AN ABSTRACT OF THE THESIS OF Steven Victor for the Master of Science in Biology presented on January 28, 2002.

Title: The effect of copper on fertilization, larval development, settlement, and metamorphosis of the reef coral *Acropora surculosa.*

Approved:_____
Dr. Robert H. Richmond, Chairperson, Thesis Committee

This study demonstrates that copper is highly toxic to gametes and larvae, and disrupts larval settlement processes of *A. surculosa.* The duration of exposure to copper had a significant effect on the toxicity level to the early developmental stages of *A. surculosa.* Comparison of EC_{50} to that of other invertebrates suggests that coral gametes are as sensitive to copper as sea urchin, with bivalves showing higher sensitivity. Although the level at which copper inhibited fertilization and larval settlement is higher than the EPA recommended 24 hour average, the level at which fertilization and survival of embryos was inhibited are well within the recommended concentration for any given time. The method used in this study is effective in showing dose/response, is practical, and inexpensive to set up. The method chosen for the fertilization bioassay maybe more ecologically relevant than other methods, where gametes were first exposed to pollutant and then combined. The artificial substrate used in the recruitment bioassay provides a standardized substrate that could be used to test the effects of variety of pollutants on coral larval settlement. However, modification of the substrate to

provide a micro-habitat could potentially aid in increasing larval settlement. Further research is needed to determine long-term effects of copper and other potential pollutants on coral embryos and larvae at low concentration. It is suggested that corals be used as indicator species for testing a variety of pollutants that may potentially enter into the marine environment because of their sensitivity to pollutants and that they represent ecologically a majority of reef organisms.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

The members of the Committee approve the thesis of Steven Victor.

Dr. Robert H. Richmond, Chairperson

Dr. Valerie J. Paul, Member

Dr. Gary R.W. Denton, Member

Dr. Ross H. Miller, Member

ACCEPTED:

DR. JOYCE MARIE CAMACHO
Dean, Graduate School and Research

THE EFFECT OF COPPER ON FERTILIZATION, LARVAL DEVELOPMENT, SETTLEMENT AND METAMORPHOSIS OF THE REEF CORAL *ACROPORA SURCULOSA*

By

STEVEN VICTOR

A thesis submitted in partial fulfillment of the
requirements for the

MASTER OF SCIENCE

IN

BIOLOGY

UNIVERSITY OF GUAM

January, 2002

ACKNOWLEDGMENTS

The following people deserves many thanks for their assistance in the laboratory and collection of corals: Walter Kelly, Sarah T. Leota, Andrew Artero, Vera Peredo, Kayleen Bausoch, Victor Bonito, "Marine technicians: Frank Cushing, Butch Irish, and Chris Bassler".

I would like to thank Rick Wood and Lucrina Concepcion for their help in copper analysis.

A very "special thank you" to Marie Peredo and Angie Duenas.

I would have not been able to come this far without the help of my mom who have always encouraged me and for always believing in me. I would like to dedicate this thesis to my mom, ELCHESEL RECHIRIKL. Special thanks to Jade Kloulchad for being a part of my life.

Funding for this project was provided by the Minority Biomedical Research Support grant # 53-J-720458-R-5 (MBRS). Special thanks to the PALAU MERIT SCHOLARSHIP for financial assistance.

The Pacific Regional Aquaculture Service Information for Education (PRAISE) was very instrumental in providing much of the needed references that were not able on islands. I wish to acknowledge the staff of PRAISE, Krisa Anderson and Lois Ann Kiel.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGMENTS ii

LIST OF TABLES v

LIST OF FIGURES vi

INTRODUCTION 1

MATERIALS AND METHODS 7

Contaminant preparations 7

Spawning and gamete collection 8

Fertilization bioassay 10

Embryonic development bioassay 12

Copper analysis 12

Larval culture 13

Larval development bioassay 14

Settlement and metamorphosis assays 17

Preparation of natural substrate 17

Preparation of artificial substrate 17

Settlement and metamorphosis bioassay 17

Data analysis 17

RESULTS 20

Inhibition of fertilization 20

Effect of copper on embryo mortality 22

TABLE OF CONTENTS (CONTINUED)

PAGE

Effect of copper on larval survival 26

Inhibition of larval settlement and metamorphosis 30

DISCUSSION 37

Inhibition of fertilization 37

Fertilization and embryo development 40

Effect of copper on larvae 42

Larval settlement and metamorphosis 43

Conclusion and future research 45

LITERATURE CITED 48

LIST OF TABLES

PAGE

Table 1. Summary of some studies on the effects of heavy metals on selected marine organisms..... 2

Table 2. Effective concentration for various developmental stages of *A. surculosa*..... 20

Table 3. Comparison of EC_{50} for fertilization in some marine organisms..... 37

Table 4. Comparison of EC_{50} for fertilization in different coral species..... 38

LIST OF FIGURES

PAGE

Figure 1. Map of Guam showing collecting sites..... 9

Figure 2. Categories of eggs and embryos used to assess the fertilization bioassays..... 11

Figure 3. Two types of substrate used for settlement and metamorphosis bioassays..... 15

Figure 4. Categories of larvae..... 16

Figure 5. Effect of copper on fertilization rates (mean ± S.E.) on gametes from *A. surculosa* after 5 hour exposure..... 21

Figure 6. Effect of copper on embryo mortality (mean ± S.E.) after 12 hour exposure..... 23

Figure 7. Loss of copper (mean ± S.E.) over 12 hour period as determined by AAS..... 24

Figure 8. Comparison of fertilization rate by time and treatment ANCOVA ($p=0.199$)..... 25

Figure 9. Effect of copper on larval survival (mean ± S.E.) after 48 hour exposure..... 27

Figure 10. Recovery of larvae (mean ± S.E.) after exposure to copper..... 28

Figure 11. Number of larvae that settled and metamorphosed after 48 hours recovery period..... 29

Figure 12. Effect of copper on larval settlement and metamorphosis (mean ± S.E.) on natural substrate..... 31

Figure 13. Effect of copper treatment on larval settlement and metamorphosis (mean ± S.E.) on artificial substrate..... 32

Figure 14 A-C. Comparison of the number of recruits in different regions of the substrate (natural substrate by Friedman's test..... 34

Figure 15 A-C. Comparison of the number of recruits in different regions of the substrate (artificial substrate) by Friedman's test..... 35

Figure 16. Comparison of mean settlement and metamorphosis by substrate type using the Sheirer-Ray-Hare extension of the Kruskal-Wallis test ($p>0.05$)..... 36

INTRODUCTION

Coral reefs are important features of many tropical islands. Reefs have provided fisheries resources for subsistence as well as providing a valuable place for reinforcing family ties and values in many tropical island societies (Richmond, 1994). Much of the nutrition, welfare, culture, employment, and recreation for tropical islanders is based on the living resources associated with coral reefs (Gillet, 1999; Hawkins and Roberts, 1994).

Coral reefs are exposed to many anthropogenic stresses which have increased in impact and range over the past decade (Dubinsky and Stambler, 1996; Done, 1992). Coastal construction is common in many tropical islands to improve infrastructures and to attract more visitors. Coastal development sometimes has a greater negative impact on coral reefs than economic benefits (Maragos, 1993). Edinger et. al. (1998) and Yap (1992) examined some of the problems threatening coral reefs in developing regions. Land based pollution is a major problem that many tropical coral reefs experience regularly. Runoff carries a wide range of pollutants that can disrupt the life cycle of many reef organisms. Discharges from mining operations in Thailand, Malaysia, Indonesia, and the Carribean basin have been suggested as the most obvious potential threat to nearby coral reefs (Howard and Brown, 1984). Destructive land practices such as land clearing, deforestation and road grading also threaten coral reefs.

A common threat facing coral reefs that has attracted increasing attention is coastal pollution from heavy metals (e.g., Johannes, 1975; Bryan, 1976). Most studies on the effects of heavy metals on marine organisms have concentrated on the more toxic heavy metals such as mercury, copper, cadmium, nickel, and zinc (Table 1). These studies demonstrated the stressful

effects of heavy metals on marine organisms, including negative impacts on fertilization, embryonic development, feeding activity, survival and growth.

Table1. Summary of some studies on the effects of heavy metals on selected marine organisms.

species tested	metals(concentration)	effects/ (duration)	Author
gastropod *Biomphalaria glabrata*	Cd (0.075-0.25 µM) Pb (0.25 -100 µM) Hg (0.25 - 1 µM)	growth and survival (6 weeks)	Allah et. al., 1997
gastropod *Babylonia lutosa*	Cu (0.02 -0.20 ppm)	movement and burrowing activity (4 weeks)	Cheung and Wong, 1999
sea urchin *Echinometra mathaei*	Cu (7 µg/l)	embryonic development (2 days)	Coglianese and Martin, 1981
reef coral *Goniastrea aspera*	Cu (20 µg/l)	fertilization (5 hours)	Reichelt-Brushett and Harrison, 1999
reef coral *Acropora millepora*	Cu (.01-1000 µg/l)	fertilization (4hours) larval metamorphosis (24 hours)	Negri, A.P. and A.J. Heyward, 2001

Marine organisms may be susceptible to stressors resulting from anthropogenic sources. Therefore, evaluating how different types of stress affect the life cycle and dynamics of field populations of marine organisms is important for understanding the current decline of coral reef health. Studies on the effects of stress, i.e., sediment and turbidity (Houk, 1999), pollution (Brown and Howard, 1985), and sewage (Pastorok and Bilyard, 1985) on coral reefs have measured coral cover and diversity because these are easily quantified estimators of coral reefs

health. However, with the increasing threat of pollution from heavy metals (Brown, 1987; Guzman and Jimenez, 1992) and the continued decline of coral cover, there is increasing concern about the early developmental stages of marine organisms which are typically more sensitive than the adult stage. Survival of the early developmental stages of coral may be an important indicator of stress because they have a direct bearing on future field populations. Toxicity is a useful tool for evaluating the effects of pollutants on these early developmental stages (Peters et. al., 1997).

There is little published information on the toxicity of pollutants to adult corals and even less information is available on early developmental stages. Most studies on the effects of pollutants on the early developmental stages of corals were evaluated on brooding corals because of the ease of obtaining their larvae. Edmonson (1946) examined the effects of freshwater (reduced salinity) on planula larvae of *Pocillopora damicornis*, *Cyphastrea ocellina*, and *Dendrophyllia manni* and found that exposure to a dilution of 1 part seawater- 3 parts freshwater caused cessation of movement in 15-20 minutes. Furthermore, larvae did not recover when returned to normal salinity.

Others have examined the effects of the most toxic trace metals on survival and recruitment of planula larvae. Goh (1991) found that survival and recruitment of planula larvae of *P. damicornis* were significantly affected by exposure to 1000 µg/l of nickel. Esquivel (1986) examined the effects of copper on the larvae of *P. damicornis* and found the EC_{50} to be 120, 115, 90, and 63 µg/l at 12, 24, 48, and 96 hours exposures, respectively. These studies were limited to the effects of pollutants on survival and recruitment of planula larvae and did not

consider the effects on fertilization. This was largely due to a limited understanding of coral reproduction and how to obtain viable coral gametes for experiments.

However, with increasing knowledge of coral reproduction and the discovery that many scleractinian corals release their gametes into the water column during limited periods annually (Heyward, 1988; Richmond and Hunter, 1990) it is now possible to perform toxicity tests of pollutants on coral gametes using laboratory based bioassays. Toxicity tests have often been used to determine the effects of pollutants on organisms and are generally performed on indicator species within a community (Peters et. al., 1997). Scleractinian corals are dominant organisms and key components of coral reef ecosystems, and therefore are useful organisms in determining the dose-response effect of potential pollutants to marine environments.

Most scleractinian corals release gametes into the water column, where external fertilization occurs. Fertilization occurs minutes to hours after the gametes are released. Fertilization will therefore be greatly affected by water quality. Richmond (1993) found that reproductive success in *A. digitifera* was affected by coastal runoff. He found a 38% decrease in fertilization success in salinity of 28.5 ppt and lateritic suspended solids of 1.28 g/l. He also found that reducing salinity to 28 ppt inhibited fertilization rate to 25% compared to 88% at 34 ppt salinity. Others have demonstrated that the presence of pollutants, such as petroleum-based products and heavy metals can inhibit fertilization and recruitment in spawning corals (Heyward, 1988; Reichelt-Brushet and Harrison, 1999; Negri and Heyward, 2000; 2001).

A variety of pollutants have been tested against marine organisms. Copper has received considerable research attention. Copper is an essential element to all living things but has been found to be toxic to many marine organisms (Heslinga, 1976; Coglianese and Martin, 1981;

Mance, 1987; Ringwood, 1992). Still, there is little information available on the toxicity of copper to corals. The effect of copper on corals is of environmental concern because there are numerous sources of copper to coral reefs. Copper is a major component of antifouling paints (Claisse and Alzieu, 1993), is found in sewer discharges (Pastorok and Bilyard, 1985), is a component of fungicides and herbicides that are used on coastal agricultural crops (Cremlyn, 1979), is used to treat wood used as construction materials for coastal waterfront structures (Brown and Eaton, 2001), and is used in heat exchangers in power plants (Stupnisek-Lisak et. al., 1998). The concentration of copper in pristine marine environments is between 0.01-0.03 µg/l (Sadiq, 1992). Given its wide use, copper poses a potential threat to marine organisms (Fang & Hong, 1999; Mance, 1987; Schmidt, 1978; Brown, 1987). Therefore, evaluating copper's effect on corals is of interest to reef management and preservation.

Heyward (1988) studied the effects of copper and zinc sulphate on fertilization rates in *Favites chinensis* and *Plagygyra ryukyuensis.* He found that a nominal concentration of 100 µg/l of copper reduced fertilization success in *F. chinensis* to less than 50%, but had no inhibitory effect on *P. ryukyuensis* fertilization. Reichelt-Brushett and Harrison (1999) found that a nominal copper concentration of 20 µg/l reduced fertilization in *Goniastrea aspera* by 41% and concentration of 200 µg/l inhibited fertilization by 99%. A similar trend was observed in a bioassay with gametes from *Acropora millepora.* It was found that copper inhibited fertilization with an EC_{50} (concentration at which fertilization is inhibited to 50%) of 17.4 µg/l (Negri and Heyward, 2001). These studies demonstrated that copper can have an inhibitory effect on fertilization rates in a few species of spawning corals. Further investigation is needed to evaluate the effects on other important reef - building spawning corals.

Since spawning periods in reef corals coincide with heavy rainfall in the tropics, it is important to evaluate how coastal runoff, which may carry a variety of pollutants including copper, can affect coral reproduction and recruitment. Most published toxicity studies in spawning corals have been evaluated on massive corals, such as *Platygyra ryukyuensis* and *Favites chinensis* (Heyward, 1988), *Goniastrea aspera* (Reichelt-Brusshett and Harrison, 1999), and *Goniastrea retiformis* (Leota, 2000). Few published studies (e.g., Richmond, 1993 for *A. digitifera*; Reichelt-Brushet and Harrison, 1999; Negri and Heyward, 2001 for *A. millepora*) have evaluated the effects of pollutants on branching *Acropora*, which include many of the major reef building coral species. The present study set out to address the effects of copper on fertilization and larval development, settlement, and metamorphosis in *Acropora surculosa.* These early developmental stages may be good indicators of the effects of pollutants on corals because they represent critical links in the life cycle of corals.

A. surculosa is found primarily at shallow depths on coral reefs throughout the Pacific. On Guam, this species inhabits reef areas close to shore where the risk of exposure to pollutants is high. It is therefore a good indicator species for evaluating the effects of pollutants on fertilization, larval development, settlement and metamorphosis in corals.

This study address the following questions: (1) Will copper affects fertilization rate when gametes are exposed to copper for 5 hours?, (2) Will embryogenesis be affected when gametes are exposed for 12 hours?, (3) Will exposure to copper for 48 hours affect larval survival, and will the larvae recover and be able to settle and metamorphose? (4) Will concurrent exposure of larvae and substrate affect the settlement and metamorphosis process?

MATERIALS AND METHODS

Deionized water was obtained from the Water and Environmental Research Institute (WERI) of the University of Guam. Seawater used for bioassays was filtered using 0.45 µm cellulose nitrate filters. All glassware used was pre-cleaned by soaking in 10% nitric acid overnight, washed with Alconox detergent, rinsed in running tap water, and then rinsing with deionized water prior to use.

All chemicals used in this study were reagent grade. The chemical form of copper that was used to make the copper stock solution was $CuSO_4 + 7\ H_2O$. Nitric acid (HNO_3) that was used as preservative (adjusting pH) was a metal analysis grade. Copper was extracted from seawater by first chelating it using pyrrolidinedithiocarbamate ammonium salt (APDC). APDC binds to dissolved copper in solution and allows it to be extracted by organic solvent. Methyl isobutyl ketone (MIBK) was used as a solvent for extracting copper from seawater. It was also used as a solvent for making copper standards for calibrating the flame atomic absorption spectrometry (AAS) (Denton, WERI, UOG pers. comm).

Contaminant preparations

A stock solution of copper (4 mg/l Cu = 15 mg $CuSO_4$ in 1L of deionized H_20) was prepared and analyzed by AAS against calibration standards to determine the exact concentration. The measured concentration was 3.98 mg/l. One ml of HNO_3 was added to the solution as a preservative. The range of copper concentrations chosen for the experiment was based on Reichelt-Brushett and Harrison (1999). The following concentrations were used: 10 ppb, 25 ppb, 50 ppb, 75 ppb, 100 ppb, and 200 ppb (ppb = µg/l). These copper

concentrations were made by adding an appropriate amount of copper from the copper stock and deionized water into a 1L volumetric flask and adding filtered seawater (FSW) to volume. The addition of appropriate amounts of deionized water to each desired concentration resulted in a 5% reduction in salinity from a normal salinity of 35 ppt. Deionized water was added to achieve the same salinity in all treatments because the addition of the appropriate amount from copper stock to make the highest concentration of solution (200 µg/l) resulted in 5% reduction in salinity. The control had a 5% reduction in salinity was well. FSW with no addition of copper was used as a reference treatment to test for the possible effect of reduced salinity on the developmental stages. In this bioassay, "concentration" refers to the nominal concentration of copper unless stated otherwise. The actual concentration of copper was determined only for the 12 hour exposure bioassay due to time constraints and limited glassware for keeping water samples.

Spawning and gamete collection

Experiments were conducted at the University of Guam Marine Laboratory. On Guam the mass spawning period of reef corals occurs among the 5th and the 10th nights after each monthly full moon from June through August (Richmond and Hunter, 1990). Gravid colonies of *A. surculosa* were collected at least one week before the predicted coral spawning periods in June and July, from Double Reef, Shark's Hole, Pago Bay, and Janum Point (Figure 1). To make sure that gravid colonies were collected, a branch of each colony was broken-off and examined for the presence of colored eggs. Colonies were maintained in aerated flow-through seawater tanks in the laboratory.

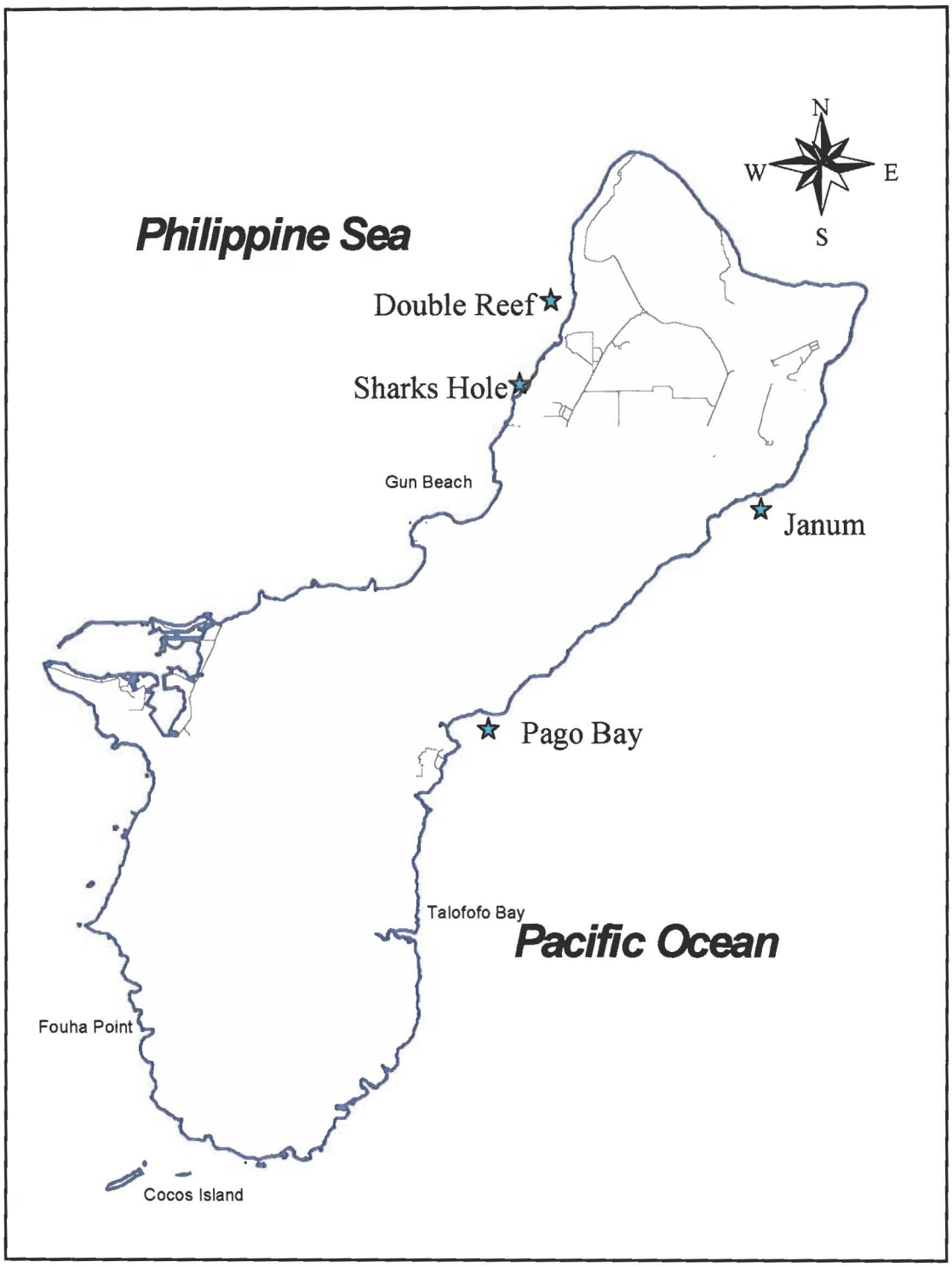


Figure 1. Map of Guam showing collecting sites.

Before a coral releases its gametes (eggs and sperm clusters), they become visible on the polyp within the corallite which is an indication that the coral is ready to spawn its gametes. This usually happens 15 - 30 minutes before a coral releases its gametes. When gametes are visible, each colony was isolated in a 14 l plastic tub filled with unfiltered seawater. After gametes were released, they were collected by gentle suction from the water surface using a 5 ml wide mouth plastic pipette (Leota, 2000).

Fertilization bioassay

Acropora surculosa is a simultaneous hermaphrodite, with little or no evidence of self-fertilization. To maximize the sensitivity of the assays, two colonies were selected for experiments. Both colonies provided eggs and sperm. Fertilization assays were carried out in 50 ml glass jars with screw on lids. Each jar contained 30 ml of the desired concentration of copper solution and 16 egg-sperm clusters (8 from each colony). Six replicate jars were used for each treatment and for the control. Jars were covered with lids and gently agitated by hand every hour for the first 3 hours to break apart the clusters.

Bioassays were performed at an ambient air temperature of ~28 °C for 5 hours to allow for maximum fertilization and early cell cleavage (Heyward, 1988). Development of fertilized eggs was terminated by the addition of 1 ml of a fixative (10 g/l sodium β-glycerophosphate, 4% formaldehyde buffered at pH 7), which maintained embryo integrity (Negri and Heyward, 2001). The eggs and embryos were assessed for fertilization the following morning under a dissecting microscope. The categories of unfertilized eggs and fertilized eggs (Figure 2 A & B) were used to assess the bioassay.

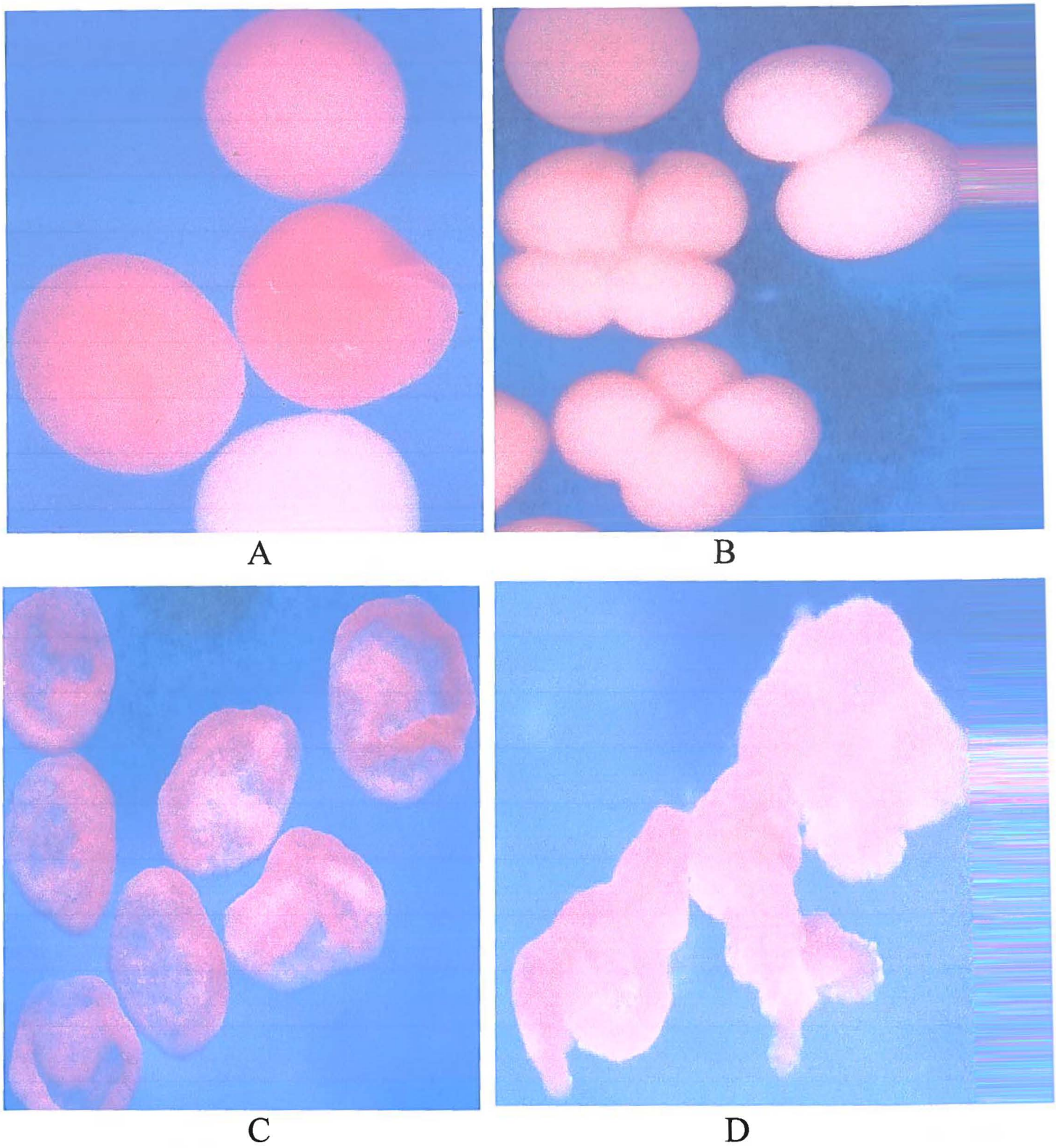


Figure 2: Categories of eggs and embryos used to assess the fertilization bioassays.
(A) unfertilized eggs, (B) fertilized eggs (3 hours) 2 & 4 cell division, (C) embryos at the blastula stage (12 hours after eggs/sperm were combined, and (D) dead embryo (12 hrs).

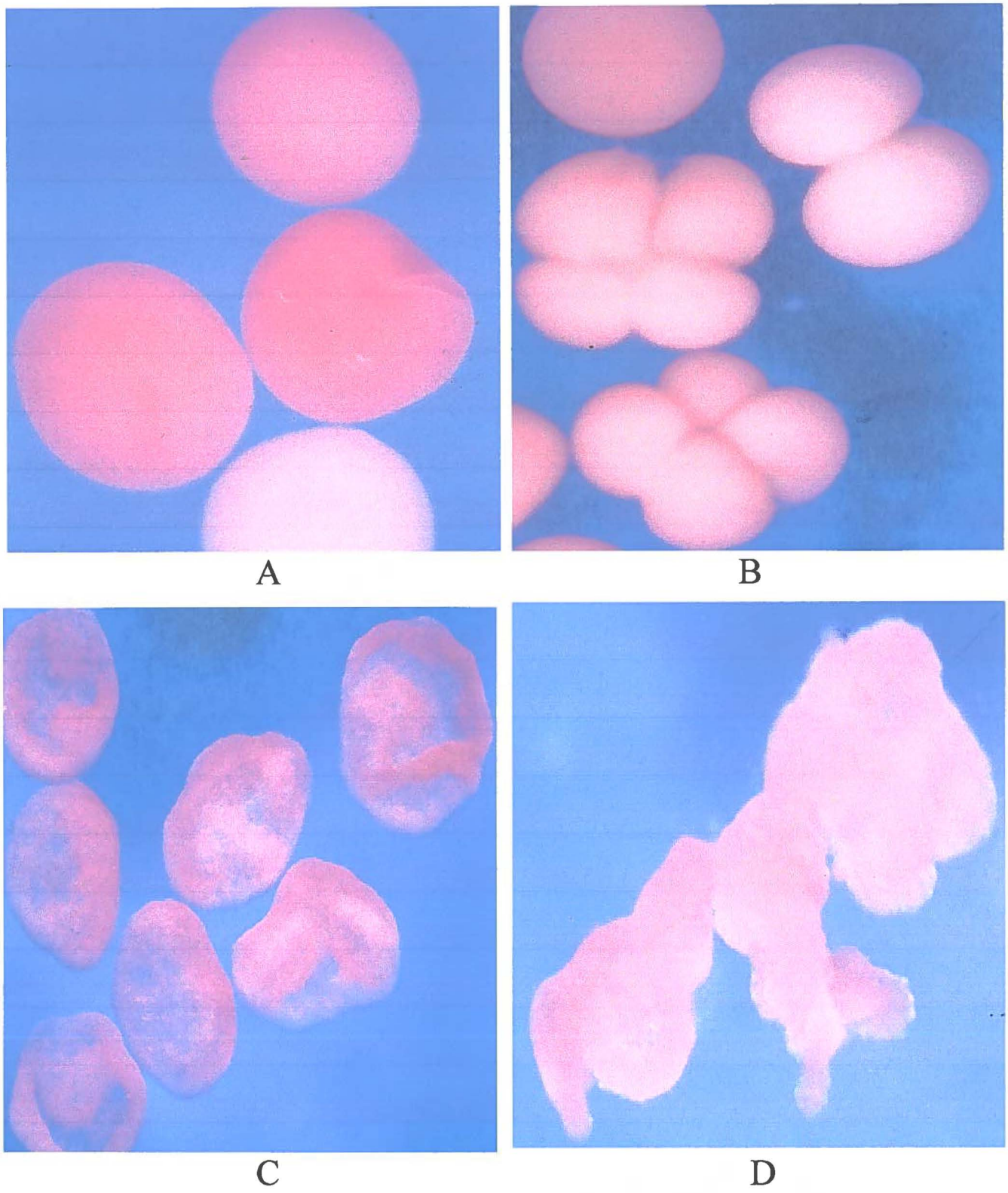


Figure 2: Categories of eggs and embryos used to assess the fertilization bioassays.
(A) unfertilized eggs, (B) fertilized eggs (3 hours) 2 & 4 cell division, (C) embryos at the blastula stage (12 hours after eggs/sperm were combined, and (D) dead embryo (12 hrs).

Embryonic development bioassay

Fertilization experiments for estimating the effects of copper on embryonic development were carried out in 250 ml beakers. Gametes were exposed to the different copper solutions and allowed to fertilize and undergo development for 12 hours. Embryonic development was assessed in terms of the survivorship of embryos after 12 hours of exposure. Survivorship was defined in terms of viable embryos. Total mortality was defined as unfertilized eggs and dead embryos. Six replicate beakers were used for each treatment. Each beaker contained 200 ml of the desired test solution and 30 egg-sperm clusters (15 clusters from each of the two colonies used). Each replicate beaker was covered with parafilm and was maintained at an ambient air temperature of ~ 28°C for 12 hours. After 12 hours, embryonic development was terminated by the addition of 3 ml fixative. The bioassay was scored immediately under a dissecting microscope. The following categories of eggs and embryos were used to score the bioassay: unfertilized eggs (Figure 2A), viable embryos (Figure 2C), and dead embryos (Figure2D).

Copper analysis

Twenty ml of the test solution in each replicate beaker was pipetted into a 25 ml volumetric flask before gametes were put into the beakers. Four replicates of each treatment were sampled for copper analysis. The samples were preserved for later analysis by adding 4.8 µl HNO_3 and refrigerated. This procedure was repeated 12 hours later. Water samples were taken before the addition of the fixative.

Copper was extracted into an organic solvent for analysis of copper in the seawater samples. Copper concentration was determined by AAS following solvent extraction. Briefly,

20 ml of the test solution were extracted with 0.5 ml of 3% APDC and 2 ml of MIBK in a 25 ml volumetric flask as previously described (Denton, WERI, UOG pers. com). After addition of the reagents, the volumetric flask was touched to a vortex mixer for 3 minutes to complete the extraction. Extraction of copper from seawater into 2 ml of solvent concentrated the copper concentration by ten times.

Deionized water was added to each flask to bring the solvent into the neck of the flask after mixing for easier aspiration by AAS. The samples were left for 12 hours to maximize extraction. After 12 hours, the organic top layer of the sample was aspirated and analyzed by AAS.

Since copper was extracted into an organic solvent, the AAS had to be calibrated with organic calibrating standards. A commercially available stock solution of copper in mineral oil was used to prepare the following calibration standards: 0.25 ppm, 0.5 ppm, 1 ppm, and 2.5 ppm (ppm = mg/l) in MIBK in a 100 ml volumetric flask. MIBK with no addition of copper was used as a blank.

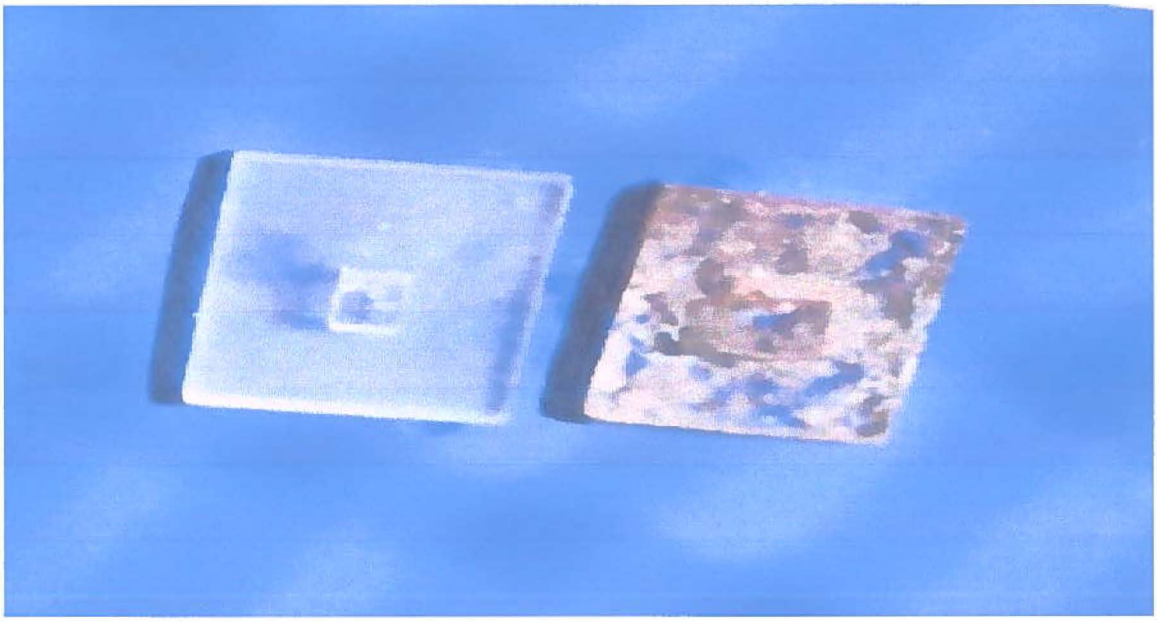
Larval culture

Following spawning, gametes were collected and mixed in 14 l tubs filled with UV sterilized seawater to enable cross-fertilization. The seawater in the tubs was aerated to allow for good mixture of eggs and sperm and covered with aluminum foil to prevent evaporation. Semi- continuous water changes in the cultures were done every day for 5 days until the larvae were competent to settle. These cultures provided larvae for recruitment bioassays.

Larval development bioassay

The larval development bioassay was performed in a 250 ml beaker. Six replicate beakers were used for each concentration to be tested. Each replicate beaker contained 200 ml of the desired test solution and 50 embryos that were at the blastula stage (approximately 18 hours post-fertilization). The bioassay was kept outside at an ambient air temperature of 28 °C during the night and 30 °C during the day for 24 hours. After 24 hours, the embryos had developed into ciliated larvae. Healthy larvae were counted initially under a dissecting microscope. The bioassay was left for another 24 hours, for a total of 48 hour exposure to copper, and then reassessed. Healthy embryos were counted in each replicate beaker by visual examination only. Healthy larvae were transferred to filtered seawater. Larvae in clean seawater were maintained outside at ambient air temperature for another 48 hours. By then the larvae were five days old and were competent for settlement. The number of larvae in each replicate beaker was counted and the seawater changed. The larvae were then given a suitable substrate (Figure 3A) for settlement. The bioassay was again maintained outside at ambient air temperature for 48 hours. Forty-eight hours after the addition of a substrate was chosen as a timely endpoint for metamorphosis. Previous observations indicated that 48 hours after larvae were given a suitable substrate, any would-be settlers had settled and metamorphosed in laboratory bioassays (S. Victor pers.obs). At this time larvae had attached and primary polyps developed (Figure 4 D). The bioassay was scored by counting the settled and metamorphosed larvae under a dissecting microscope.

A



B

Figure 3: Two types of substrate used for settlement and metamorphosis bioassays. (A) Natural substrate (the quarter is used for scale) and (B) artificial substrate (4.5 cm x 0.75 cm x 6 cm). On the left is the substrate before it was soaked in seawater and on the right is after 3 weeks in seawater.

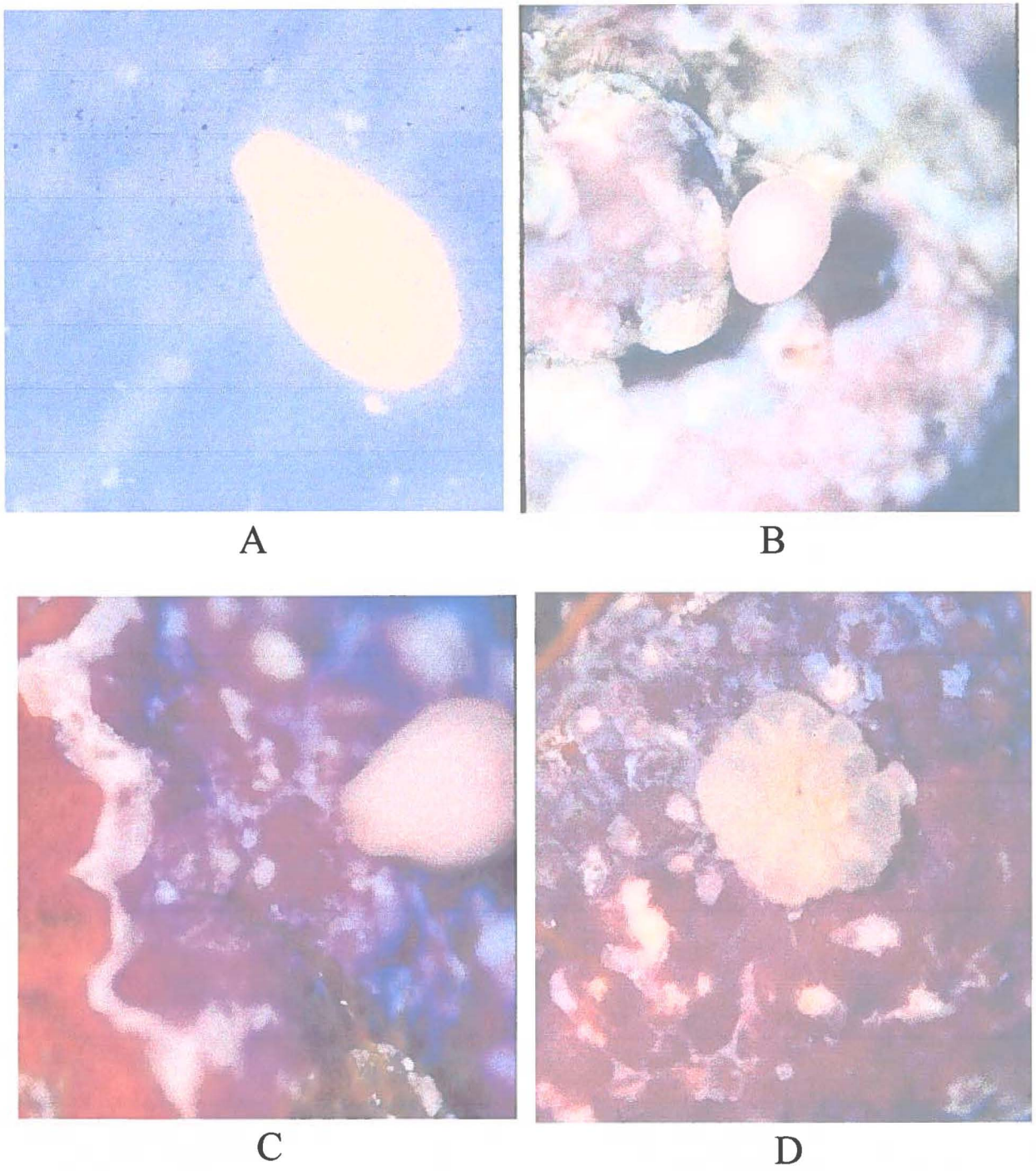


Figure 4. Categories of larvae. (A) free- swimming, (B) settled but not attached, (C) settled and attached to the substrate, and (D) recruit.

Settlement and metamorphosis assays

Preparation of natural substrate - Pieces of rubble were collected off the Pago Bay Reef flat in eastern Guam (Figure 1). Rubble pieces were of varying sizes. However, only rubble of almost the same size were used (Figure 3 A). Rubble pieces were covered with crustose coralline algae. Prior to use in the bioassay, rubble was cleaned by brushing off sediments and epiphytes with a toothbrush.

Preparation of artificial substrate - Plexiglass recruitment tiles (Figure 3 B) were prepared prior to spawning and soaked in a seawater tank with flowing aerated seawater. Tiles were soaked for approximately three weeks until they were used. After three weeks, 30%-50% of each tile was covered with crustose coralline algae.

Settlement and metamorphosis bioassay- The experiments were carried out in 250 ml beakers. Eight replicates were used for each treatment. Each replicate beaker contained 200 ml of test solution, 30 larvae, and the artificial or the natural substrate. A bioassay was carried out first with 4-day old larvae using natural substrate. The next day, a bioassay with 5-day old larvae using artificial substrate was conducted. The bioassay was maintained outside at ambient air temperature for 48 hours. The bioassay was scored based on the following: free-swimming larvae (Figure 4A), settled but not metamorphosed (Figure 4B & C), and settled and metamorphosed (Figure 4D).

Data analysis

The numbers of fertilized eggs, viable embryos, mortality (unfertilized eggs + dead embryos) and settlement and metamorphosis were converted to percentages. Fertilization and mortality data was analyzed using 1-way ANOVA. Before analysis, they were arcsine

transformed and analysis was run using Number Crunching Statistical Software (NCSS, 2000). Transformation of the data was necessary to make the data fit assumptions of ANOVA. Post-hoc tests using Tukey-Kramer were carried out to examine differences between means. Comparison of fertilization rate for 5 hours vs. 12 hours among treatments was carried out using analysis of covariance (ANCOVA). T-test between the means for 5 hours and 12 hours within each treatment was carried out after ANCOVA.

Since in the larval development assay, the same individuals were measured over time it would have been appropriate to use a repeated measure ANOVA on the data if it had met the assumption of constant variance. It did not, so a non-parametric equivalent of 1-way ANOVA was used. Since the data were collected at three different times during the experiment, they were separated with respect to the time they were collected and analyzed. The data were separated based on the following: 1. Exposure for 48 hours, 2. recovery period, and 3. settlement and metamorphosis. Each part of the data was analyzed using a Kruskal-Wallis test. Post-hoc tests using the Kruskal-Wallis Z-test were carried out to examine difference between means.

Total number of settled and metamorphosed larvae for the bioassays with natural and artificial substrates were analyzed using a Kruskal-Wallis test. Multiple comparison among means were carried out using Kruskal Wallis Z-test. Friedman's test was used to test for the effect of treatment and the different regions on substrate on settlement and metamorphosis. Kruskal Wallis Z-test was used to examine differences among percent settlements on the different regions. The Sheirer-Ray-Hare Extension of the Kruskal-Wallis test was used to test for interaction between the two types of substrate on settlement and metamorphosis. The test

rank the data and then analysis of Two-Way ANOVA is carried out (Sokal and Rohlf, 1995).

Mean and standard error (SE) values were calculated for each data set and plotted. Effective concentration at which 50% of the test subjects are affected (EC_{50}) for fertilization, embryonic, and larval development were estimated using probit analysis (NCSS 2000).

RESULTS

Inhibition of fertilization

Fertilization rates after 5 hours of exposure was 98% in FSW and 99% in the control (Figure 5). There was no significant difference in mean fertilization rate between the control and FSW suggesting that a 5% reduction in salinity did not have any effect on gametes. There was a significant effect of copper treatment on mean fertilization rates (Figure 5). Copper inhibited mean fertilization to 10% at the lowest copper concentration (10 µg/l) tested. This was significantly different from the control and FSW (Figure 5). Fertilization rate was inhibited to 20% and 10% in 100 µg/l and 200 µg/l, respectively. There was no significant difference in mean fertilization rate between the two highest concentrations of copper. Copper inhibited fertilization in 50% of *A. surculosa* gametes relative to the control at a nominal concentration of 38.4 µg/l (Table 2).

Table 2. Effective concentration for various developmental stages of *A. surculosa.*

EC_{50} (µg/l)	95% Conf. limits	Stage	Time (hours)
38.4	32 - 43	gametes	5
12.9 *	11.3 - 14.5	gametes	12
54.5	44.6 - 67.2	larvae	24
30.0	23.9 - 36.4	larvae	48

* = measured concentration.

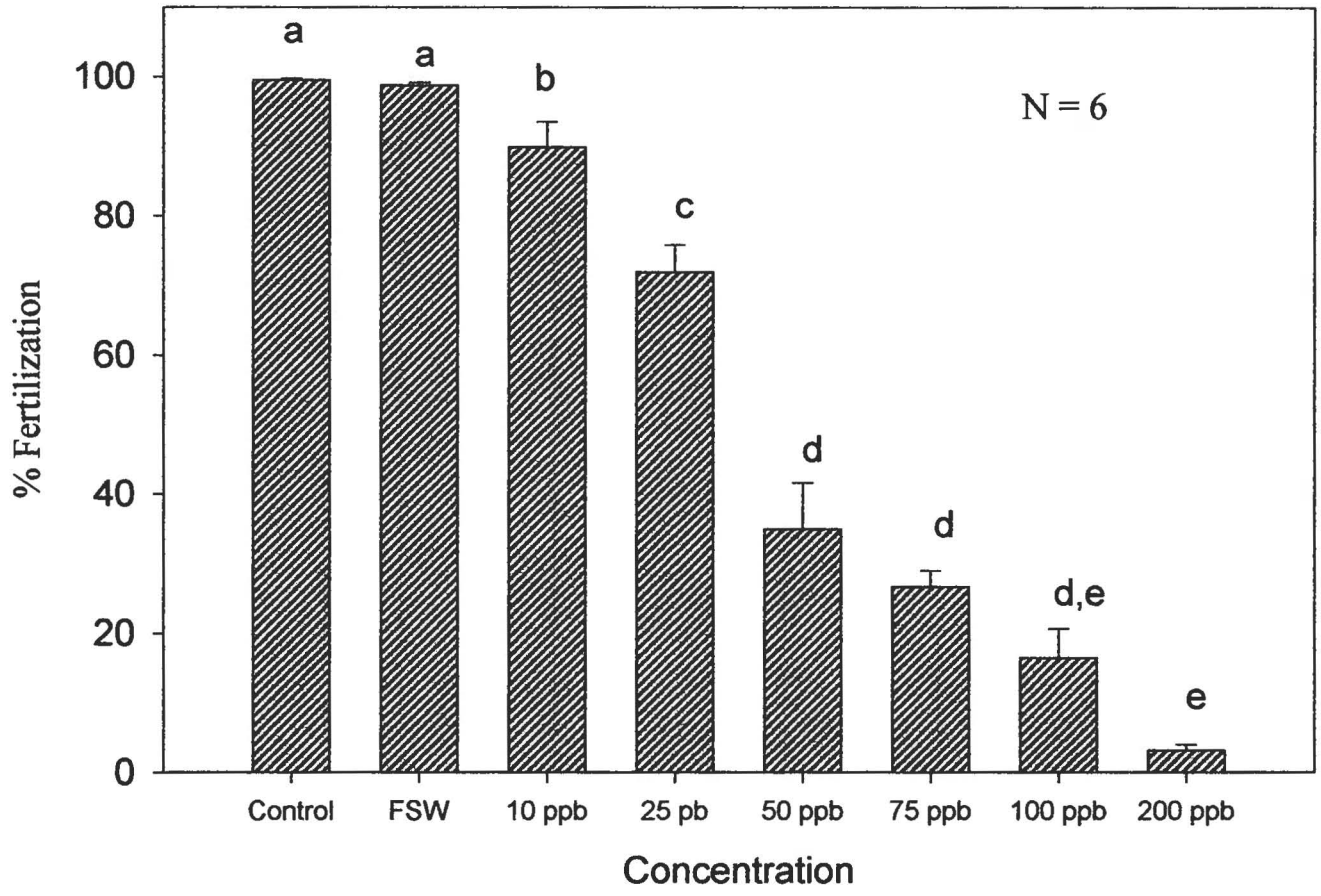


Figure 5. Effect of copper on fertilization rates (mean ± S.E.) on gametes from *A. surculosa* after 5 hour exposure. Significant treatment effect by ANOVA ($p<0.001$). Letters above each bar show significant groupings by Tukey-Kramer ($p<0.05$). N is the number of replicate per treatment.

Effect of copper on embryo mortality

There was less than 40% embryo mortality after 12 hours of gamete exposure in the control and in FSW (Figure 6). No significant difference on mortality was detected between the control and FSW. After 12 hours embryos had reach the blastula stage (Figure 2C). Embryos that were affected by copper had started to degenerate after 12 hours (Figure 2D). There was a significant effect of copper treatments on mortality after 12 hours of exposure (Figure 6). It should be noted here that mortality was estimated from the number of unfertilized eggs and dead embryos that were fertilized after the first 5 hours of gamete exposure to copper solution. Inhibition of fertilization correlates to high mortality because there were no embryos to begin with. So the observed effect of copper on mortality may be exaggerated due to inhibition of fertilization. Copper killed 50% of gametes and embryos that were fertilized at the beginning of gametes' exposure to copper at a measured concentration of 12.96 µg/l (Table 2).

The measured copper concentration in each treatment was between 10-15% more than the calculated value based on the copper stock solution (Figure 7). After 12 hours, there was about 52-65% recovery of copper in all concentrations, except 10 ppb, where 90% of dissolved copper was recovered (Figure 7).

The fertilization rate estimated for the 12 hours exposure was not significantly different from fertilization rate for the 5 hours exposure of gametes, except for the mean fertilization rates for the 200 µg/l treatment (Figure 8). However, none of the fertilized eggs in the 12 hour exposure bioassay developed into embryos.

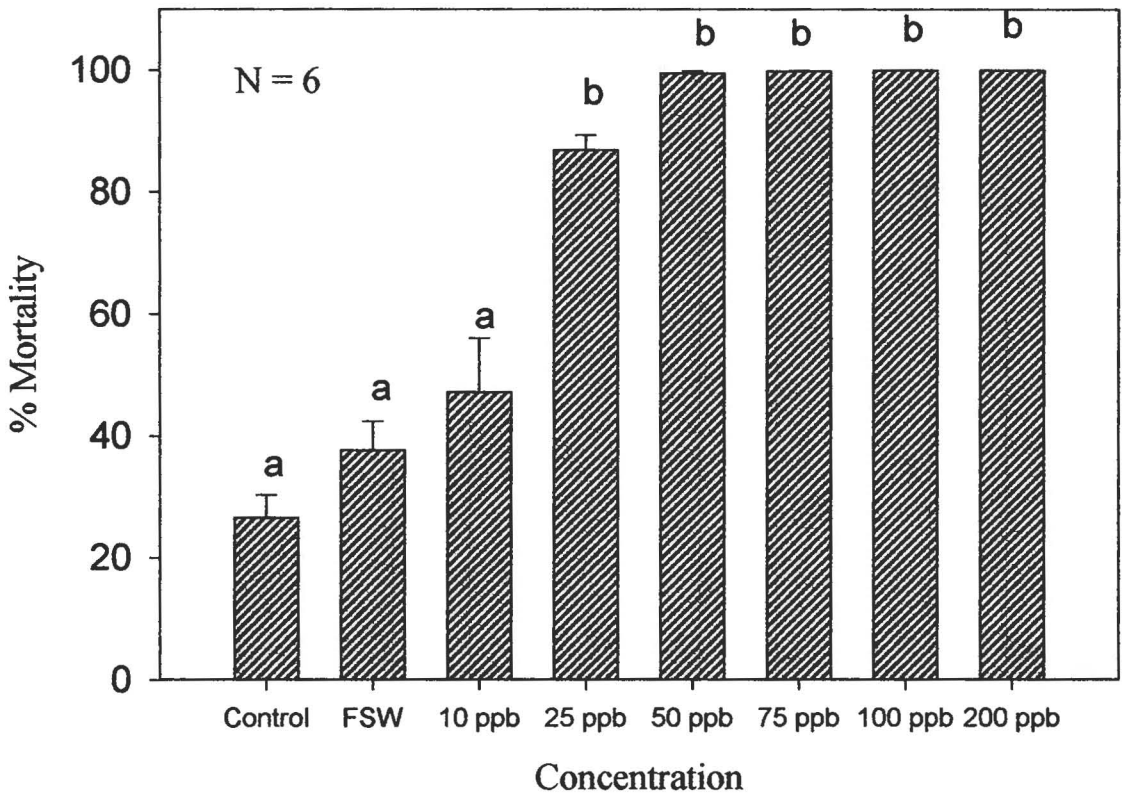


Figure 6. Effect of copper on embryo mortality (mean ± S.E.) after 12 hour exposure. Significant treatment effect by ANOVA ($p<0.001$). Letters above each bar show significant groupings by Tukey-Kramer ($p<0.05$). N is the number of replicates per treatment.

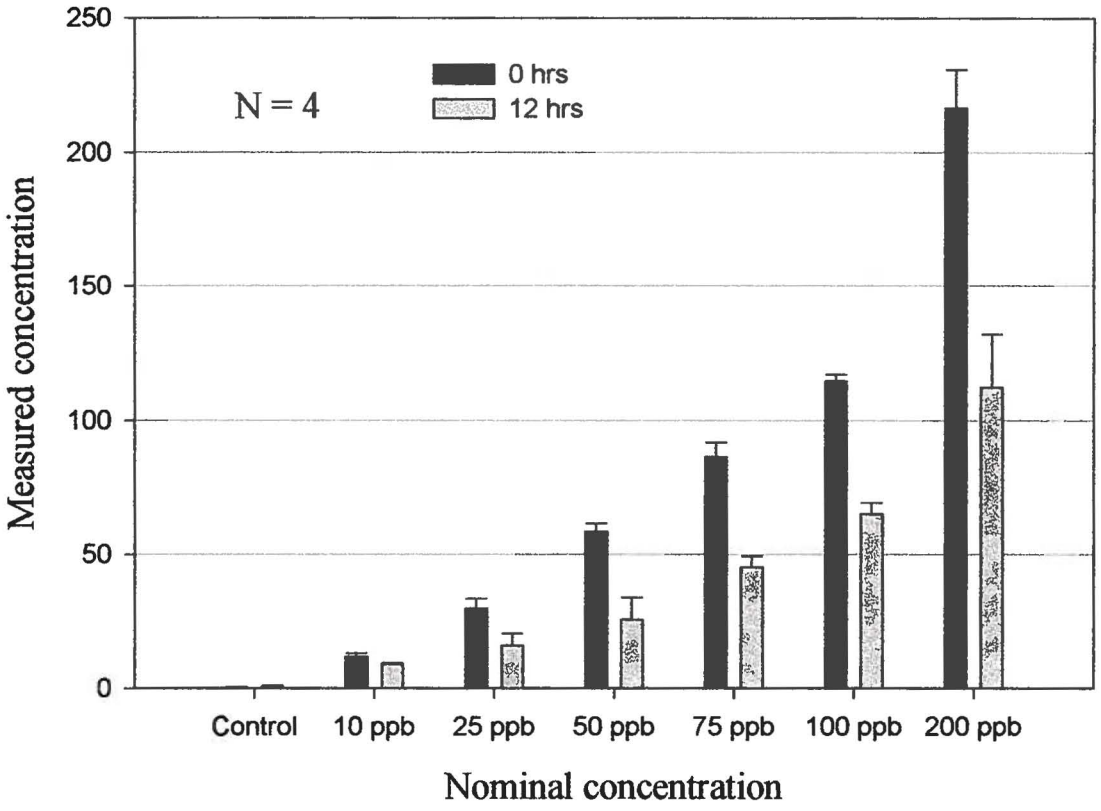


Figure 7. Loss of copper (mean ± S.E.) over 12 hour period as determined by AAS. N is the number of samples analyzed per treatment.

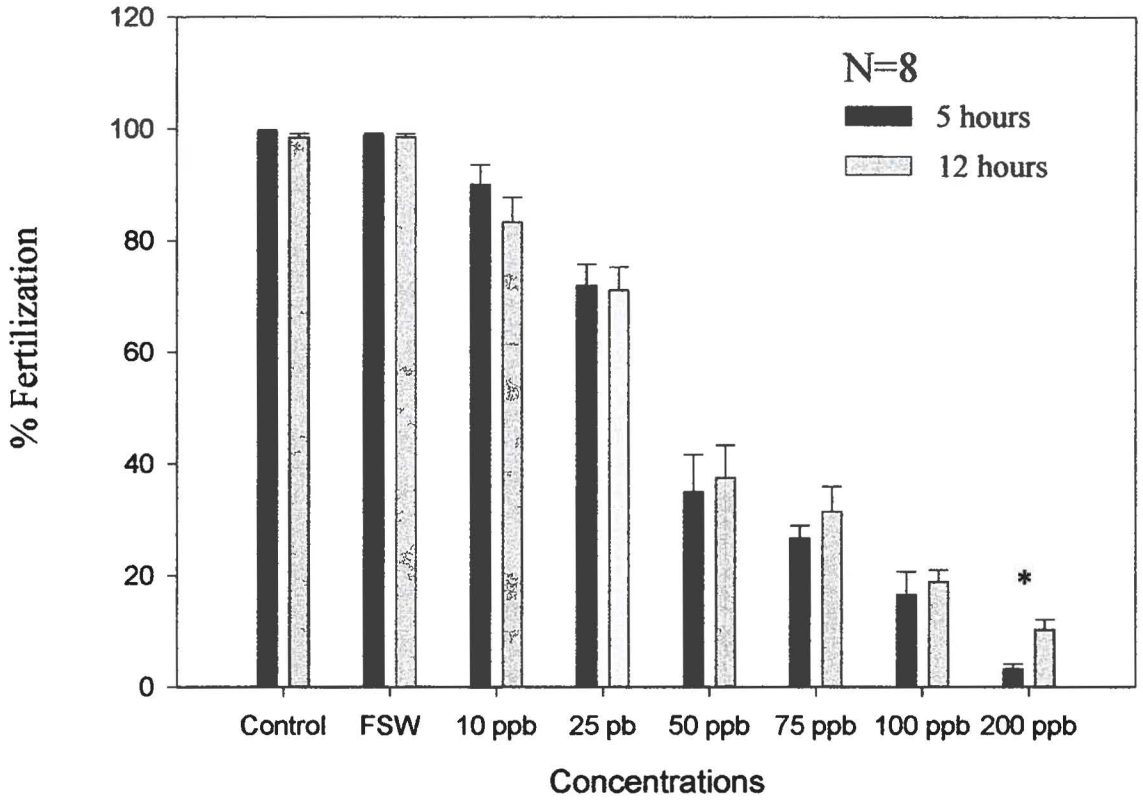


Figure 8: Comparison of fertilization rate by time and treatment ANCOVA ($p=0.199$).
* indicate significant difference on fertilization rate by time by t-test ($p<0.05$).
N is the number of replicate per treatment.

Effect of copper on larval survival

In many species of *Acropora*, the embryo becomes a ciliated larva 24 hours after fertilization (Babcock, and Heyward, 1986). In this bioassay, 12 hour old embryos (blastula stage) were exposed to copper treatments. Embryos in the 200 µg/l copper treatment all died within the first 24 hours of exposure. However, in all the other treatments embryos developed into ciliated larvae.

There were 86% and 78% survival rate in the control and FSW, respectively after the first 24 hours. There was statistically significant treatment effect on the development of the embryos into larvae (Figure 9). There was no significant difference of copper treatment at 10 µg/l and 25 µg/l on larval development compared to the control. Copper at 50 µg/l significantly decreased survival of embryos during exposure. Less than 10% of the embryos successfully developed into the ciliated larval stage in 75 µg/l and 100 µg/l (Figure 9). The observed effects of copper were (1) abnormal development of larvae (2) larvae were not ciliated compared to the healthy ones, (3) contraction, and (4) dead. The EC_{50} for 24 hour and 48 hour exposure to copper was 54.5 µg/l and 30.0 µg/l, respectively (Table 2).

Larvae that survived 48 hours of exposure to copper were returned to normal seawater for another 48 hour to estimate their recovery rate. There were 80% and 70% recovery of larvae in the control and in FSW, respectively (Figure 10). There was significant effect of treatment on the recovery of larvae. Recovery rates for all copper treatments above 10 µg/l were 50% - 66%. There was 85% recovery in 10 µg/l. The recovery rates for larvae that were exposed to 75 µg/l and 100 µg/l was significantly different from the control (Figure 10).

After the 48 hour recovery period, larvae were given a substrate to recruit onto for

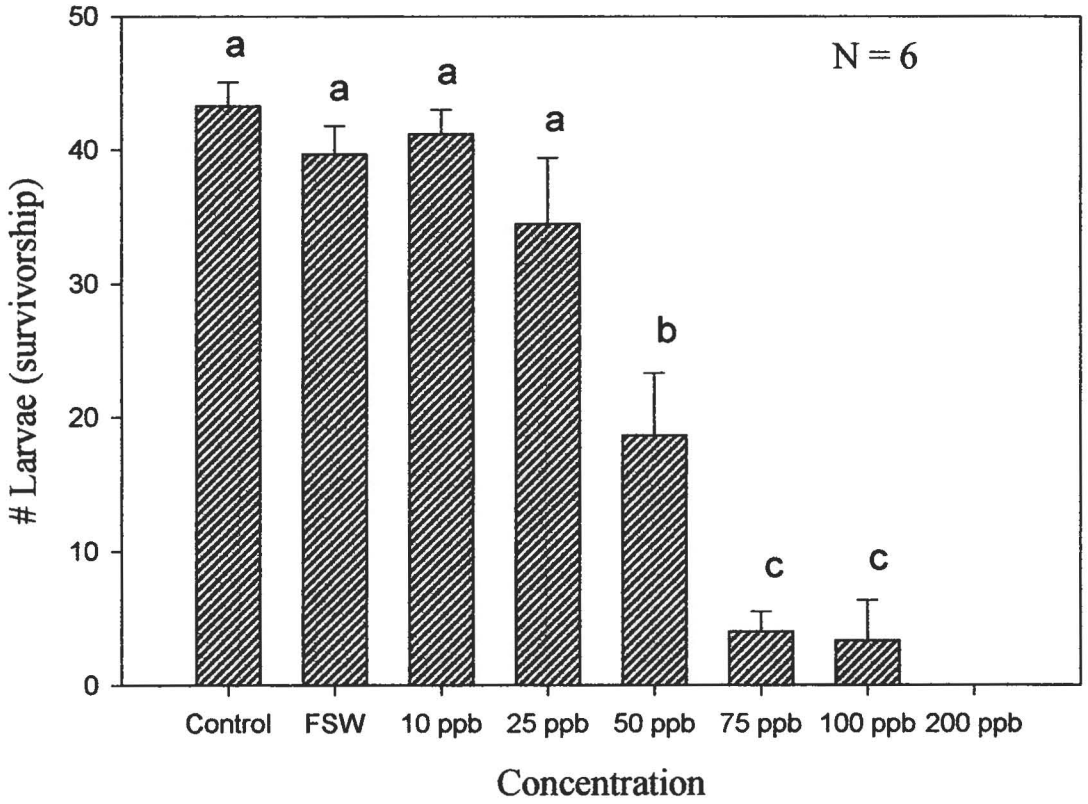


Figure 9 . Effect of copper on larval survival (mean ± S.E.) after 48 hour exposure. There were 50 larvae at the beginning of the bioassay. Significant treatment effect by Kruskal Wallis test ($p<0.001$). Letter above each bar show significant groupings by Kruskal Wallis Z-test ($p<0.05$). N is the number of replicates per treatment.

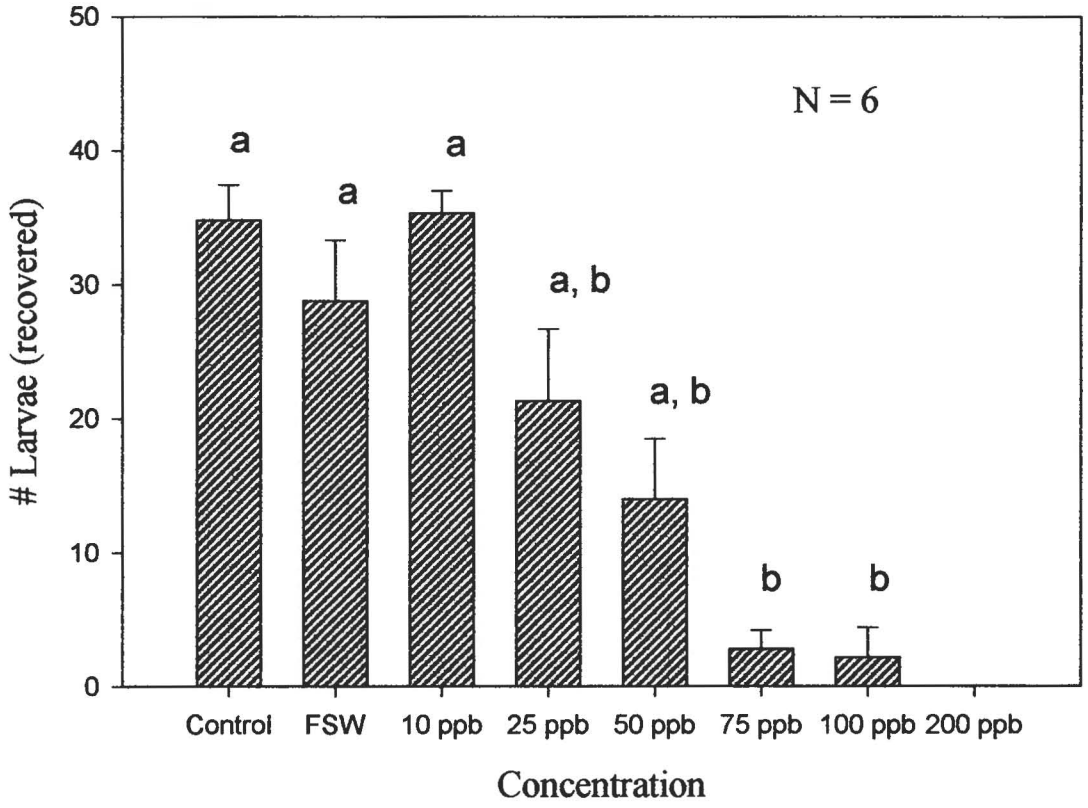


Figure 10. Recovery of larvae (mean ± S.E.) after exposure to copper. Significant treatment effect by Kruskal Wallis test ($p<0.001$). Letters above each bar shows significant groupings by Kruskal Wallis Z-test ($p<0.05$). N is the number of replicates per treatment.

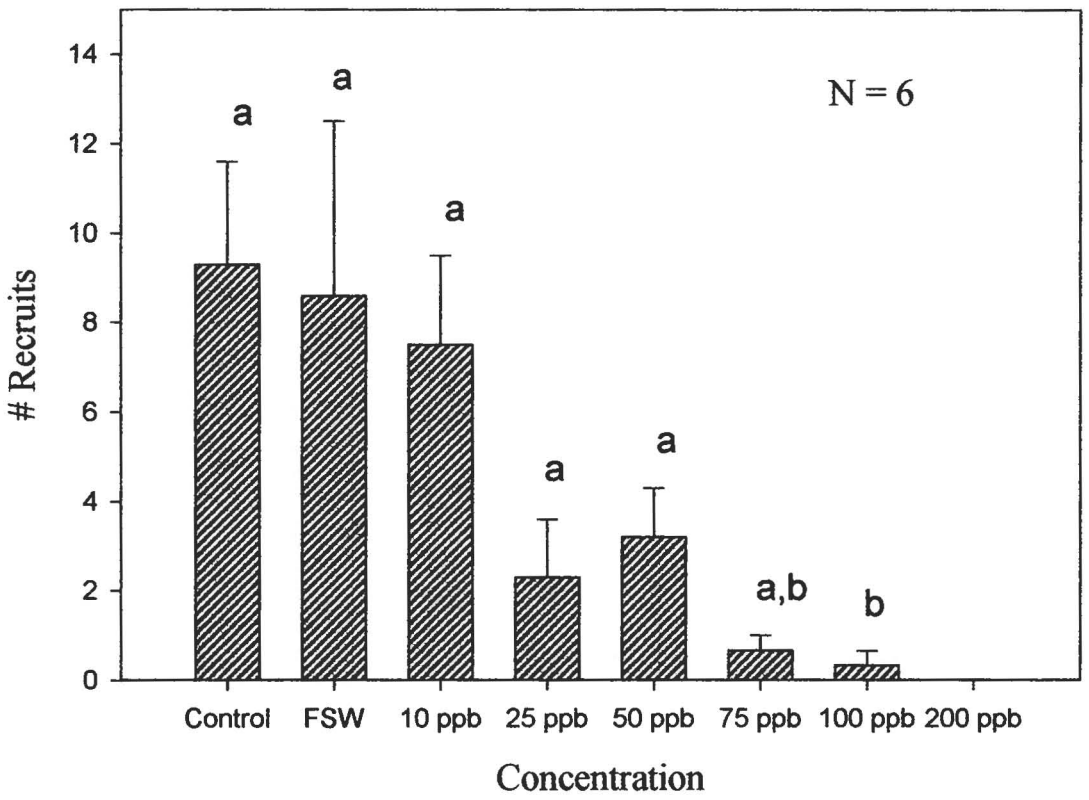


Figure 11. Number of larvae that settled and metamorphosed after 48 hours recovery period. Significant treatment effect by Kruskal Wallis test ($p<0.005$). Letters above bar shows significant groupings by Kruskal Wallis Z-test ($p<0.05$). N is the number of replicate per treatment.

another 48 hours. There were 26% and 28% settlement and metamorphosis in the control and in FSW, respectively (Figure 11). There was a significant effect of exposure to copper treatments on settlement and metamorphosis(Figure 11). The only significant difference of copper concentration on settlement and metamorphosis was observed between the control and 100 µg/l.

Inhibition of larval settlement and metamorphosis

In the settlement and metamorphosis assay with natural substrates there were 24% and 30% successful settlement and metamorphosis in FSW and control, respectively (Figure 12). There was a significant effect of treatment on settlement and metamorphosis. Settlement and metamorphosis in 10 µg/l was 40%, which was not significantly different from the control. Mean settlement and metamorphosis in 25 µg/l and 50 µg/l did not differ significantly from the control but were significantly different from 75 µg/l and 100 µg/l. There was a significant difference in settlement and metamorphosis rate between the control and 75 µg/l and 100.

Settlement and metamorphosis in the bioassay with artificial substrate were 26% and 18% in the control and FSW, respectively (Figure 13). The number of recruits on natural substrate that was used as a reference treatment to test the effect of larval age on settlement and metamorphosis was not significantly different from the control with ($p>0.05$). There was a significant effect of treatment on settlement and metamorphosis. Settlement and metamorphosis in the 10 µg/l was 31%, which was not different from the control. Mean settlement and metamorphosis in 25 µg/l and 50 µg/l did not differ significantly from the control. However, mean settlement and metamorphosis in 25 µg/l was significantly different from 75 µg/l and 100 µg/l. Mean settlement and metamorphosis in 25 µg/l and 50 µg/l did not

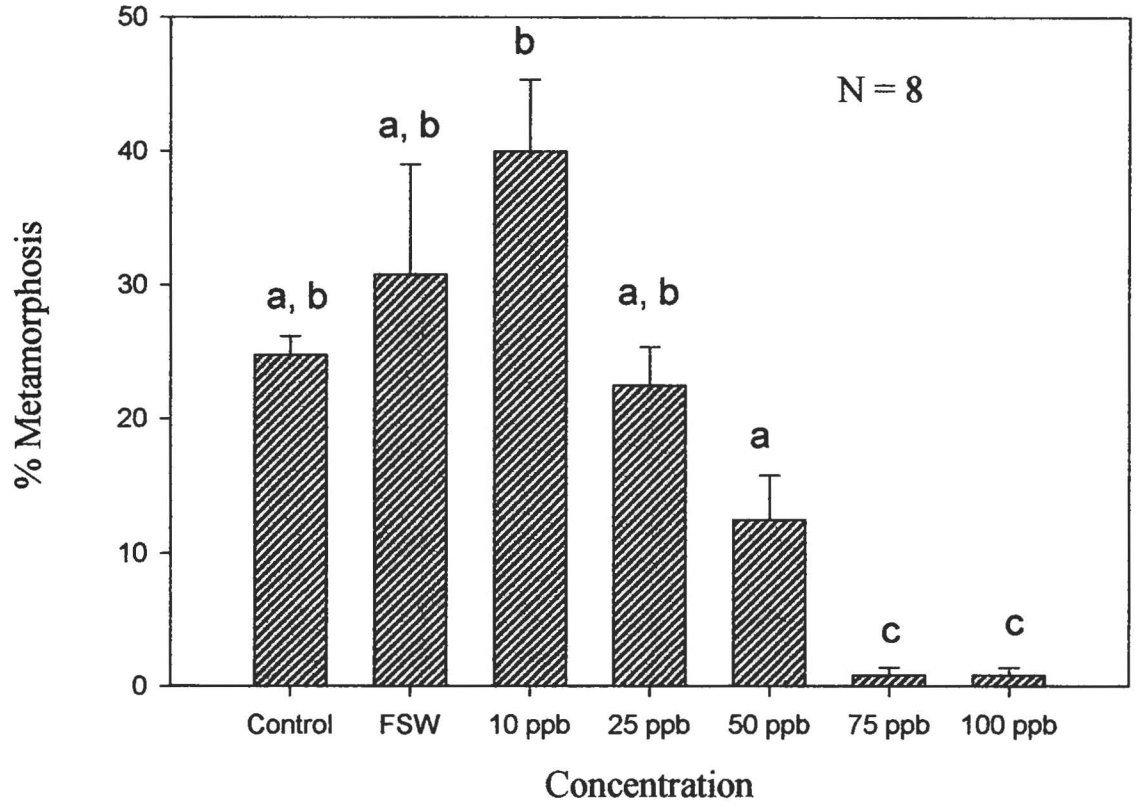


Figure 12. Effect of copper on larval metamorphosis (mean ± S.E.) on natural substrate.
Significant treatment effect on number of metamorphosed larvae by Kruskal Wallis test ($p<0.001$).
Letters above each bar shows significant groupings by Kruskal Wallis Z-test ($p<0.05$).
N is the number of replicates per treatment.

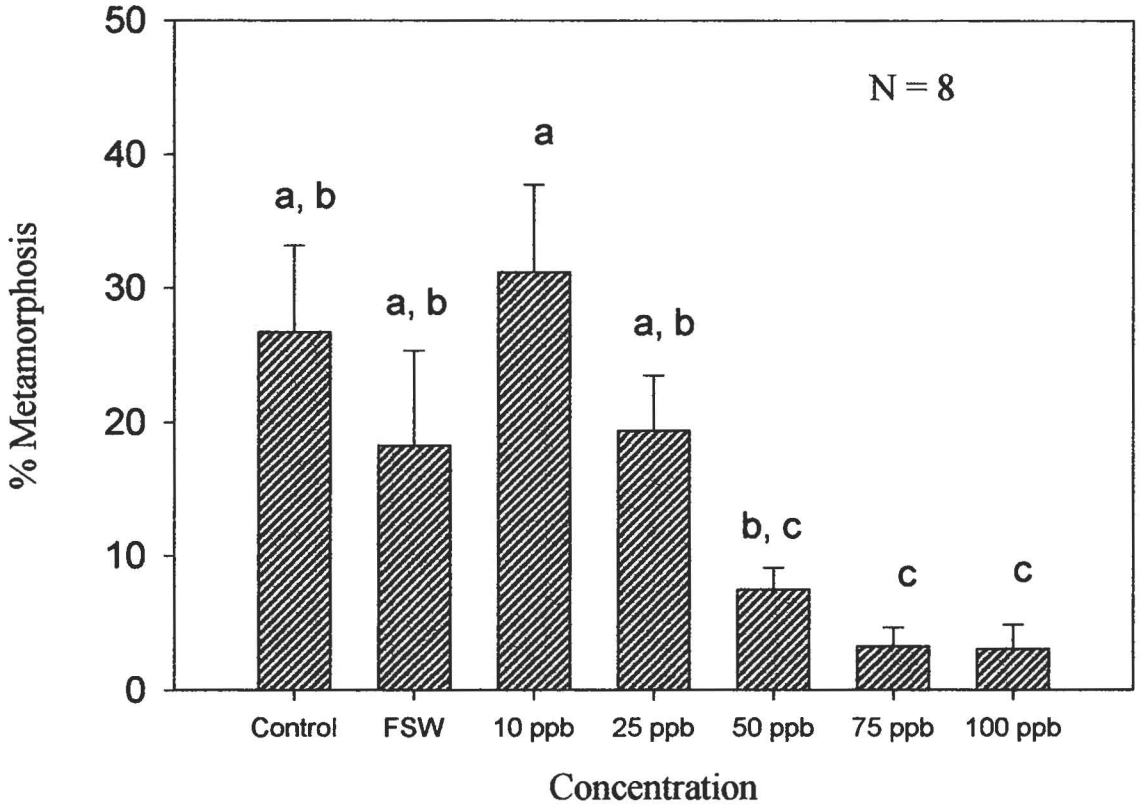
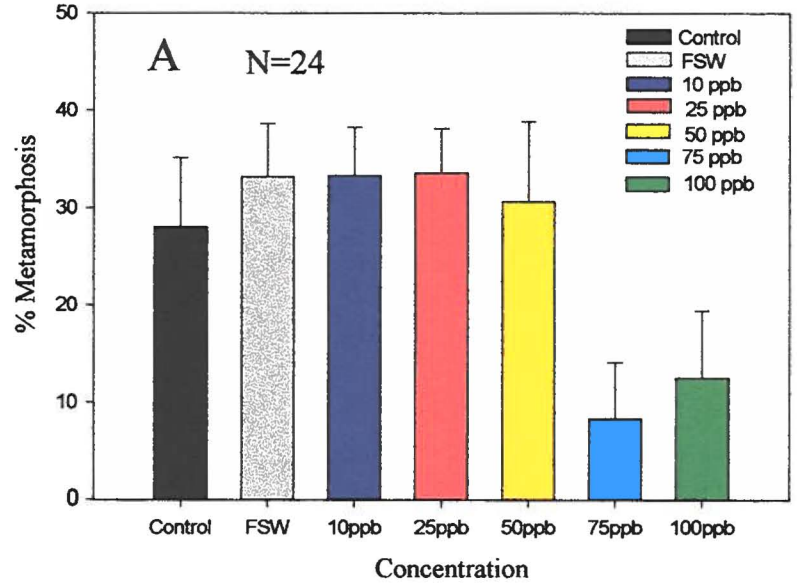
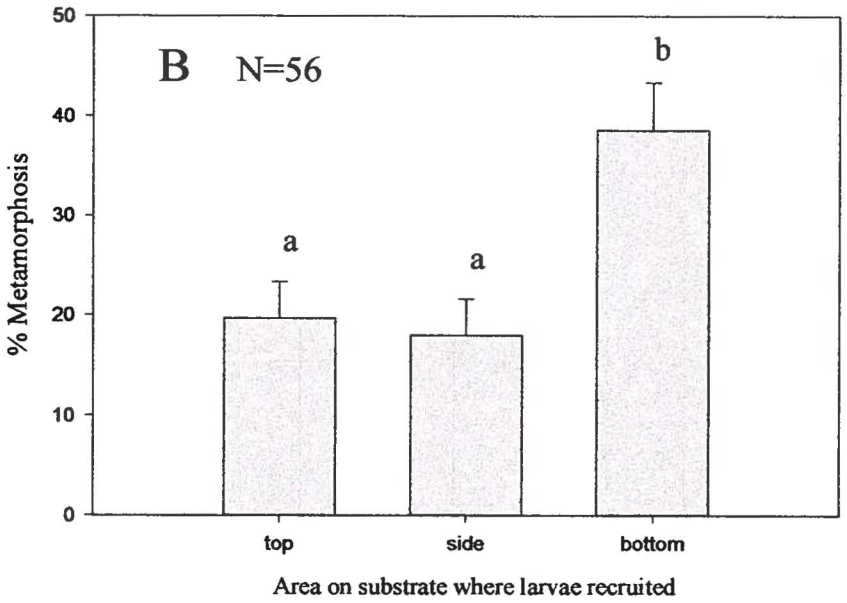


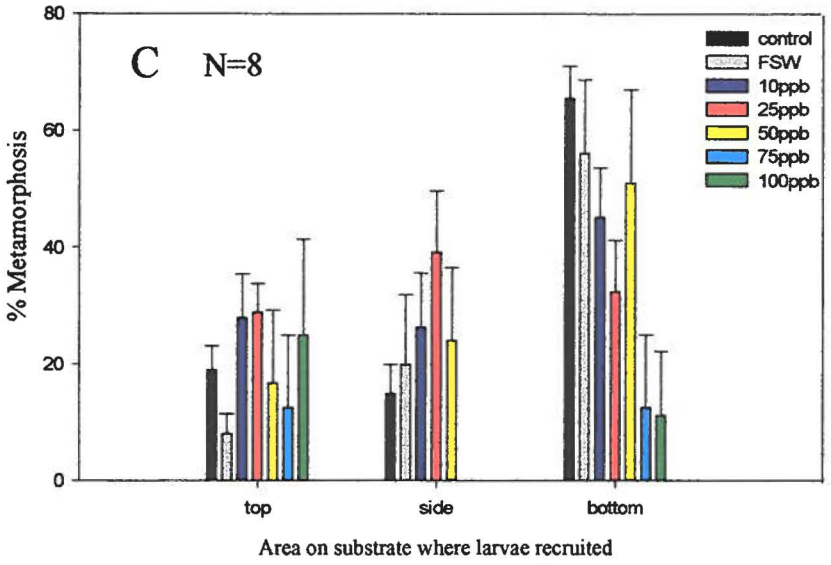
Figure 13. Effect of copper treatment on larval metamorphosis (mean ± S.E.) on artificial substrate. Significant treatment effect on the number of metamorphosed larvae by Kruskal Wallis test ($p<0.001$). Letters above each bar shows significant groupings by Kruskal Wallis Z-test ($p<0.05$). N is the number of replicates per treatment.

differ significantly from the control but were significantly different from 75 µg/l and 100 µg/l. There was a significant difference in number of recruits between the control and 75 µg/l and 100 µg/l.

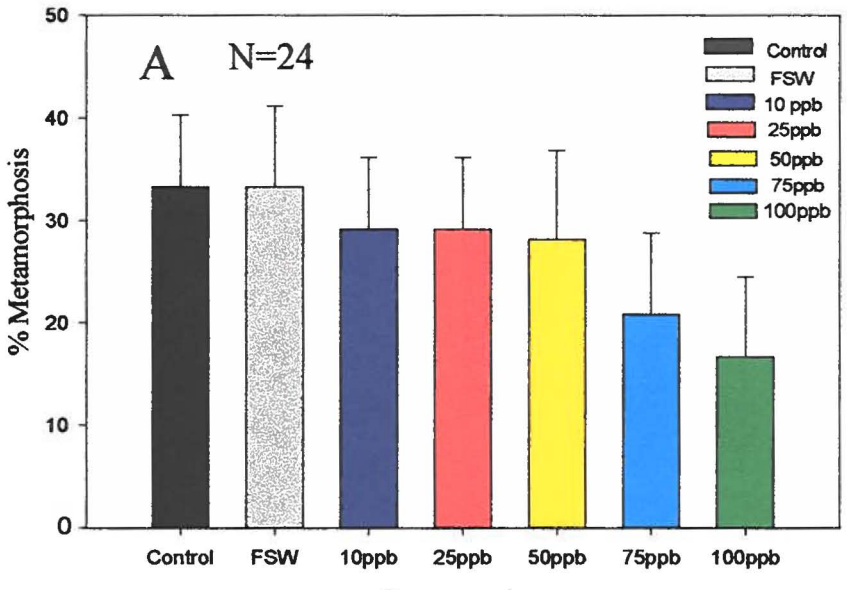
The majority of recruits in both bioassays was found on the undersurface of the substrate (Figures 14 and 15). There was no significant effect of treatment on where larvae settled and metamorphosed on the substrate surface ($p = 0.105$ - natural and $= 0.215$ - artificial substrate). However, there was significantly high number of recruits on undersurface of the plexiglass compared to the numbers on the side and bottom (Figure 15). This trend was not as obvious on natural substrate. There was no significant difference on mean settlement and metamorphosis between the two types of substrates at each treatment level (Figure 16).

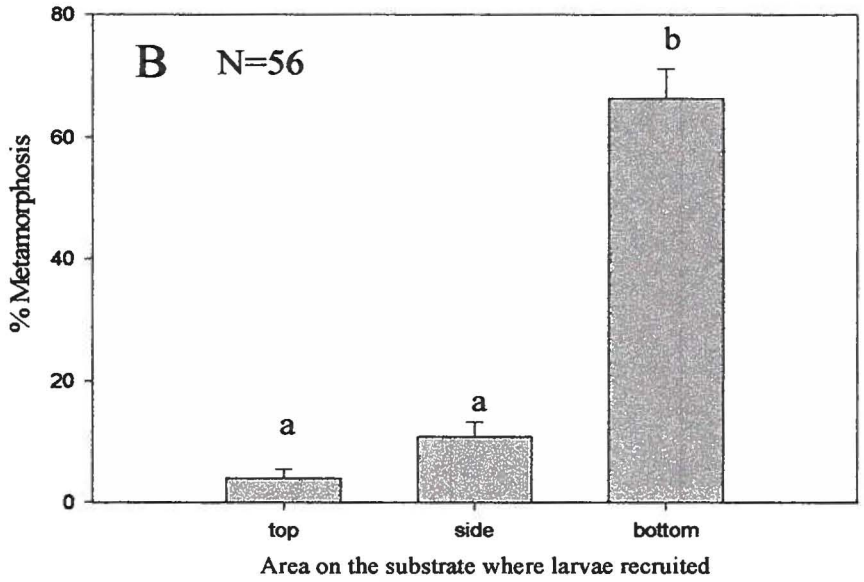


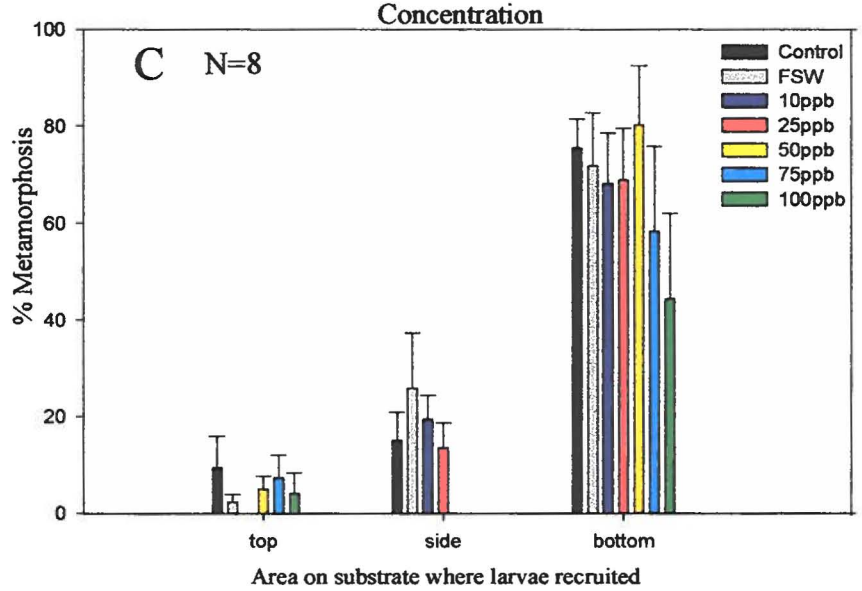




Figures 14 A-C. Comparison of the number of recruits in different regions of the substrate (natural substrate) by Friedman's test. A. Number of recruits by treatments ($p=0.105$). N is the pooled samples size for each treatment by orientation. B. Number of recruits by where they settled on the substrate ($p<0.001$) . Letters above each bar indicate significant grouping by Kruskal-Wallis Z-test ($p<0.05$). N is the pooled sample size for settlement site for all treatments. C. Comparison of settlement by site and treatment. N is the sample size for each treatment.







Figures 15 A-C. Comparison of the number of recruits in different regions of the substrate (artificial substrate) by Friedman's test. A. Number of recruits by treatments ($p=0.213$). N is the pooled sample size for each treatment by orientation. B. Number of recruits by where they settled on the substrate ($p<0.001$). Letter above each bar indicate significant groupings by Kruskal-Wallis Z-test ($p<0.05$). N is the pooled sample size for settlement site for all treatments. C. Comparison of settlement by site and orientation. N is the sample size for each treatment.

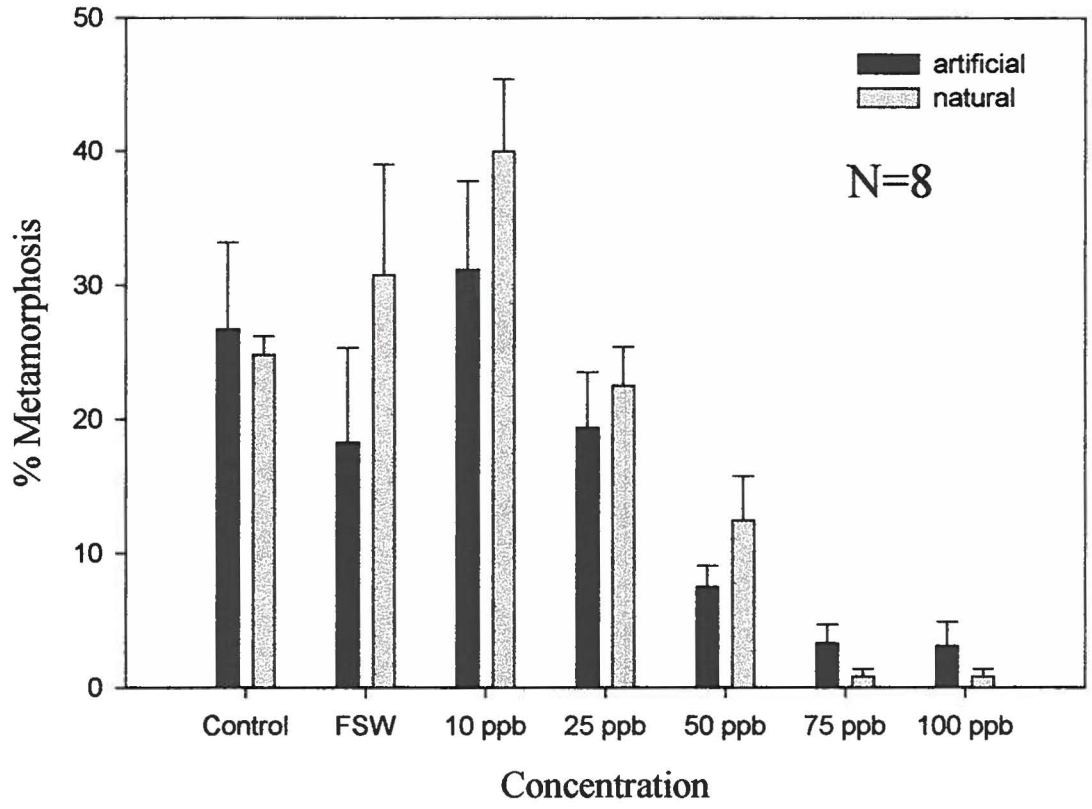


Figure 16. Comparison of mean settlement and metamorphosis by substrate type using the Sheirer-Ray-Hare extension of the Kruskal Wallis test ($p>0.05$). N is the number of replicates per treatment.

DISCUSSION

Inhibition of fertilization

The U.S. Environmental Protection Agency (USEPA) guidelines state that the total recoverable copper to protect saltwater aquatic life is 4 µg/l as a 24 hour average. In addition the concentration should not exceed 23 µg/l at any time (USEPA, 1980). In open ocean or unpolluted seawater, copper concentration has been found to be 0.33 µg/l or less (Schmidt, 1978) but in polluted water values as high as 29.2 µg/l have been reported (Sadiq, 1992). Heavy metal discharges into the marine environment associate with sediments and accumulate to concentrations higher than in the water column (Lee and Jones, 1984). Elevated copper levels have been reported in sediments around Australian coral reefs (Reichelt and Jones, 1994). Disturbance to these sediments can mobilize copper into the water column and may affect aquatic organisms (Calamano et. al., 1992). Copper has been shown to inhibit fertilization in various marine organisms (Table 3).

Table 3. Comparison of EC_{50} for fertilization in some marine organisms.

species	EC_{50}	Authors
sea urchin (*E. mathaei*)	14 µg/l	Ringwood, 1992
bivalve (*I. californucum)*	55 µg/l	Ringwood, 1992
coral (*G. aspera)*	14.5 µg/l	Reichelt-Brushett & Harrison 1999
fish (*A. affinis)*	109 µg/l	Anderson et. al., 1991

The result of this shows that copper exerted a significant effect on fertilization rate in *A. surculosa*. The result is consistent with what has been found in previous studies using gametes from other coral species (Heyward, 1988; Reichelt-Brushett and Harrison, 1999; Negri and Heyward, 2001. However the estimated EC_{50} for gametes in *A. surculosa* in this study is twice the concentration for other coral species that have been tested (Table 4).

Table 4. Comparison of EC_{50} for fertilization in different coral species.

Species	EC_{50}	exposure time	Author
Goniastrea aspera	14.5 µg/l	5 hours	Reichelt-Brushett & Harrison, 1999
Acropora millepora	17.4 µg/l	4 hours	Negri and Heyward, 2001
Acropora surculosa	38.4 µg/l	5 hours	This study

The differences in the EC_{50} may result from species differences in sensitivity to copper and/or the variation in methodology. In bioassay used in this study gametes were exposed to copper and allowed to separate and fertilize as bundles from the two colonies used for the bioassay. In previous studies, bundles were separated outside of the test container and then exposed to copper, and combined 30 minutes later for fertilization. This method would have allowed for longer handling of gametes which may stress them and render them susceptible to pollutants. In this bioassay the handling artifact was avoided by combining gametes immediately

after they were spawned into the test container with the copper solution and allowed to separate by gentle agitation. This method may be more ecologically relevant because gametes may be exposed to pollutants as bundles in the natural environment. The type of method used could have an effect on sensitivity of gametes to copper and may lower the estimated EC_{50} value. This may happen because when eggs/sperm bundles are exposed to copper, the sperm are inside the egg cluster. So the copper may adhere to the eggs and lowering the concentration at which the sperm may be exposed to when the bundles break apart.

The toxicity of copper to aquatic organisms is related to the copper free ion (Crecilius et. al., 1982; Eriksen et al., 2001; Young et. al., 1979). However, copper may be complexed to carbonate and hydrogen ions (Pagenkopf et. al., 1974) and organic matter (Bately and Gardener, 1978). Its toxicity will therefore be reduced in seawater and in the presence of organic matter, such as the egg. Copper has been shown to damage sperm of other species through oxidative stress (Loyd et. al., 1997) but this mechanism has not been demonstrated in corals. This mechanism needs further investigation to ascertain whether direct exposure of sperm to copper would have had an effect on the estimated EC_{50}. This may help in separating out the effects due to variation in methodology and the actual species differences in sensitivity to copper.

Fertilization in broadcast spawning corals reach maximum rate between four and six hours after the gamete bundles have separated (Oliver and Babcock, 1992). For this reason, 5 hours seems an appropriate endpoint to determine the effect of pollutant on fertilization rate. However, 5 hours is a rather acute measure of the effect of a pollutant on early developmental stages since fertilized eggs have to go through several stages before they are recruited into a population. Although fertilization itself is an important determining factor for successful

reproduction in corals, development of embryos and subsequent larval maturation and competency are just as important in determining the fate of the success of reproduction. However, there is a lack of toxicity data about these developmental stages for spawning corals.

Fertilization and embryo development

The result of this experiment showed that prolonged exposure of gametes during fertilization and embryogenesis for 12 hours in copper solution had a significant effect on number of embryos that survive to the blastula stage (Figure 6). This may be more of an effect on the inhibition of fertilization than toxicity to embryos. The EC_{50} estimated for the 12 hour exposure was 12.9 µg/l (Table 2). This value is three times lower than the value for five hours exposure and suggests that exposure time exerts a significant effect on copper toxicity in early developmental stages. Studies with other invertebrates report lower EC_{50} to embryos and larvae than gametes following a longer exposure to pollutants (Ringwood, 1992; Vaschenko et. al., 1999). A longer exposure time may be ecologically relevant because there are coral reef areas where there is less water movement and matter residence time exceeds 5 hours. Up to 45% of material, such as larvae and sewage may still be present 10 days after they are released in a typical reef as predicted by a model proposed by Black et. al., 1990.

The fertilization rate estimated from the 12 hour exposure was not significantly different from mean fertilization rate for the 5 hour exposure bioassay (Figure 8). Although, there was a significant difference in fertilization rate at the 12 hours vs 5 hours exposure for the highest copper concentration (200 µg/l), all fertilized eggs were not able to undergo cleavage. There were higher proportions of unfertilized eggs than fertilized eggs at concentrations above 25 µg/l. It appears that sperm were not able to successfully fertilize the eggs. This may not be

an effect of sperm dilution because there were very high fertilization rates in controls having the same number of gametes as the treatments. This seems to reinforce the theory that toxicity of copper to coral gametes lies within the sperm. The effect of heavy metal on the fertilization capability of spermatozoa in marine organisms have been demonstrated (Vaschenko et. al, 1999; Anderson et. al, 1991). If the sperm becomes stressed as a result of being exposed to copper, it would be less likely to successfully fertilize an egg. This is shown in the higher concentration where there were sign of fertilization but the fertilized eggs didn't undergo successful cleavage. The sperm must penetrate the egg's membrane to fuse with its nucleus in successful fertilization (Alberts et. al., 1983), but if the sperm is oxidatively stressed, it may not have the ability to reach the nucleus.

Reduced salinity did not have significant effect on number of embryos that survived after 12 hour gamete exposure. However, the 5% reduction in salinity may have had an effect on the total dissolved copper (Figure 7) thus affecting its toxicity. More copper ions becomes bio available as salinity is reduced due to the presence of less chloride ions. It has been shown that reduction in salinity correlates with increased copper toxicity (Ozoh, 1994; Bugenyi and Lutalo-Bosa, 1990; Anderson et. al., 1995).

The very high mortality of embryos resulting in the treatments may be an added effect of the experimental set up and the effects of copper. Since fertilization was inhibited when gametes were exposed to copper, lower fertilization rates correlated to high mortality. A more appropriate method for estimating the effect of copper on mortality of embryos alone would be to expose fertilized eggs to copper treatments. The result of this bioassay nevertheless demonstrate that duration of exposure and concentration of copper would exert a significant

effect on the early developmental stages in corals and would possibly have detrimental effects on populations and coral reef ecosystems.

Effect of copper on larvae

Larval development has been studied in brooding coral, *P. damicornis*, which is a zooxanthellate larvae that may respond differently to copper than azooxanthellate larvae. In a study using *P. damicornis* planulae, Esquivel (1986) showed that the larvae is more resistant to copper than the adult colony. In another study, Negri and Heyward (2001) found that the fertilization process in *A. millepora* was more resistant than larval metamorphosis process to a petroleum products. Different coral species may respond differently to pollutants in their developmental stages.

Delayed development of larvae has been reported for other invertebrates as a result of exposure to pollutant (Coglianese and Martin, 1981; Mortimer and Miller, 1994; Nice et. al., 2000). In this study, it was observed that copper had an effect on larval development and survival after 48 hour exposure as blastula embryos (Figure 9A). The copper seemed to have slowed the development process of the blastula into larvae, so after 48 hour exposure, the affected larvae have not formed cilia and the body appeared deformed. Esquivel (1986) studied the effect of copper on larvae of *P. damicornis* and found the same response of larvae to copper, where larvae contracted and lose motility after exposure. Others have reported similar response of coral larvae to other pollutants (Epstein et. al., 2001; Rinkevich and Loya, 1979). The mechanism of copper toxicity to coral larvae is not clearly understood. However, it has been suggested that one possible effect is that copper may absorb to the membrane and interfere with respiratory exchange and osmoregulation (Esquivel, 1986). This seems plausible given that

larvae are lecithotrophic (rely on own energy reserve), so the uptake of copper via food material does not represent a significant metal input.

Exposure to pollutants has been shown to affect recovery of larvae (Goh, 1991). In this bioassay, recovery was not significantly affected after exposure to copper at each treatment level. It should be clarified here that treatment had a significant effect on the survivorship of larvae and therefore significant difference on the mean between treatments was observed. However, the mean number of larvae that recovered at each treatment level compared to the number of larvae that survived the exposure to copper did not differ significantly. This seems to suggest that the larvae that survived the exposure to copper seemed to offer some resistance. It is known that marine organisms have detoxifying mechanisms that allows them to regulate heavy metals (Mouneyrac et. al., 1998).

The result of this bioassay suggests that coral larvae are less sensitive to copper than gametes and embryos. This conclusion is generally applicable to other pollutants. It has been shown that sensitivity of aquatic organisms to pollutant decreases as the developmental stage becomes more developed. This has been demonstrated in other organisms, such as sea urchins, shrimp, crabs , fish, gastropod (Chen and Lin, 2001; Coglianese and Martin, 1981; Connor, 1973; Heslinga, 1976; Johnson, 1988; Mortimer and Miller; 1994; Nice et. al., 2000). It should be noted that here that a reported EC_{50} may for larvae may be lower than gametes but it must be understood that exposure time will significantly affect the toxicity of copper to larvae.

Larval settlement and metamorphosis

Settlement and metamorphosis in this bioassay was below 40% in the control (Figures 10 & 12). Similar levels of recruitment were observed in other laboratory bioassays studying the

effects of pollutants on coral settlement (Epstein et. al, 2000; Reichelt-Brushett and Harrison, 2000). Copper significantly affected larval settlement in the bioassays with natural and artificial substrates relative to controls at 75 and 100µg/l. This observed effect of copper on larval settlement and metamorphosis was also observed in a study with *A. millepora* where copper inhibit metamorphosis at 80 µg/l (Negri and Heyward, 2001). In a study with *A. tenuis*, larval settlement was inhibited at much lower concentration (42 µg/l -measured concentration) (Reichelt-Brusshet and Harrison, 2000). It is important to note at this point that there was no observed direct effect of copper on larval metamorphosis. It has been shown that larval metamorphosis can be affected by pollutants (Epstein et. al., 2000). All the larvae that settled successfully metamorphose as described by Negri and Heyward (2001) as a firmly attached with obvious mesenteries radiating from the mouth region. The only effect that was observed in this bioassay is toxicity of copper to larvae.

Other studies have investigated the effect of different pollutants on coral larval settlement, including nutrients and sedimentation (Hunte and Wittenberg, 1992); petroleum products (Epstein et. al., 2000; Rinkevich and Loya, 1977, 1979; Te, 1991); nickel (Goh, 1991). All these studies showed that larval settlement was inhibited as a result of toxicity of pollutant to the larvae, where the larvae became deformed, lost normal swimming behavior, contracted , and the tissue started to degenerate after exposure to pollutant. These responses of coral larvae to pollutants reduce their ability to find settlement sites and subsequent metamorphosis is therefore inhibited. The observed effect is directly at the larval viability to successfully survive. Wisely (1963) suggested that the terms pre- attachment mortality and post-attachment mortality be used to describe the effect of copper observed on larval settlement.

Because there is no obvious evidence that has been shown that copper inhibits metamorphosis or repel larvae from potential settlement sites.

Although, substrate had no effect on the total number of larvae that successfully settled and metamorphosed (Figure 14), the site on the substrate at which the larvae recruited was significantly different. In the natural substrate, there was no obvious difference on where the larvae recruited (Figure 11) but in the bioassay with artificial substrate, majority of the larvae recruited to the undersurface (Figure 13). Although, it is a known phenomenon that larvae will preferentially settle and metamorphose on the undersurface of a substrate (Birkeland et. al, 1981; Goreau et. al., 1981.), the obvious trend observed in the natural substrate may be due to the effect of the substrate itself. The natural substrate was flat and smooth on both surfaces, but on the undersurface a small piece of plexiglass was glued to it as a spacer to allow for space between the substrate and the bottom of the beaker. Most of the recruits were found on that piece, which seems to suggest that the larvae may not like the flat surface and may prefer substrate that are rough and provide micro-habitats. The preferential settlement of larvae on rough surfaces has been demonstrated in field a field experiment where coral recruits correlated positively with surface irregularity (Carleton and Sammarco,1987).

Conclusion and future research

This study provides a look at the effect of copper on gametes, embryos, larval development, settlement and metamorphosis of a common spawning Pacific coral, *A. surculosa*. Past studies have examined only the effect of copper on gametes, settlement and metamorphosis in a few spawning corals. The differences in EC_{50} between this study and the previous studies may be a result of species differences in sensitivity to copper and/or the variation in

methodology. Nonetheless, the results show that coral gametes are highly sensitive to copper in seawater. It is therefore, suggested that corals be used as indicator species for testing the effect of variety of potential pollutants to marine environments.

The mechanism on toxicity of copper to coral gametes is not clearly understood, yet. It has been suggested that toxicity may lie within the sperm cells. Therefore, future research should expose sperms cells to copper and combine with clean eggs for fertilization. The vice versa should be done to determine wether exposure of eggs alone will have any effect on the fertilization process.

Although, the result of the embryo bioassay shows almost the same sensitivity of embryos to copper as the gametes, the embryo may be less sensitive than gametes. The way the bioassay was set up may have exaggerated the effect of copper on embryo mortality. The very high mortality in all treatments may be correlated to inhibition of fertilization. Mortality was defined by pooling the numbers of unfertilized eggs and dead embryos. Since exposing gametes to copper solution resulted in inhibition of fertilization, there were few embryos to begin with that may have resulted in high mortality. It is suggested that a more appropriate experimental set up to test the effect of copper on embryogenesis and mortality would be to exposed fertilized eggs to copper solution.

The use of artificial substrates in a laboratory bioassay is a relatively new approach. However, it has proven its usefulness by providing a standardized substrate where the effect of pollutant can be quantified. This is particularly important because some pollutants are hydrophobic and therefore will adhere to the substrate and may affect the necessary cues that coral larvae must sense before it settles and metamorphose. Since natural substrates are not

uniform, the quantity of a potential pollutant that may adhere to them may vary and therefore the effect will be different. Future research using on settlement and metamorphosis should investigate the competency period for a given species to determine the appropriate time for setting up settlement and metamorphosis bioassays. Modification of the substrate used in this study to provide micro-habitats and soaking for longer period of time for colonization of coralline algae may help in increasing the number of coral settlement.

LITERATURE CITED

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1983. Molecular Biology of The Cell. Garland Publishing, Inc. New York, p. 801-809.

Allah, A.T.A., Wana, M.Q.S., and Thompson, S.N. 1997. Effects of heavy metals on survival and growth of *Biomphalaria glabrata* Say (Gastropoda: Pulmonata) and interaction with schistosome infection. J. moll. Stud. 63: 79-86.

Anderson, B.S., Hunt, J.W, Piekarski, W.J., Phillips, B.M., Englund, M.A., Tjeerdema, R.S., and Goetzl, J.D. 1995. Influence of salinity on copper and azide toxicity to larval topsmelt Atherinops affinis (Ayres). Arch. Environ. Contam. Toxicol. 29: 366-372.

Anderson, B.S., Middaugh, D.P., Hunt, J.W. and Turpen, S.L. 1991. Copper toxicity to sperm embryos and larvae of topsmelt *Atherinops affinis*, with notes on induced spawning. Marine Environmental Research 31: 17-35.

Babcock, R. and Heyward, A.J. 1986. Larval development for certain gamete-spawning scleractinian corals. Coral Reefs 5: 111-116.

Bately, G.E. and Gardner, D. 1978. A study of copper, lead, and cadmium speciation in some estuarine and coastal marine waters. Estuarine and Coastal Marine Science 7: 59-70.

Birkeland, C., Rowley, D, and Randall, R.H. 1981. Coral recruitment patterns at Guam. Proc. 3rd Int. Coral Reef Symposium 1: 15.21.

Black, K.P., Gay, S.L, and Andrews, J.C. 1990. Residence time of neutrally-buoyant matter such as larvae , sewage, or nutrients on coral reefs. Coral Reefs 9: 105-114.

Bryan, G.W. 1976. Some Aspects of Heavy metal tolerance in Aquatic Organisms. Lockwood, A.P.M. (ed.), Cambridge University Press, pp. 7-34.

Brown, B.E. 1987. Heavy metal pollution in coral reefs. In: Human impacts on coral reefs: facts and recommendations. Salvat, B. (ed.). Antenne Museum E.P.H.E., French Plynesia 120-134.

Brown, C.J. and Eaton, R.A. 2001. Toxicity of chromated copper arsenate (CCA)-treated wood to non-target marine fouling communities in Langstone Harbour, Portsmouth, UK. Marine Pollution Bulletin 42(4): 310-318.

Brown, B.E. and Howard, L.S. 1985. Assessing the effects of "Stress" on coral reefs. Advances in Marine Biology 22: 1-63.

Bugenyi, F.W.B. and Lutalo-Bosa, A.J. 1990. Likely effects of salinity on acute copper toxicity to the fisheries of Lake George-Edward basin. Hydrobiologia 208: 39-44.

Calamano, W., Ahlf, W., and Bening, J.C. 1992. Chemical mobility and bioavailability of sediment-bound heavy metals influenced by salinity. Hydrobiologia 235/236: 605-610.

Carleton, J.H. and Sammarco, P.W. 1987. Effects of substratum irregularity on success of coral settlement: quantification by comparative geomorphological techniques. Bulletin of Marine Science 40(1): 85-98.

Chen, J.C and Lin. C.H. 2001. Toxicity of copper sulfate for survival, growth, molting, and feeding of juveniles of the tiger shrimp, *Penaeus monodon*. Aquaculture 192: 55-65.

Cheung, S.G. and Wong, L.S. 1999. Effect of copper on activity and feeding in the subtidal prosobranch *Babylonia lutosa* (Lamarck) (Gastropoda: Buccinidae). Marine Pollution Bulletin 39(1-12): 106-111.

Coglianese, M.P. and Martin, M. 1981. Individual and interactive effects of environmental stress on embryonic development of the Pacific oyster *Cassostrea gigas* I. The toxicology of copper and silver. Marine Environmental Research 5: 13-21.

Connor, P.M. 1973. Acute toxicity of heavy metals to some marine larvae. Marine Pollution Bulletin 2: 190-193.

Claisse, D. and Alzieu, C. 1993. Copper contamination as a result of antifouling regulation. Marine Pollution Bulletin 26: 395-397.

Cremlyn, R. 1979. Pesticides Preparation and Mode of Action. Wiley, London.

Crecelius, E.A., Hardy, J.T., Gibson, C.I., Schmidt, R.L., Apts, C.W., Curtissen, J.M., and Joyce, S.P. 1982. Copper bioavailability to marine bivalves and shrimp: relationship to cupric ion activity. Marine Environmental Research 6: 13-26.

Done, T.J. 1992. Phase shift in coral communities and their ecological significance. Hydrobiologia 247: 121-132.

Dubinsky, Z. And Stambler, N. 1996. Marine pollution and coral reefs. Global Change Biology 2: 511-526.

Edinger, E.N., Jompa, J., Limmon, G.V., Widjatmoko, W. Amd Risk, M. 1998. Reef degredation and coral biodiversity in Indonesia: effects of land-based pollution, destructive fishing practices and change over time. Marine pollution Bulletin 36(8): 617-630.

Edmonson, C.H. 1946. Behavior of coral planula under altered saline and thermal conditions. In: Occassional papers of Bernice P. Bishop Museum, Honolulu, Hawaii 18(19): 283-304.

Epstein, N., Bak, R.P.M., and Rinkevich, B. 2000. Toxicity of third generation dispersants and dispersed Egyptian crude oil on the Red Sea coral larvae. Marine Pollution Bulletin 40(6): 497-503.

Eriksen, R.S., Mackey, D.J., van Dam, R., Nowak, B. 2001. Copper speciation and toxicity in Macquarie Harbour, Tasmania: an investigation using copper ion selective electrode. Marine Chemistry 74: 99-113.

Esquivel, I. 1986. Short term copper bioassay on the planula of the reef coral Pocillopora damicornis. In: Jokiel, P.L., Richmond, R.H., and Rogers, R.A. (eds). Coral reefs Pop. Biol. Technical report 37. Hawaii Institute of Marine Biology, Coconut Island, HI.

Fang, T.H. and Hong, E. 1999. Mechanisms influencing the spatial distribution of trace metals in surficial sediments of the South-Western Taiwan. Marine Pollution Bulletin 38(11): 1026-1037.

Gillet, B. 1999. Voices from the village: a comparative study of coral resource management in the Pacific Islands. In: Fisheries Newsletter No. 89: 26-32.

Goh, B.P.L. 1991. Mortality and settlement of *Pocillopora damicornis* planula larvae during recovery from low levels of nickel. Pacific Science 45(3): 276-286.

Goreau, N.I., Goreau, T.J., and Hayes, R.L. 1981. Settling, survivorship and spatial aggregation in planulae and juveniles of the coral *Porites porites* (Pallas). Bulletin of Marine Science 3(2): 424-435.

Guzman, H.M. and Jimenez, C.E. 1992. Contamination of coral reefs by heavy metals along the Caribbean Coast of Central America (Costa Rica and Panama). Marine Pollution Bulletin 24(11): 554-561.

Hawkins, J.P. and Roberts, C.M. 1994. The growth of coastal tourism in the Red Sea: present and future effects on coral reefs: Ambio 23(8): 503-509.

Heslinga, G.A. 1976. Effects of copper on the coral reef echinoid, *Echinometra mathaei.* Marine Biology 35: 135.

Heyward, A.J. 1988. Inhibitory effects of copper and zinc on fertilization in corals. In: Proc. 6th Int. Coral Reef Symposium, Australia 2: 299-303.

Houk, P. 1999. Coral Community Structure changes in response to development activities and natural bleaching: Iwayama Bay, Republic of Palau. Master's thesis. Marine lab, University of Guam, Mangilao, Gu.

Howard, L.S. and Brown, B.E. 1984. Heavy metals and reef corals. Oceanogr. Mar. Biol. Ann. Rev. 22: 195-210.

Johannes, R.E. 1975. Pollution and degradation of coral reef communities. In: Wood, E.J.F., and R.E. Johannes (eds.). Tropical Marine Pollution. Elsevier Scientific Publishing Company, Oxford, p.1-181.

Johnson, D. 1988. Development of *Mytilus edulis* embryos: a bioassay for polluted waters. Marine Ecology Progress Series 46: 135-138.

Hunte, W. and Wittenberg, M. 1992. Effects of eutrophication and sedimentation on juvenile corals. Marine Biology 114: 625-631.

Lee, G.F. and Jones, R.A. 1984. In: K.L. Dickson, A.W. Maki, and W.A. Brungs (eds.). Fate and effects of sediment bound chemicals in aquatic systems, Permagon Press, Elmsford, NY, pp. 1-34.

Leota, S.T. 2000. Effect of a pesticide (Chlorpyrifos: Organophoshate) on larval recruitment of Gonisastrea retiformis (Cnidaria: Scleractinia). Master's thesis. Marine lab, University of Guam, Mangilao, Gu.

Loyd, D.R., Phillips, D.H., and Carmicheal, P.L. 1997. Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. Chem. Res. Toxicol. 10: 393-400.

Mance, G. 1987. Pollution threat of heavy metals in aquatic environments. In: Mellanby, K. (ed.). Pollution Monitoring Series, Elsevier Applied Science, New York.

Maragos, J.E. 1993. Impact of coastal construction on coral reefs in the US-Affiliated Pacific Islands. Coastal Management 21: 235-269.

Mouneyrac, C. Amiard, J.C., and Amiard-Triquet, C. 1998. Effects of natural factors (salinity and body weight) on cadmium, copper, zinc, and metallothionein-like protein levels in resident populations of oysters *Carssostrea gigas* from a polluted estuary. Marine Ecology Progress Series 162: 125-135.

Mortimer, M.R. and Miller, G.J. 1994. Susceptability of larval and juvenile instars of the sand crab, Portunus pelagicus(L.), to seawater contaminated by chromium, nickel, and copper. Aust. J. Mar. Freshwater Res. 45: 1107-1121.

Number Crunching Statistical Software (NCSS) 2000.

Negri, A.P. and Heyward, A.J. 2001. Inhibition of coral fertilisation and larval metamorphosis by tributyltin and copper. Marine Environmental Research 51: 17-27.

Negri, A.P. and Heyward, A.J. 2000. Inhibition of fertilization and larval metamorphosis of the coral *Acropora millepora* (Ehrenberg, 1834) by petroleum products. Marine Pollution Bulletin 41(7-12): 420-427.

Nice, H.E., Thorndyke, M.C., Morritt, D., Steele, S., and Crane, M. 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. Marine Pollution Bulletin 40(6): 491-496.

Oliver, J. and Babcock, R. 1992. Aspects of the fertilization ecology of broadcast spawning corals: sperm dilution effects and in situ measurements of fertilization. Bioll. Bull. 183: 409-417.

Ozoh, P.T.E. 1994. The effect of salinity, temperature and time on the accumulation and depuration of copper in ragworm, *Hediste diversicolor* (Nereis) (O.F. Muller). Environmental Monitoring and Assessment 29: 155-166.

Pagenkopf, G.K., Russo, R.C., and thurston, R.V. 1974. Effect of complexation on toxicity of copper to fishes. Journal Fisheries Research Board of Canada 31(4): 462-465.

Pastorok, R.A. and Bilyard, G.R. 1985. Effects of sewage pollution on coral reef communities. Marine ecology progress series 21: 175-189.

Peters, E.C., Gassaman, N.J., Firman, J.C., Richmond, R.H., and Power, E.A. 1997. Ecotoxicology of tropical marine ecosystems. Environmental Toxicology and Chemistry 16(1): 12-40.

Reichelt, A.J. and Jones, G.B. 1994. Trace metals as tracers of dredging activity in Cleveland Bay - filed and laboratory studies. Aust. J. Mar. Freshwater Res. 45: 1237-1257.

Reichelt-Brushett, A.J., and Harrison, P.L. 2000. The effect of copper on the settlement success of larvae from the scleractinian coral Acropora tenuis. Marine Pollution Bulletin 41(7-12): 385-391.

Reichelt-Brushett, A.J. and Harrison, P.L. 1999. The effects of copper, zinc, and cadmium on fertilization success of gametes from scleractinian reef corals. Marine Pollution Bulletin 38(3): 182-187.

Richmond, R.H. 1994. Coral Reef Resources: pollution impacts. In: Forum for Applied Research and Public Policy Spring 1994: 54.57.

Richmond, R.H. 1993. Effects of coastal runoffs on coral reproduction. In: Proceedings of the Colleqium on Global Aspects of Health, Hazard, and History 360-364.

Richmond, R.H. and Hunter, C.L. 1990. Reproduction and recruitment of corals: comparisons among the Caribbean, the tropical Pacific, and the Red Sea. Marine Ecology Progress Series 60: 185-203.

Ringwood, A. 1992. Comparative sensitivity of gametes and early development stages of a sea urchin *Echinometra mathaei* and bivalve species *Isognomon californicum* during metal exposures. Archives of Environmental Contamination and Toxicology 22: 288-295.

Rinkevich, B. and Loya, Y. 1979. Laboratory experiments on the effects of crude oil on the Res Sea coral *Stylophora pistillata.* Marine Pollution Bulletin 10: 328-330.

Rinkevich, B. and Loya, Y. 1977. Harmful effects of chronic oil pollution on a Red Sea scleractinian coral population. In Proc. 3rd Int. Coral Reef Symposium 585-591.

Sadiq, M. 1992. Toxic Chemistry in Marine Environments. Marcel Dekker Inc., New York, p.1-390.

Schmidt, R.L. 1978. Copper in the Marine Environment Part I. U.S. Department of Energy.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. W.H. Freeman and Company, New York, p. 1-887.

Stupnisek-Lisac, E., Bozic, A.L., and Cafuk, I. 1998. Low toxicity copper corrosion inhibitors. Corrosion 54(9): 713-720.

Te, F.T. 1991. Effects of two petroleum products on *Pocillopora damicornis* planulae. Pacific Science 45(3): 290-298.

U.S. Environmental Protection Agency (US EPA). 1980. Ambient Water Quality Criteria for Copper. EPA 440/5-80-036, Criteria and Standard Division, Washington, DC.

Vaschenko, M.A., Zhang, Z.P., Lam, P.K.S., and Wu, R.S.S. 1999. Toxic effects of cadmium on fertilizing capability of spermatozoa, dynamics of the first cleavage and pluteus formation in the sea urchin *Anthocidaris crassispina* (Agassiz). Marine Pollution Bulletin 38(12): 1097-1104.

Wisely, B. 1963. Detection and avoidance of a cuprous oxide antifouling paint by bivalve and gastropod larvae (Mollusca). Aust. J. Mar. Freshwater Res. 14(1): 60-69.

Yap, H.T. 1992. Marine environmental problems. Experiences of developing regions. Marine Pollution Bulletin 25(1-4): 37-40.

Young, J.S., Gurtisen, J.M., Apts, C.W., and Receliu, E.A. 1979. The Relationship between the copper complexing capacity of seawater and copper toxicity in shrimp zoeae. Marine Environ. Res.