrusting a hard coral substratum of a mixed marine community of epibionts. However, it is not clear whether the cue that induces metamorphosis in this

species is chemical, bacterial, textural, or a (possibly hierarchical) combination of all of these factors. The choice of an appropriate substratum is essential for survival of each individual, due to the sessile nature of the adult coral colony (Negri, 2001). The value of this research lies in informed coral reef management. On a large scale, it is larval production and subsequent recruitment that is essential for maintaining reef populations (Richmond, 1993). Determination of the factors mediating this recruitment in coral planulae is not only important in understanding adult distributions, but can help relieve pressure from coral harvesting for the burgeoning aquarium industry, aid studies ranging from various coral stressors to the role of recruitment in reef communities, and have applications in anti-fouling efforts as well as reef rehabilitation.

Materials and Methods

The laboratory experiments discussed here were conducted from March through August 2004. They were performed on *Leptastrea purpurea*, a small scleractinian coral that can commonly be found in the Indo-Pacific on reef flats and reef slopes to 50 m.

Collection and maintenance of adult corals. The coral colonies as well as CCA-encrusted substrata were collected from Cocos Lagoon and Luminao reef flat, with additional CCA collected from Pago Bay. *Leptastrea purpurea* colonies can be picked up by hand in these areas as they are settled on dead *Acropora* branch fragments. After return to the laboratory, adults were removed from their substrata with a chisel and hammer (to increase planulae collection efficiency through maximum colony density) and maintained in 9 to 12-liter plastic tubs containing aerated seawater. The seawater in the tubs was changed daily when planulae were collected. Between experiments, the adult colonies were kept in an open, flow-through seawater table.

Collection of planulae. Larvae were collected by washing them off of the colony and tub surfaces with a stream of water from a large pipette, thus suspending them in the seawater contained in the tub. This water then was filtered through a 90um-mesh sieve and the contents of the sieve were washed into a 250 ml glass bowl. Individual planulae were hand-picked from the surrounding sediment (trapped within fleshy algae on remaining substrate or entering from the seawater system when filling the tubs) with the aid of a dissecting microscope and a glass Pasteur pipette, cleaned of debris and placed in ml 250 ml bowls with fresh seawater until use for experiments.

Experiment design. Ten planulae were added to individual polystyrene petri dishes containing 10 ml unfiltered seawater and the treatment substratum. Five treatment replicates and five seawater controls were used for each experiment. Positive controls were 1-2 cm chips from live rock encrusted with crustose coralline algae collected from the site of the adult coral colonies, and the negative control was 1-2 cm chips of sun-bleached crustose coralline algae that have dried under the transparent lanai roof to prevent fresh-water exposure from rain. Three chips of substrate were placed in each petri dish. Petri dishes were maintained on the lanai of the Marine Laboratory at the University of Guam at ambient air temperature (27-31 C) and indirect sunlight. Experiments were conducted for 24 hours without water change. The petri dishes were set up in a randomized block design to increase the probability of independence among replicates. The endpoint for scoring metamorphosis was 16-24 hours, following Heyward and Negri (1999), except when otherwise noted.

Chemical Extracts. CCA were removed from their substrata by chipping them with a chisel and hammer. The chips of CCA were then combined, weighed, placed in an Erlynmeyer flask that was filled with methanol (or 1:1 ethyl acetate/hexane for the extraction of non-polar compounds) until the chips were completely covered. The mixture was be constantly stirred for 1 hour, decanted, dried by a rotary evaporator to remove the solvent, and then extracted again with solvent for 24 hours. After removing the solvent again, a third extraction (of 4 hours) was performed to remove potential extractable morphogens, and then freeze-dried to remove the remaining solvent. Chemical extracts were stored in the freezer and used within that week.

Inert (sun-bleached) CCA chips were coated with the extract by dissolving the crude extract in 2 ml of solvent and than pipetting the mixture evenly onto the entire surface of the chips over a period of 1 hour, allowing the chips to dry before turning them to coat the underside to mimic the live CCA. The chips were then left to further dry under an exhaust hood for a minimum of 30 minutes before testing for morphogenesis. Natural concentration of the extract was determined by dividing the mass of the CCA chips prior to extraction by the mass of the entire substrate before removal of the CCA, and then multiplying this by the mass of the chips that were to be coated. Both natural and five-fold concentrations were tested; the latter concentration was used to counteract any losses inherent in this procedure.

Data Treatment. Data was recorded as the total percentage of larvae settled per day on the substratum of interest among the replicates. Missing larvae were considered dead and included in the percent metamorphosis calculations. For bar graphs, percentages +SE will be presented. Statistical differences were tested using ANOVA and

Tukey-Kramer Multiple Comparison Test (Number Cruncher Statistical Systems), which tests for all pairwise differences between the means of each treatment and control.

I. SUBSTRATE PREFERENCES

Exp. 1; Larval Response to Crustose Coralline Algae. This experiment was designed to determine if a cue emitted from the treatment substratum was responsible for attracting larvae to suitable substrata. Crustose coralline algae-encrusted rock chips, approximately 1 cm high and 2 cm long were cleaned of debris and put in 10ml polystyrene petri dishes with 10 ml of seawater. Similar sized chips of sun-bleached crustose coralline algae skeleton were used as an optional substrate for settlement, and petri dishes containing only seawater were used as controls. Three pieces of test substrata were placed in each of the treatment petri dishes, and 10 planulae were pipetted into each of 5 replicates.

Exp. 2; Response to various types of CCA. This assay tested the response of the planulae to the presence of two species of CCA (*Hydrolithon reinboldii* and *Paragonalithon conicum*) and to the patchy mix of CCA on the *Acropora* branch substrate on which *L. purpurea* is often found. *H. reinboldii* and *P. conicum* were collected from Pago Bay, whereas the *Acropora*-encrusting CCA mix were collected from the same site as the adult coral colonies, Cocos Lagoon and Luminao reef flat.

Exp. 3; Response to various substrata. Substrata tested were *Acropora* skeleton (boiled to remove tissue and then cooled to ambient temperature), glass petri dishes, *Goniastrea edwardsii* coral skeleton chips, ceramic settlement plate chips, and polystrene petri

dishes. These substrata were chosen as they are all implicated in various studies to induce scleractinian metamorphosis.

II. GENERAL REQUIRMENTS

Exp. 4; Effects of coral water and CCA water on metamorphosis. This experiment was designed to determine whether the larval settlement cue exists as a waterborne product or as a surface-bound substance on bacterial films or CCA itself. Water was taken from tubs of corals or CCA after soaking for 72 hours. Treatments were *L. purpurea* coral water, coral water and sun-dried CCA, seawater, seawater with sun-dried CCA, *Hydrolithon* water, and *Paragonalithon* water. The positive control was live CCA chips. This assay consisted of 3 replicates, with 8 larvae in each replicate.

Exp. 5; Effects of amount of zooxanthellae on metamorphosis. For this experiment, planulae were separated, using visual inspection, into two groups having either high numbers of zooxanthellae or low numbers of zooxanthellae (*i.e.* dark vs. pale color). Fifteen planulae were homogenized in 0.1 ml filtered (0.2 um) seawater and 2 counts on each of 4 homogenates were made for each group with a hemocytometer to find the average number of zooxanthellae contained by each of these two groups. Live CCA and sun-bleached CCA were offered as substrates. This assay had 3 replicates, with 10 larvae per replicate.

Exp. 6: Textural requirement of planulae. As recorded for other invertebrate larvae, I have observed that planulae of *Leptastrea purpurea* often appear to prefer irregular

substrate to settle upon. This may be due to their need to avoid UV radiation, or there may be a greater density of microbes in nooks and crevices. This assay was designed to test if the larvae can respond to a purely textural cue. It consisted of 5 replicates with 10 larvae each. Treatments were etched polystyrene petri dishes, and sun-bleached beach rock chips. Controls were live CCA chips and seawater.

Exp. 7. Effect of Age on Competency for Metamorphosis and Substratum Choice. Planulae were pooled in a 250 ml bowl with a 1-2 day water change. At the appropriate day (day 1, 7, and 14), 50 larvae (n=10, 5 replicates) were removed for the assay. They were offered the choice polystyrene petri dishes, CCA and sun-bleached CCA for substrates.

III. Chemical Nature of the Metamorphic Cue

Experiment 8: Polar crude extract effect on metamorphosis.

This experiment had 5 replicates with 10 larvae per replicate. The positive control was live CCA chips with chips of sun-bleached CCA coated in methanol (to demonstrate that the solvent itself did not negatively effect metamorphosis), the negative control was sun-dried CCA skeleton, and treatments were methanol extracts of *Hydrolithon* at a natural concentration and at a five-fold concentration. The second polar extract assay tested two other species of CCA, all at concentrations five times normal concentrations.

Exp. 9: Non-polar extract effects on metamorphosis.

In experiments 8A and B, methanol extracts of the crustose coralline algae *Hydrolithon reinboldii*, *Paragonilithon conicum*, and a mixed extract of species from both these

genera (found naturally growing together on dead *Acropora* branches) did not induce *Leptastrea purpurea* to settle. However, the cue may be lipophilic rather than alcohol or water-soluble and thus require an extraction with ethyl acetate and hexane rather than methanol and deionized water. Thus, This experiment was performed as above, with the ethyl acetate and hexane used as solvents. The extract was tested at a five-fold concentration.

IV. EFFECT OF BIOFILM ON METAMORPHOSIS

Exp. 10A: 3 Day Old Biofilm. 3 replicates, n=8. 3 treatments were tested (biofilm on glass, biofilm on beach rock, clean glass) plus the negative control (clean beach rock), and positive control (reef rock). Biofilm was allowed to develop on substrates for three days prior to use in assay.

Exp 10B. 2-week Biofilm on chips vs autoclaved chips

5 replicates, n=10. CCA, autoclaved CCA, sun-bleached CCA, autoclaved sun-bleached CCA, seawater, 2-week biofilm on sun-bleached CCA, autoclaved 2-week biofilm on sun-bleached CCA.

Exp. 11. Response to inert substrates with 1 month old biofilm. Different substrata used in preliminary experiments were tested for metamorphic inducing properties after a two–week biofilm has been allowed to develop in seawater. Polystyrene petri dishes, glass petri dishes, *Acropora* skeleton, sun-bleached crustose coralline algae skeleton, a

Goniastrea coral skeleton and ceramic settlement plates were used as substrata, along with crustose coralline algae as a positive control.

Exp. 12. Response to antibiotic treatment. Above substrata were used, in addition to antibiotic treatment (3 antibiotics). Live CCA were treated with antibiotics as well in addition to untreated CCA used as a positive control.

Exp. 13. Response to AHLs. In order to test whether *L. purpurea* can be induced to settle after exposure to synthetic AHL's, 3 types of AHL's, which are normally produced by a diverse assemblage of Gram-negative bacteria, were tested. These chemical signals (HHL, OHHL, and ODDHL) each have a different chain length, which may cause differential larval response. The chemicals were pipetted onto autoclaved sundried CCA chips (to create a chemical gradient and to provide a suitable textural substrate) and placed into 10ml unfiltered seawater for a final concentration of 50um of each AHL. Along with these chemicals, positive live CCA control and negative sun-bleached CCA control were also tested.

Exp. 14: Response to furanones. Furanones are a class of low molecular weight diffusible compounds that inhibit the AHL regulatory pathway through reduction in LuxR protein concentration, competitive interactions with AHL for LuxR sites, and ultimately cause LuxR conformational changes (Manefield *et al.*, 2002). First isolated in the red alga *Delisea pulchra* (Maximilien *et al.*, 1998), they can be used to . quench biofilm development and thus aid in determining the role of biofilm for the settlement of

L. purpurea. However, the extent of this inhibition is dependent on the furanones involved, the bacterial phenotypes, and the substrate used. In this experiment, ceramic chips were incubated for two weeks in a furanone mixture (C4 and C8) that was dissolved in 1 ml of acetone and then added to 100 ml of unfiltered seawater. As a positive control, ceramic chips were incubated in unfiltered seawater for the same length of time, and sterile ceramic chips were used as a negative control. The furanone mixture and seawater controls were both changed daily. After two weeks, the larvae were added to the petri dishes.

RESULTS

I. SUBSTRATE PREFERENCES

Exp. 1; Larval response to Crustose Coralline Algae. The larvae settled in significantly higher numbers in the presence of CCA (50%), and no metamorphosis occurred on the sun-dried CCA or seawater control (Figure 1).

Exp. 2; Response to various types of CCA. There was a high amount of metamorphosis in response to this patchy mix of CCA (97%) as well as to *Hydrolithon reinboldii* (99%), whereas the CCA of the genus *Paragonalithon* did not induce a response that was significantly different from the negative control (Figure 2).

Exp. 3; Response to various substrata for settlement. Substrates testerd were *Acropora* skeleton (boiled to remove tissue and then cooled to ambient temperature), glass petri dishes, *Goniastrea edwardsii* coral skeleton chips, ceramic settlement plate chips, and

polystrene petri dishes. However, none of them significantly affected metamorphosis for *L. purpurea* (Figure 3).

II. GENERAL REQUIRMENTS

Exp. 4; Effects of coral water and CCA water on metamorphosis. The results of this experiment suggest that the morphogen(s) that induce *L. purpurea* to metamorphose are not water-soluble, or that physical contact with the live substratum is required for metamorphosis of this species (Figure 4).

Exp. 5; Effects of amount of zooxanthellae on metamorphosis. Planulae which had higher numbers of zooxanthellae responded more successfully to a cue from the CCA substrate, indicating that the lower level of zooxanthellae may present energetic constraints that preclude successful settlement (Figure 5).

Exp. 6: Textural requirement of planulae. There was no response to this etched surface (Figure 6).

Exp. 7. Effect of Age on Competency for Metamorphosis and Substratum Choice.

No significant difference in response occurred from Day 1 until Day 14.

III. Chemical Nature of the Metamorphic Cue

Exp. 8: Polar crude extract effect on metamorphosis.

No significant response occurred with any of the methanol extracts of CCA, which indicates that the morphogen(s) for *L. purpurea* are likely bacterial in nature (Figures 8 and 9).

Exp. 9: Non-polar extract effects on metamorphosis.

Since no metamorphic activity was generated by the extracts, it suggests that the cue associated with crustose coralline algae is not found chemically in the alga itself, and may be instead related to the presence of crustose coralline algae-specific bacteria that colonize the algal surface (Figure 10).

V. EFFECT OF BIOFILM ON METAMORPHOSIS

Exp.10A: 3 Day old Biofilm. Although there appeared to be a small response, it was not significantly different from the negative control (Figure 10).

Exp.10 B. 2-week Biofilm on chips vs autoclaved chips. A 2-week old biofilm produced a response almost as strong as that for live CCA itself, whereas the autoclaved live CCA chips induced no metamorphosis (Figure 11).

Exp. 11. Response to inert substrates with 1 month old biofilm.

Exp. 12. Antibiotic treatment.

Exp 13; Response to AHLs. (RESULTS PENDING)

Exp. 14: Response to Furanone–incubated substrate (RESULTS PENDING).

DISCUSSION

Effective inducers of coral larvae metamorphosis have been identified by previous researchers as extractable chemicals from various species of CCA, coral rubble and skeletons of *Goneastrea*, biofilm communities, and single strains of bacteria. Negri, *et al.* (2001) determined that either multiple inducers for metamorphosis exist or specific inducers originate from a variety of sources. It appears that the inducer(s) is specific to the individual species as well. Negri has found that for *Acropora millepora*, crustose coralline algae skeleton as well as autoclaved crustose coralline algae induce metamorphosis, while this was not the case for *Leptastrea purpurea,* even after soaking in autoclaved seawater to remove terrestrial inorganics and maintain water quality. Chemical extracts showed no activity for this species, while for the *Acropora* species they were highly active from both crustose coralline algae and *Goniastrea* skeleton. Methanol has been used effectively in extracting morphogens from CCA (Heyward & Negri 1999) but may be not be useful in extracting those produced from bacteria (Negri et al. 2001). The phenomenon of marine larvae appearing to have a common response to CCA but different fine-scale response to chemicals and bacteria may be due to marine invertebrate larvae sharing a common primary receptor type but different secondary transduction pathways that lead to metamorphosis (Negri 2001). This makes ecological sense as it may direct larvae to a suitable habitat while reducing competition for space.

Effect of Age on Competency and Substrate Choice

While brooded larvae are able to settle more quickly than spawning corals, they are also more likely to have a greater competency period due to their large energy reserves (Richmond, 1989). *Pocillipora damicornis* larvae, which are brooded, had a competency period of 100 days, whereas the competency period for *Acropora tenuis* lasted only 20 days (Richmond, 1989). Wilson & Harrison (1998) found that competency for three scleractinian species ranged from 26-78 days. While most larvae tend to settle early during the competency period, delayed settlement and metamorphosis would be an advantage in unsuitable habitats. During this time, a stringent requirement for a specific cue may be reduced, as larvae that do not settle will ultimately not survive. However, the time frame of this experiment was likely too short to test this hypothesis.

Antibiotics.

Negri *et al.* (2001) found that antibiotics did not significantly reduce the morphogenic ability of CCA for coral, although previous researchers found otherwise for crown-of-thorns larvae (Johnson and Sutton, 1994).

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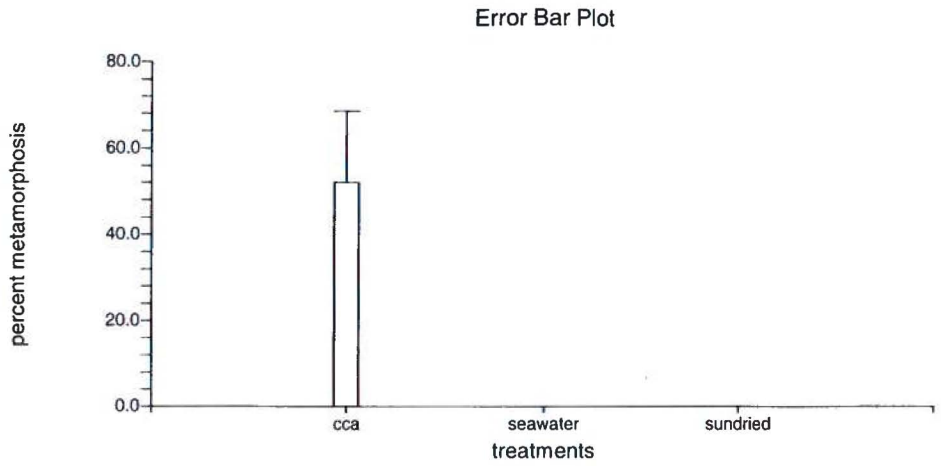
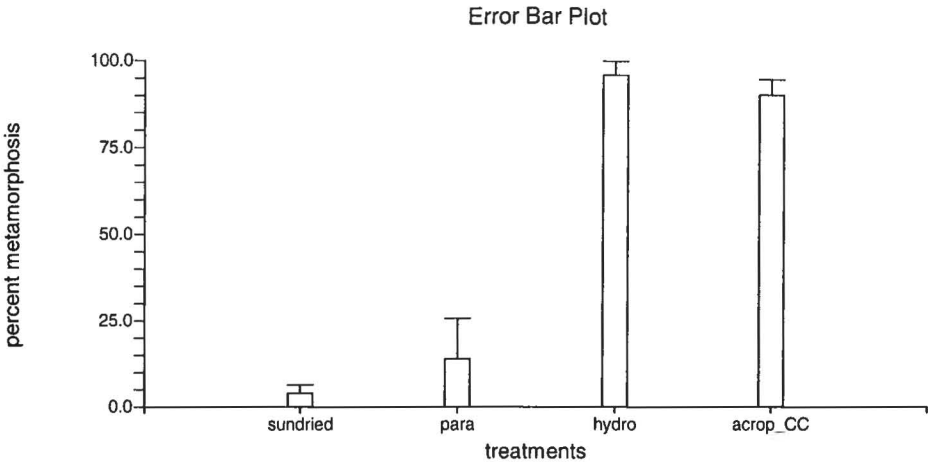


Figure 1. Exp. 1: Larval Response to Crustose Coralline Algae. Probability Level =0.029, Power = 0.456.



Analysis of Variance Table

Source Term	DF	Sum of Squares (Alpha=0.05)	Mean Square	F-Ratio	Prob Level	Power
A (...)	3	35620	11873.33	53.36	0.000000*	1.000000
S(A)	16	3560	222.5			
Total (Adjusted)	19	39180				
Total	20					

Φιγυρε 7. Term significant at alpha = 0.05

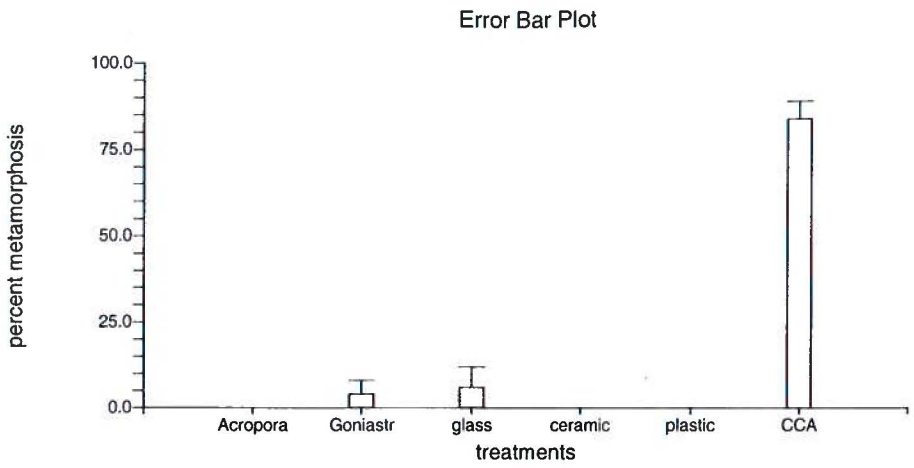
Tukey-Kramer Multiple-Comparison Test

Response: acrop_CCA,hydro,para,sundried_CCA
Term A:

Alpha=0.050 Error Term=S(A) DF=16 MSE=222.5 Critical Value=4.0461

Group	Count	Mean	Different From Groups
sundried_CCA	5	4	acrop_CCA, hydro
para	5	14	acrop_CCA, hydro
acrop_CCA	5	90	sundried_CCA, para
hydro	5	96	sundried_CCA, para

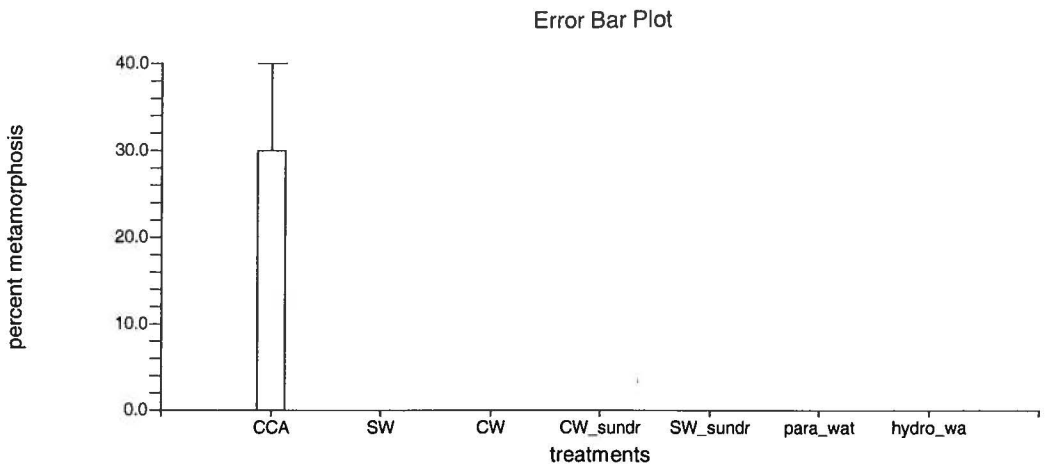
Figure 2. Exp. 2: Response to Various Types of CCA.



Alpha=0.050 Error Term=S(A) DF=24 MSE=0.65 Critical Value=4.3727

Group	Count	Mean	Different From Groups
Acrop_skel	5	0	CCA
ceramic	5	0	CCA
plastic	5	0	CCA
Goniastrea_skel	5	0.4	CCA
glass	5	0.6	CCA
CCA	5	8.4	Goniastrea_skel, ceramic, plastic, glass

Figure 3. Exp. 3; Response to various substrata.



Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A (...)	6	2314.286	385.7143	9.00	0.000380*	0.997872
S(A)	14	600	42.85714			
Total (Adjusted)	20	2914.286				
Total	21					

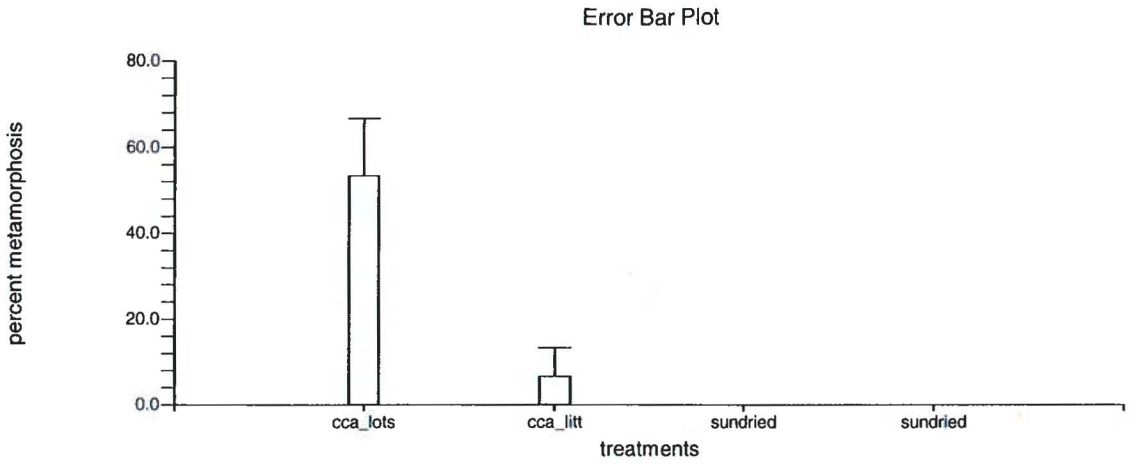
Φιγυρε 7. Term significant at alpha = 0.05

Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=14 MSE=42.85714 Critical Value=4.8290

Group	Count	Mean	Different From Groups
SW	3	0	CCA
CW	3	0	CCA
hydro_water	3	0	CCA
SW_sundried_CCA	3	0	CCA
CW_sundried_CCA	3	0	CCA
para_water	3	0	CCA
CCA	3	30	SW, CW, hydro_water, CW_sundried_CCA, para_water SW_sundried_CCA

Figure 4. ***Exp. 4; Effects of coral water and CCA* water on metamorphosis.**



Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A (...)	3	59.66667	19.88889	11.93	0.002530*	0.981046
S(A)	8	13.33333	1.666667			
Total (Adjusted)	11	73				
Total	12					

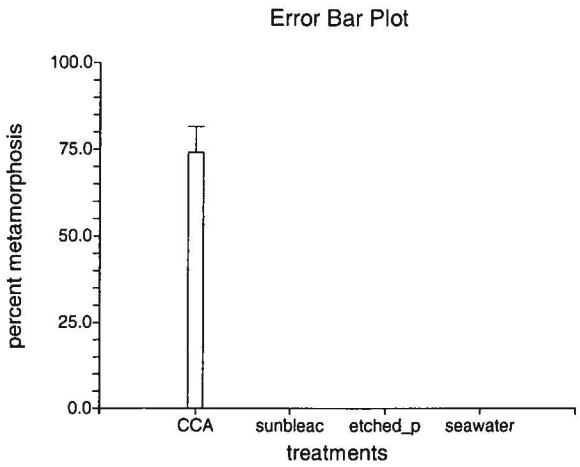
Φιγυρε 7. Term significant at alpha = 0.05

Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=8 MSE=1.666667 Critical Value=4.5288

Group	Count	Mean	Different From Groups
sundried_cca__lots	3	0	cca_lots
sundried_cca__little	3	0	cca_lots
cca_little	3	0.6666667	cca_lots
cca_lots	3	5.333333	sundried_cca__lots, sundried_cca__little cca_little

Figure 5. Exp. 5; Effects of amount of zooxanthellae on metamorphosis.

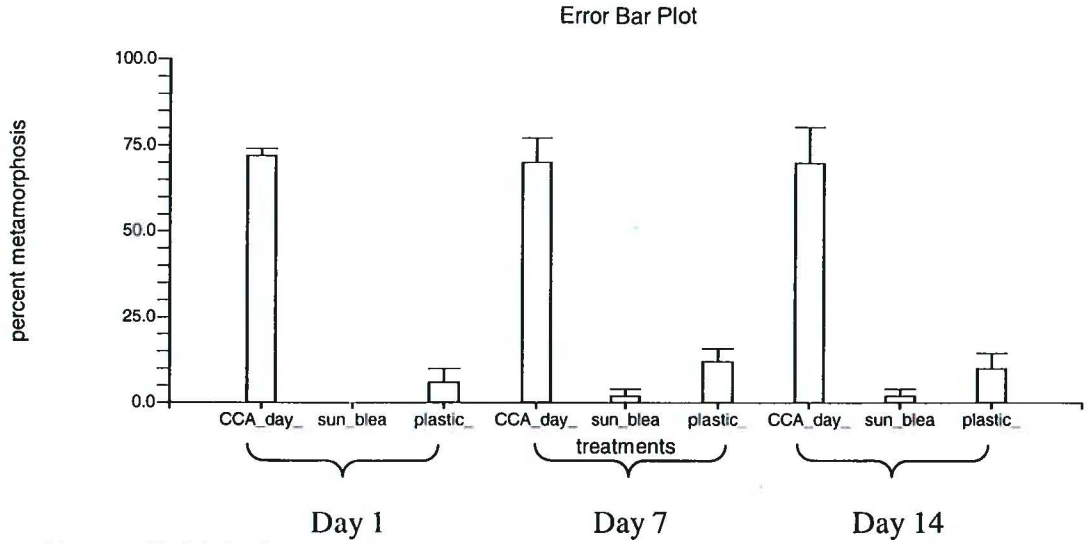


Analysis of Variance Table

Source Term	DF	Sum of Squares (Alpha=0.05)	Mean Square	F-Ratio	Prob Level	Power
A (...)	3	20535	6845	97.79	0.000000*	1.000000
S(A)	16	1120	70			
Total (Adjusted)	19	21655				
Total	20					

Φιγυρε 7. Term significant at alpha = 0.05

Figure 6. Exp. 6: Textural requirement of planulae.



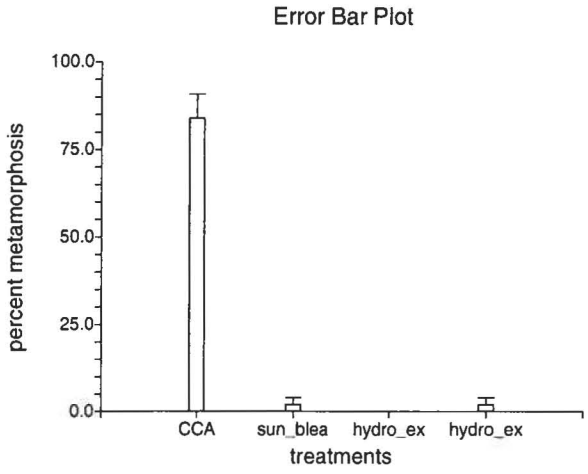
Tukey-Kramer Multiple-Comparison Test

Response:
CCA_day_1,CCA_day_14,CCA_day_7,plastic_day_1,plastic_day_7,sun_bleached_CCA_day_1, sun_bleached_CCA_day_7,sun_bleached_day_14
Term A: Alpha=0.050 Error Term=S(A) DF=32 MSE=126.25 Critical Value=4.5811

Group	Count	Mean	Different From Groups
sun_bleached_CCA_day_1	5	0	CCA_day_14, CCA_day_7, CCA_day_1
sun_bleached_day_14	5	2	CCA_day_14, CCA_day_7, CCA_day_1
sun_bleached_CCA_day_7	5	2	CCA_day_14, CCA_day_7, CCA_day_1
plastic_day_1	5	6	CCA_day_14, CCA_day_7, CCA_day_1
plastic_day_7	5	12	CCA_day_14, CCA_day_7, CCA_day_1
CCA_day_14	5	70	sun_bleached_CCA_day_1 sun_bleached_day_14 sun_bleached_CCA_day_7, plastic_day_1 plastic_day_7
CCA_day_7	5	70	sun_bleached_CCA_day_1 sun_bleached_day_14 sun_bleached_CCA_day_7, plastic_day_1 plastic_day_7
CCA_day_1	5	72	sun_bleached_CCA_day_1 sun_bleached_day_14 sun_bleached_CCA_day_7, plastic_day_1 plastic_day_7

Figure 7. Exp. 7. Effect of Age on Competency for Metamorphosis and Substratum Choice.

A)

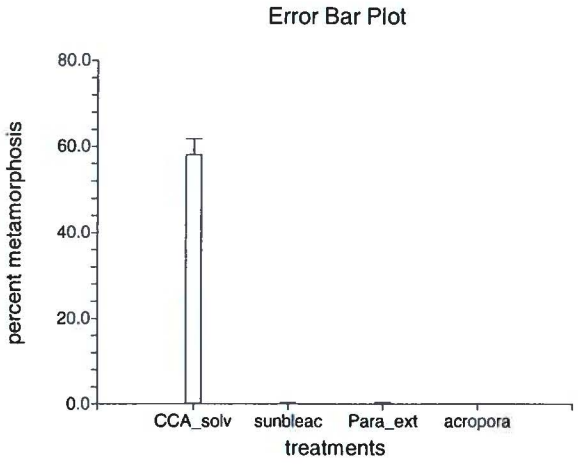


Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=16 MSE=0.675 Critical Value=4.0461

Group	Count	Mean	Different From Groups
hydro_extract	5	0	CCA
hydro_extract_x_5	5	0.2	CCA
sun_bleached_CCA	5	0.2	CCA
CCA	5	8.4	hydro_extract, hydro_extract_x_5 sun_bleached_CCA

Figure 8. Exp. 8A: Polar crude extract effect on metamorphosis.

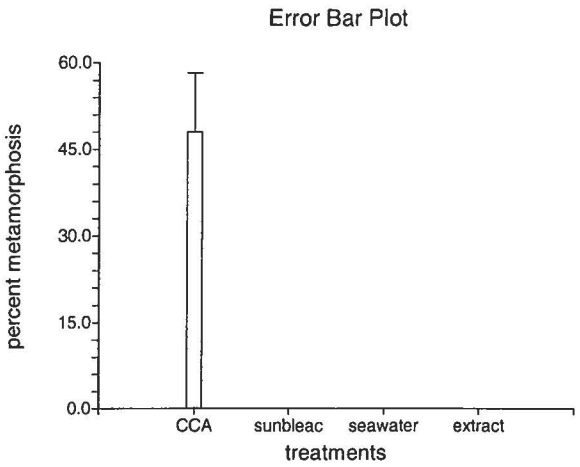


Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=16 MSE=17.6 Critical Value=4.0461

Group	Count	Mean	Different From Groups
Acropora_cca_extract	5	0	CCA_solvent_on_sbCCA
Para_extract	5	0.2	CCA_solvent_on_sbCCA
sunbleached_cca	5	0.2	CCA_solvent_on_sbCCA
CCA_solvent_on_sbCCA	5	58	acropora_cca_extract, Para_extract sunbleached_cca

Figure 8.Exp. 8B. Polar crude extract effect on metamorphosis.

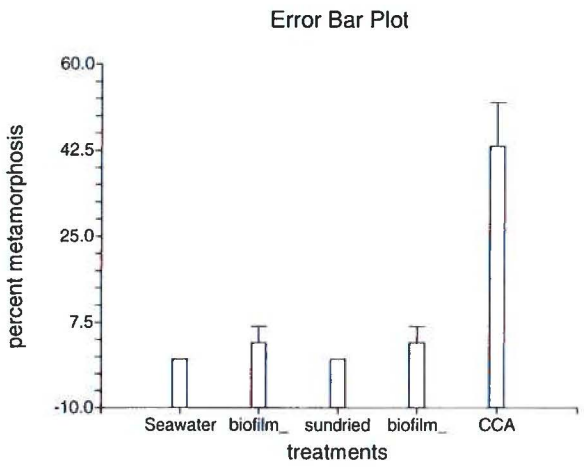


Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=16 MSE=130 Critical Value=4.0461

Group	Count	Mean	Different From Groups
extract	5	0	CCA
seawater	5	0	CCA
sunbleached_CCA	5	0	CCA
CCA	5	48	extract, seawater, sunbleached_CCA

Figure 9.Exp. 9: Non-polar extract effects on metamorphosis.



Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A (...)	4	42	10.5	17.50	0.000164*	0.999891
S(A)	10	6	0.6			
Total (Adjusted)	14	48				
Total	15					

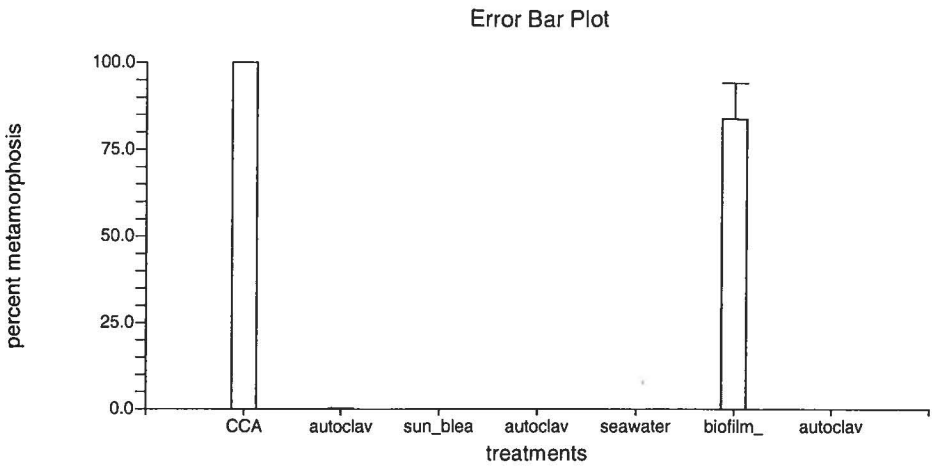
Φιγυρε 7. Term significant at alpha = 0.05

Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=10 MSE=0.6 Critical Value=4.6543

Group	Count	Mean	Different From Groups
sundried_CCA	3	0	CCA
Seawater	3	0	CCA
biofilm_sundried_CCA	3	0.3333333	CCA
biofilm_glass	3	0.3333333	CCA
CCA	3	4.333333	sundried_CCA, Seawater biofilm_sundried_CCA, biofilm_glass

Figure 10. Response to 3 Day Old Biofilm.



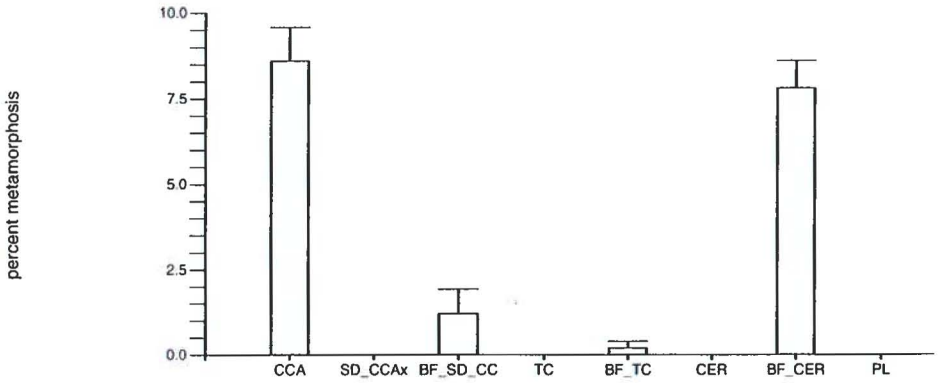
Tukey-Kramer Multiple-Comparison Test

Alpha=0.050 Error Term=S(A) DF=28 MSE=75.74286 Critical Value=4.4861

Group	Count	Mean	Different From Groups
autoclaved_sbCCA	5	0	biofilm_sbCCA, CCA
sun_bleached_CCA	5	0	biofilm_sbCCA, CCA
autoclaved_biosbCCA	5	0	biofilm_sbCCA, CCA
seawater	5	0	biofilm_sbCCA, CCA
autoclaved_CCA	5	0.2	biofilm_sbCCA, CCA
biofilm_sbCCA	5	84	autoclaved_sbCCA, sun_bleached_CCA autoclaved_biosbCCA, seawater autoclaved_CCA
CCA	5	100	autoclaved_sbCCA, sun_bleached_CCA autoclaved_biosbCCA, seawater autoclaved_CCA

Figure 11. Response to a 2 week old biofilm.

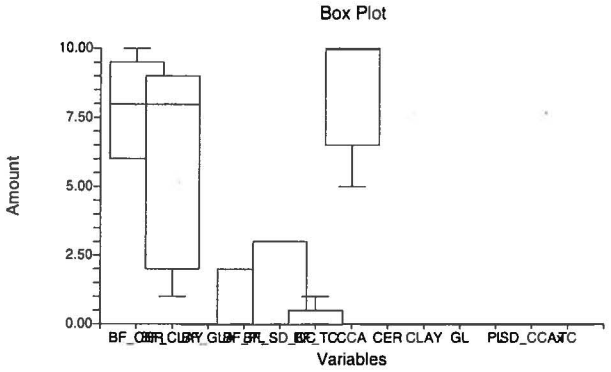
1+ month biofilm



Analysis of Variance Report

Page/Date/Time 1 8/14/2004 10:21:41 PM
Database E:\longterm biofilm data.S0
Response
BF_CER,BF_CLAY,BF_GLA,BF_PL,BF_SD_CCA,BF_TC,CCA,CER,CL
D_CCAx,TC

Box Plot Section



Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
	(Alpha=0.05)				
A (...)	12	631.8461	52.65385	26.23	0.00000(
S(A)	52	104.4	2.007692		
Total (Adjusted)	64	736.2462			
Total	65				

* Term significant at alpha = 0.05

Tukey-Kramer Multiple-Comparison Test

Response:
BF_CER,BF_CLAY,BF_GLA,BF_PL,BF_SD_CCA,BF_TC,CCA,CER,CLAY,GL,PL,S
Term A:

Alpha=0.050 Error Term=S(A) DF=52 MSE=2.007692 Critical Value=4.9085

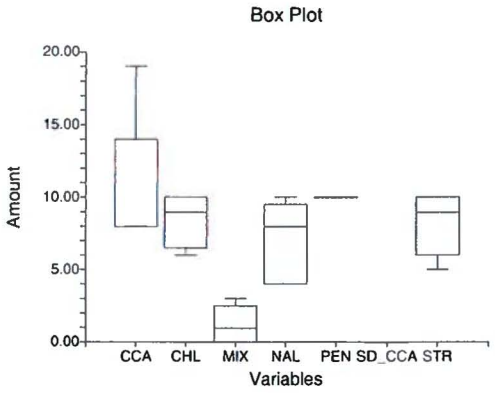
Group	Count	Mean	Different From Groups
SD_CCAx	5	0	BF_CLAY, BF_CER, CCA
CLAY	5	0	BF_CLAY, BF_CER, CCA
TC	5	0	BF_CLAY, BF_CER, CCA
PL	5	0	BF_CLAY, BF_CER, CCA
CER	5	0	BF_CLAY, BF_CER, CCA
BF_GLA	5	0	BF_CLAY, BF_CER, CCA
GL	5	0	BF_CLAY, BF_CER, CCA
BF_TC	5	0.2	BF_CLAY, BF_CER, CCA
BF_PL	5	0.8	BF_CLAY, BF_CER, CCA
BF_SD_CCA	5	1.2	BF_CLAY, BF_CER, CCA
BF_CLAY	5	6	SD_CCAx, CLAY, TC, PL, (
BF_GLA, GL			
			BF_TC, BF_PL, BF_SD_C(

Figure 12. Response to 1 + month biofilm.

Analysis of Variance Report

Page/Date/Time 1 8/14/2004 10:58:21 PM
Database E:\antibioticdata.S0
Response CCA,CHL,MIX,NAL,PEN,SD_CCA,STR

Box Plot Section



Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	P
	(Alpha=0.05)					
A (...)	6	522.6857	87.11429	14.87	0.000000*	1.
S(A)	28	164	5.857143			
Total (Adjusted)	34	686.6857				
Total	35					

* Term significant at alpha = 0.05

Tukey-Kramer Multiple-Comparison Test

Response: CCA,CHL,MIX,NAL,PEN,SD_CCA,STR
Term A:

Alpha=0.050 Error Term=S(A) DF=28 MSE=5.857143 Critical Value=4.4861

Group	Count	Mean	Different From Groups
SD_CCA	5	0	NAL, STR, CHL, PEN, CCA
MIX	5	1.2	NAL, STR, CHL, PEN, CCA
NAL	5	7	SD_CCA, MIX
STR	5	8.2	SD_CCA, MIX
CHL	5	8.4	SD_CCA, MIX
PEN	5	10	SD_CCA, MIX
CCA	5	10.4	SD_CCA, MIX

Notes:
This report provides multiple comparison tests for all pairwise differences between the means.