Through the system, two mixtures of brachyuran zoeae were exposed to three concentrations of primary-treated sewage for 24 hours. A 24-hour LC50 was determined through analyses of the data.

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The culture of crab larvae in the laboratory has been the source of a great deal of research. Brachyurans, including the land crab, kelp crabs, and shore crabs have received the greatest amount of attention, possibly due to their economic value. Many species of brachyuran larvae have been subjected to salinity/temperature combinations (Costlow and Bookhout, 1968; Young and Hazlett, 1978), different feeding regimens (Anger, Klaus, Dawirs, Anger, and Costlow, 1981), and rearing in a variety of environmental systems. Some experiments are designed to examine the physiology of the larvae (Levine and Sulkin, 1979), describe the different stages in larval development (Costlow, et. al 1968), examine larval behavior (Sulkin, Van Heukelem, Kelly, and Van Heukelem, 1980), or investigate the possible advantages of a new culturing system (Costlow, et. al 1960; Buchanan, Myers, and Caldwell, 1975). This research investigated possible effects of antibiotic and feeding level.

The survival of larvae of the mangrove crab, Ucid cordatus, was studied in the laboratory. The larvae were reared in a specially designed culturing setup from first zoea to megalopa, at which point the experiment was terminated. Larvae were held under two different feeding levels and the conditions of antibiotic treatment or non-antibiotic treatment. The same culturing system was later

used for a 24-hour range finding bioassay using a mixture of species of crab larvae to assess toxicity levels in Puerto Rican primarily treated sewage. Data from the bioassay are presented to establish critical limits for future bioassays with crab larvae.

LITERATURE REVIEW

General culture technique of brachyuran decapods dictates the use of Artemia salina nauplii as the sole source of food (Roberts, 1975). Brine shrimp are easily hatched in a period of 48-72 hours, and are small enough in the nauplius stage to be handled by the mouthparts of the crab zoeae. Experimental work with the xanthid crab has shown the optimum feeding level of Artemia to be a food density of five nauplii per ml of seawater (cited in Levine, et. al., 1973). Feeding regimen is variable from report to report, ranging from three days weekly (Buchanan, et al., 1975) to daily feeding (Costlow, et. al., 1968; Levine, et. al., 1979).

Brachyuran larvae have been raised in a variety of rearing systems, both static and free-flowing, most systems surveyed being static. Young, et. al., (1981) separated zoeae into individual compartments of large trays, which were kept in environmental chambers under constant temperature and a fixed light schedule. Costlow, et. al. (1968) and Buchanan, et al. (1975) grouped larvae 10-20 per container. Costlow used an Eberbach variable speed shaker to maintain larvae, while Buchanan utilized a reverse-flow flowing water system.

Finally, Levine (1979) and Sutkin, et al. (1980) grouped up to 200 larvae in a single container and maintained them under constant temperature and light conditions. Antibiotics have been...

The text is widely used in culturing marine invertebrates. In the literature surveyed, limited information was found which compared the effect of antibiotics against two non-antibiotic controls. As stated in Sulkin et al. (1980), egg and larval survival are improved, with no effect on larval behavior, through the use of antibiotics (unpublished data). Sulkin's set-up used antibiotics continuously in the system, as contrasted with antibiotic axenization, a process whereby eggs and larvae are exposed to massive doses of antibiotics for brief periods of time. The advantage of axenization is that long-term effects of antibiotics, such as larval growth inhibition, are no longer critical factors (O'Agostino, 1975). In addition to reporting the findings of the culturing experiment, this paper also presents the results of a short exploratory bioassay conducted with a mixture of crab zoeae of several species. Bioassays are effective means to determine the toxicity of industrial wastes to a particular organism. The purpose of this 24 hr bioassay was to determine a concentration of sewage effluent which is lethal to 50% of the test organisms (a 24 hr LC50). Range finding, or exploratory bioassays function to determine concentrations of effluent which will be critical in future bioassays of longer endurance. One concentration in a range should kill all of the test organisms (a maximum dilution to be used), and one concentration should kill no organisms (a minimal dilution to be used).

Materials and Methods

Larvae Culturing:

A single ovigerous female mangrove crab was collected from the mangrove-lined channel of Joyuda Lagoon, a tropical lagoon on the west coast of Puerto Rico. The specimen was taken to the

laboratory, where its abdomen was removed, intact with pleopods and eggs. Pleopods were removed with their clusters of eggs intact and immersed in two successive water baths (250 ml) for one minute in each bath. Wash water (salinity 35 p.p.t., and temperature 27-41 °C) had been passed through a 6-micron filter. Gentle

Swirling of the eggs, by grasping the pleopod stem of each cluster, allowed debris to be washed free. Ninety percent of the 995 were then transferred with forceps to an antibiotic bath (5.5 mg Nitrofurazone, 2.3 mg Furazolidone, and .18 mg Methylene blue in one liter of water), and 10% were transferred to one liter of non-antibiotic water. Immersion time was 30 minutes. The antibiotic axenization method was based on Peltier (1978), which suggested 3-5 mg of nitrofurazone/ 1L for 30-60 minutes immersion. The antibiotic mixture used throughout the experiment was a commercial brand marketed as Furan-2. According to manufacturer specifications, each Furan-2 capsule contains 60 mg nitrofurazone, 25 mg furazolidone, and 2 mg methylene blue. The capsules also contain about 600 mg of a filler, so to partition a given mass of nitrofurazone, a simple proportionality using the mass of the entire capsule contents was used.

Following washing, the eggs were placed in the culturing system. The system consisted of two identical set-ups, each of the following design. Four, 502 ml plastic, circular containers were housed in a five-gallon insulated aquarium. Each container had approximately 35% wall area and 65% lid area removed and covered with 202-micron mesh, which was secured with silicone rubber. This allowed enough water to circulate through each container so that it would turn over in volume in less than an hour with 250 ml/minute flow into the aquarium. Containers floated, so they were anchored to the bottom with plastic bars. Water flow to the system was maintained by a single jet to the bottom of the aquarium (Figure 1). The system was located in an outdoor sheltered area.

Ten percent of the antibiotic eggs were transferred to containers 1-4 and placed in aquarium "A". These four containers were treated with antibiotic throughout the remainder of the experiment, and they were always maintained in the same aquarium. Non-antibiotic eggs went to containers 5-8 in aquarium "B", and the surplus antibiotic eggs...

The text was revised to:

Went to a five-gallon flowing water batch culture. Twenty-four hours later, containers were examined, and all unhatched eggs and larvae were removed to leave only 20 larvae per container. Since there was such limited survival in the non-antibiotic containers, all larvae for these containers were drawn from the batch culture. Thus all larvae in the culturing experiment hatched from antibiotically axenized eggs. Due to a lack of Artemia, the first feeding of the zoeae and formal initiation of the experiment were delayed to 48 hours after the initial introduction of the eggs to water. At 48 hours, time zero for the experiment, larvae were counted in each container. If a container held less than 20 zoeae, it was restocked from the batch culture. Since eggs were continually hatching in the batch culture up to this time, the beginning larval age could range from a few hours to almost 48 hours. Larvae were fed three times weekly (MMF), and on these same days, they were transferred to clean containers and antibiotically axenized, if from the appropriate group. Containers were removed from the aquarium and carefully rinsed down before and after opening. Larvae were individually transferred, via a mouth pipette, to a clean container, and during this process, larval live and dead counts were made with missing larvae counted as dead. Clean containers sat in two enameled trays, each holding two liters of water, one with antibiotic and the other without. The latter served as a control for this part of the experiment. Due to the mesh walls of

the containers, water from the trays circulated into them, filling them to approximately 80 ml. Immersion was for 40 minutes and was done outside to keep the bath temperature close to that of the culture temperature. The dirty containers from which the larvae were removed were washed in an Alconox solution, followed by a thorough tap, distilled, and sea water rinse. Antibiotic levels used ranged from...

The corrected text is as follows:

The range was 4.08-5.51 mg of nitrofurazone/l, averaging 4.65 mol/l. Correspondingly, average furazolidone and methylene blue concentrations were 1.94 mg/l and 0.156 mol/l, respectively. Following immersion, the containers were floated in the aquarium until fed, then capped and secured to the aquarium bottom. Feeding was at two levels: one group of zoeae received 1000 Artemia/container/feeding session, while the other group received 600 Artemia/container/feeding session. Each feeding group included two antibiotic and two non-antibiotic containers, thus providing two repetitions for each experimental condition.

The Artemia were cultured in one-gallon mason jars in room temperature water (27 °C; salinity 35 p.p.t.) under gentle aeration. On each feeding day, a small fluorescent lamp was placed near the 48-96 hr culture, and within 15 minutes, large numbers of nauplii and pre-zoeae were collected via pipette. Two, one ml subsamples were taken from the jar of collected brine shrimp and counted. Using the shrimp/ml estimates from the subsampling, appropriate volumes from the jar of Artemia were determined for feeding. Since the set-up was outside, it was subject to temperature fluctuations induced by the ambient temperature. Thermograms made with a Ryan thermograph on two separate occasions (four days each) showed a temperature of 27.8-42.8 °C on the first occasion and 29.5-49 °C for the second. There was a definite thermal cycle present in a 24 hr period. Since the flow into the aquariums was unregulated, it also varied greatly. Aquarium "A" flow was 229-461 ml/min., and aquarium "B" flow was 248-494 ml/min. Salinity of the water, which was unfiltered, ranged from 35-35.5 p.p.t. Data collected on the larvae were for mortality and appearance of megalopa, the first post-zoeal stage. When a megalopa appeared, it was removed from the experiment and counted as a larva that survived. A 24 hr range finding bioassay was conducted in the flow-through system. Zoeae were field collected in a half meter plankton net tow (202 micron mesh) from

"Zoeae from the Joyuda Lagoon were held in the lab for 24 hours without the addition of food. The water quality in the lagoon at the time of collection was 28.2 °C, 33 p.p.t., pH 8.2, and 0.0, <4 p.p.m. During the 24-hour holding period, a flow of bioassay dilution water (approximately 150 ml/min) was maintained to the holding bucket. The quality of this water appears in Table 1 under "Control". The collected zoeae were a mixture of species, all of the superfamily Brachyrhynca. The bioassay set-up consisted of four pairs of five-gallon aquariums, each pair receiving one of four concentrations of toxicant. The toxicant was primarily treated sewage outfall from Arecibo, Puerto Rico. Each aquarium held one flow-through container of the same design as mentioned in the previous section. This set-up was in accordance with APHA, AVMA, and WPC (1976) which indicates that true replicates must be made in which there is no water connection between the replicate containers. The concentrations of effluent used were 0 (control), 2.5, 5, and 10% by volume. Dilutions were made by adjusting the effluent flow rates with peristaltic pumps which drew the toxicant from 50-gallon barrels. The toxicant was mixed with dilution water, flow rate also regulated with peristaltic pumps, outside of the aquariums in Y-connectors and a length of tubing

(Figure 2). Water quality was monitored twice during the study (Table 1). Zoeae were drawn from the holding bucket with a mouth pipette and transferred to containers. Thirty-five zoeae of approximately the same size were placed in each container and fed a mixture of Artemia nauplii (200) and eggs (1400) before being introduced to the bioassay. Twenty-four hours later, containers were removed, rinsed free of effluent to stop the bioassay, and poured into counting chambers. Dead and live counts were made under low magnification, with missing larvae counted as dead. Data were plotted on semilogarithmic paper for the --Page Break-- TABLE 1. Water quality data for 24-hour range Dibrachyuran zoeae, Puerto Rico."

The following is the corrected text:

Finding bioassay on assorted toxicants was primarily treated sewage from Arecibo. Treatment dissolved repetition oxygen salinity at a number 3 post. One control 1 55 2.43 era Control 385 2128 era 3 360 27:43 era 3 33 273 era 50: 350 28:13 here 33 2 x0 28:28 here 2 ws 212 here ve 2 35.0 7838 here constant 1 36.0 28.622 hours, Generator 2 36.9 28:35 by hours 3 i 30°0 27:67 4 hours 0 2 i 30 27°30 by hours 50 i: 35.0 28:10 24 hours 30 2: 3 28.18 2 hours zs: ' 5.0 2812 by hours 25 2 33 B10 35.0 28.12 2 hours.

Purpose of graphical interpolation. This plotting method is used to estimate the 24 hr LC50 by plotting the concentration of effluent (& by volume) on a logarithmic scale against percent mortality on an arithmetic scale. A straight line is drawn between every two consecutive points, and the LC50 concentration is estimated from the line connecting the points which bracket the 50% mortality level on the arithmetic scale. The LC50 sewage concentration is simply read from the log scale (APHA, et al., 1976).

Results: Larvae Culturing

The culturing experiment endured for a period of 24 days after which all larvae had either died or reached megalopa (Table 2, Figures B and 4). Data for repetitions of a given condition were summed (thus 49 zoeae at time zero for each condition) and analyzed using a 2X2 chi-squared test for deviation from random expectation (Sokal and Rohlf, 1969). The results were shown to be statistically insignificant at the 95% confidence level. Since this is the case, results will be presented as trends. The only group of zoeae which produced megalopa were the antibioticly treated larvae. On day 13, only two non-antibiotic animals were alive compared to 13 living antibiotic animals. This illustrates the trend that, except for day 9, there were always more antibiotic than non-antibiotic animals alive. On day 9, there were an equal number of animals alive from both conditions. A total of five megalopa were produced from the antibiotic animals, making final mortality.

Ucides cordatus larvae were fine, at feeding level 1000 (1000 Artemia Satina Fea were given to each group of twenty animals on H¥F). Each curve represents the sum of dead animals from both replicates. Asterisks represent megalopa at the indicated day.

Table 3. Mortality of brachyuran zoeae involved in the 24-hour range finding bioassay. Percent mortality represents the total number of dead larvae. From each concentration (number dead out of initial 70 larvae), the toxicant used was primary-treated sewage from Arecibo, Puerto Rico. Concentration of Effluent (% by volume) is noted.

100% survival, and each successive effluent concentration shows decreasing survival, with mortality at 10% effluent approaching 100%. Data were analyzed using graphical interpolation (Figure 5), and the 24 hr LC50 value obtained was 6.723% effluent.

Figure 5. The concentration of Sewage (% by volume) in a semilogarithmic plot of brachyuran bioassay results showing the 24 hr LC50 value.

Discussion on Larvae Culturing: Even though the results of this culturing experiment are statistically insignificant using a G test, the trends observed are worthy of note. The fact that only antibiotic zoeae reached megalopa and always had a greater number of animals alive at a given time suggests positive results. Furthermore, the hatching of non-antibiotic eggs was visibly poorer than antibiotic hatching. I would definitely recommend the use of antibiotics in culturing crab larvae. Further research should investigate the effects of various antibiotics and their optimum concentrations to further improve survival of larvae.

Upon review of the literature, several distinct flaws of this research became apparent. Feeding problems were the major flaw, followed by factors beyond experimental control inherent in the laboratory water system, and possible handling technique problems. Feeding problems were two-fold. Primarily, an initial

The starvation period could have accounted for a large percentage of the mortality. In experiments with several species of brachyurans, initial starvation periods of 24-48 hours had profound effects upon larval survival, even if zoeae were re-fed adequately following starvation (Anger, et al. 1981). Starvation during the first 48 hours increased mortality before and during molting from zoea 1 to zoea 2, and also increased mortality of the first stage zoea after the first re-feeding. It also significantly lengthened the first zoeal stage and the length of development to megalopa by almost 50%. My experiment started 48 hours following egg immersion, so zoeae could have been starving for any period of time up to 48 hours prior to their first feeding. Clearly, initial starvation of larvae can have a very detrimental effect on the outcome of the experiment.

The second feeding problem was insufficient food density. Food levels were somewhat based on Buchanan, et al. (1975), but later literature review indicates that these levels were probably too low. As cited in Levine, et al. (1973), the optimum density of nauplii for the xanthid crab was five nauplii/ml, and these were daily feedings. The 1000 level feeding brought food density to two nauplii/ml, and the 600 level feeding was close to one nauplius/ml, and feeding was only three times weekly. Underfeeding probably explains the disappearance of some animals through cannibalism (witnessed on occasion) and perhaps also significantly contributes to the high mortality through starvation.

The second major source of problems came from the inadequacies of the laboratory water system. On one occasion, water flow had been accidentally shut-off and the water had completely drained from the aquarium, leaving very little water for the zoeae. Secondly, the water is not sterilized, so it may be rich in organics. The storage tank in which system water is held may accumulate debris and organic matter, and coupled with the warm temperature of the water, this could provide a breeding ground for harmful organisms.

Favorable environment for bacterial growth. Since there were no sterilizers or water filters smaller than 202 microns, bacteria were possibly a factor in the high larval mortality. When dead zoea were examined, they were loaded with ciliate bacteria grazers such as Uronema spp. In the literature reviewed, most setups relied on thoroughly filtered sterilized water for their systems.

The third source of problems may have been in handling technique. Larvae were transferred individually in a Pasteur pipette from container to container. The problem with this technique may be abuse to the zoea through over handling. The zoea's defense to disturbance is rolling into a ball, but its dorsal spine is still exposed. Fracturing the spine could make the larva very susceptible to infection. The rim of the pipette's bore may have damaged some larvae in this manner. From this research, the most important information gained was probably, 'how to do it better next time.' For future culturing attempts with crab larvae, I can make the following suggestions:

1. Use antibiotic axenization for eggs and larvae, perhaps on a schedule of four times weekly. Three times weekly still allowed a good number of bacteria to thrive as evidenced by the abundance of bacteria grazers on the dead organisms.
2. Feed larvae the suggested density of five nauplii/ml/day. This should ensure limited starvation and cannibalism. Feed Artemia in the nauplius (48-72 hours) stage. They are nutritionally more valuable than prezoeae and are easily handled by the crab zoeae.
3. Monitor hatching closely, keeping the number of eggs/container less than 300. When hatching is first observed, introduce food to the containers to avoid the lethal effects of early starvation.
4. Sterilize and filter system water so that the bacterial problem can be minimized.
5. Conduct pre-experiments to determine recoverability of zoeae with different handling techniques (pipetting, pouring).

In summary, the results of the range finding are useful in

"Evaluating bioassay methods for brachyuran larvae, and determining their sensitivity to Puerto Rican sewage toxicants. They indicate that the maximal bioassay sewage concentration used in future brachyuran bioassays with Arecibo sewage effluents probably need not exceed 10% by volume. In addition to information concerning sewage concentrations, the handling technique of pouring the flow-through container contents into a counting chamber was proven to be very effective in recovering the organisms from the containers. This is important information because handling assay organisms via a mouth pipette is potentially toxic to the experimenters. Recoverability was 100% in all control containers indicating that holding methods don't themselves contribute to larval mortality.

LITERATURE CITED

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1976. Standard Methods for the Examination of Water and Wastewater. Fourteenth Edition, American Public Health Association, Washington, D.C. pp. 683-686.

Anger, Klaus, R.R. Dawirs, V. Anger and J.D. Costlow. 1981. Effects of early starvation periods on zoeal development of brachyuran crabs. Biol. Bull., Vol. 161, pp. 199-212.

Buchanan, D.V., M.J. Myers and R.S. Caldwell. 1975. Improved flowing water apparatus for the culture of brachyuran crab larvae. J. Fish. Res. Board Can., Vol. 32, pp. 1880-1883.

Costlow, J.D., Jr. and C.G. Bookhout. 1960. A method for developing brachyuran eggs in vitro. Limnology and Oceanography, Vol. 5, pp. 212-215.

1968. The complete larval development of the land crab, Cardisoma guanhumi Latreille in the laboratory (brachyura, gecarcinidae), Crustaceana, Sup. 2 pp. 259-280.

1968. The effect of environmental factors on development of the land crab Cardisoma guanhumi Latreille. Am. Zoologist, Vol. 8, pp. 399-410.

DiAgostino, A. 1975. Antibiotics in culture of invertebrates. Culture of Marine Invertebrate Animals. Walter Smith and Matoira Chaney, Eds. Plenum Press, N.Y. pp."

109-131, Levine, O.G. and S.D. Sulkin. 1979. "Partitioning and utilization of energy during the larval development of the xanthid crab, Rhithropanopeus li Gould." J. Exp. Mar. Biol. Ecol., Vol. 40.

B.B Peltier, W. 1978. "Methods for measuring the acute toxicity of effluents to aquatic organisms." Environmental Monitoring and Support Laboratory, Office of Research and Development. U.S.E.P.A., Cincinnati, Ohio. EPA-600/4-78-012. p. 11.

Roberts, M.M., Jr. 1975. "Culture techniques for decapod crustacean larvae." Culture of Marine Invertebrate Animals. Walter Smith and Matoira Chenley, Eds. Plenum Press, N.Y. pp. 209-219.

Sok RAR. and F.J. RoAIF. 1969. Biometry. W.H. Freeman and Company, San Francisco, CA. pp. 588-592.

Sulkin, S.D., W. Van Heukelen, O. Kelly and L. Van Heukelen. 1980. "The behavioral basis of larval recruitment in the crab Callinectes sapidus Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis." Biol. Bull., Vol. 159. pp. 402-417.

Young, A.M. and T.L. Hazlett, III. 1978. "The effect of salinity and temperature on the larval development of Clibanarius vittatus Bose (Crustacea: Decapoda: Diogenidae)." J. Exp. Mar. Biol. Ecol. Vol. 34, pp. 131-141.