EFFECT OF HEAVY METALS ON SPAWNING AND HATCHING OF *Penaeus indicus* IN KWA ZULU-NATAL (AMATIKULU PRAWN FARM)

By

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DECLARATION

This is to declare that the results reported in this thesis are original research that I carried out at the Amatikulu Prawn Farm from May-July 2003 and it has not been previously submitted for any degree at any other university. I carried out all the data analysis procedures with the help of my colleague Mr. D. Y. Ghebrehiwet at the Zoology Department of University of Cape Town.

Mebrahtu Tesfaghiorghis Hagos

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ABSTRACT

Generally aquaculture continues to suffer from pollution and this has resulted in the decline of production. The effect of pollution can be directly on the organisms cultured causing immediate death or retarding their growth and making them vulnerable to a variety of diseases, or indirectly by reducing their reproductive capacity. This study was carried out to investigate the effect of zinc and lead on the hatched eggs, and zinc and copper on spawning and hatching of eggs from mature female *Penaeus indicus*. The experiment for each heavy metal was conducted separately.

In the post-spawning experiment, hatched eggs were counted and exposed to 0.0, 0.1, 1.0, 10.0 and 100 ppm of zinc and lead to see the effect of the heavy metals on hatching of the eggs and survival of the hatched eggs. Concentrations of 0.0, 0.1 and 1.0 ppm of zinc and lead did not show significant difference of hatch rate among each other but 10.0 and 100.0 ppm of zinc and lead gave significantly lower hatch rate as compared with the lower concentrations. Hatch rate completely failed at 100.0 ppm of both heavy metals.

In the pre-spawning experiment, ready-to-spawn females were exposed to 0.0, 0.01, 0.1, 1.0 and 10.0 ppm of zinc and copper to investigate the effect of the heavy metals on spawning and hatching rate. Concentrations of 0.1, 1.0 and 10.0 ppm of zinc and copper gave significantly lower hatch rate as compared to 0.0 and 0.01 ppm of the heavy metals. Hatch rate completely failed at 0.1, 1.0 and 10.0 ppm of the heavy metals.

In the EDTA experiment, ready-to-spawn females were exposed to 0.1 ppm of zinc and copper (in all tanks) and 0.0, 0.1, 1.0 and 10.0 ppm of EDTA in the case of zinc experiment and 0.0, 0.1 and 10.0 ppm of EDTA in the case of copper experiment. EDTA treatment for zinc and copper-polluted spawning tanks showed that there was significant difference in hatch rate between the EDTA treated and non-EDTA treated tanks, though there was no significant difference between the different EDTA treated tanks. Hatch rate completely failed in the non-EDTA treated tanks. There was also no significant difference in spawned eggs in all the EDTA and non-EDTA treated tanks.

1. INTRODUCTION

Aquaculture is an ancient practice, particularly in Asia, and has received increasing attention throughout the world over the past half century, because of its contribution to increasing food production. It has now become a major industry with numerous training centres and rapid technology transfer in many areas of the world. The increasing demand for seafood cannot, in the long run, be met only by intensifying the exploitation of wild stocks. Therefore, the present world production from aquaculture should be increased many times, but the necessary scientific know how needs to be clearly understood and implemented (Davenport *et al*., 2003).

However, there are so many factors that have to be considered when developing aquaculture facilities. Water quality is one of the most important of these. Water quality has a wide scope and encompasses all physical, chemical and biological variables that affect aquacultural productions. Most pond management procedures and efforts are aimed at improving chemical and biological conditions in ponds, as physical factors often are not controllable. Physical factors can only be improved through wise site selection, good design and construction practices, and management of ponds in harmony with existing climatic and geologic regimes (Boyd, 1990). Species selection is one of the major factors that determines the effect of physical factors on any given aquaculture development.

Shrimp farmers who rely on wild stocks for availability of post-larvae face a lot of problems. The problems include fluctuations on the availability of wild broodstock, on a day-to-day basis, as well as seasonal one, coupled with variable spawning performance and diseases. They may also cause substantial ecological effects by overexploiting wild stocks of postlarvae, or through associated by-catch of non-target species. These result in postlarvae supply often not being synchronized with demand of farms for grow-out production schedules (Boyd, 1990). Thus setting up a hatchery can minimize or even solve such problems. Even in a hatchery there are a lot of problems associated with availability of post larvae, natural mortality, diseases, fluctuation of physical parameters and some chemicals from the water itself, such as heavy metals.

Fairly high concentrations of heavy metals have been reported in surface waters of many nations. Thus the study of the toxicity of heavy metals to shrimp and other aquacultural species is important (Boyd, 1990). A variety of heavy metals are found as both naturally and anthropogenically-derived components of the estuarine environment. These metals may exist in several oxidation states with different reaction potentials, depending on their specific chemistries. The toxicity of heavy metals is related primarily to the dissolved, ionic form of the metal rather than the total concentration of the metal (Treece and Fox, 1993).

Estuaries and brackish waters, where the larval development of Penaeid shrimps normally takes place, are subject to heavy metal pollution through industrial effluents and domestic sewage disposals (Chinni *et al.*, 2000). Some may be acted upon by estuarine microbes to produce alkyl-metallic compounds that can be accumulated by estuarine species and are potent toxicants (Treece and Fox, 1993). The effect of a substance, in this case the heavy metals, on reproductive potential and growth is just as important as the direct toxicity of the substance. Unfortunately, most studies only include data on direct toxicity (Boyd, 1990).

Embryos and larvae of aquatic species show, especially during certain developmental phases, a high sensitivity towards toxic agents (Luckenbach *et al.*, 2001). Rosenthal and Alderdice (1976) also concluded that gametogenesis, embryogenesis and larval transition from an endogenous to an exogenous source of food are the most sensitive stages in the life cycle. Thus water quality of any aquaculture facility must be managed properly so that its production can achieve sustainability (Tilley *et al.*, 2002). In aquaculture, it would be advisable to maintain the concentration of a potential toxicant below the level that is known to have any adverse effect on the reproduction, health, and growth of the cultured species (Boyd, 1990).

South Africa has a long coastline on both the Indian and Atlantic Oceans. The country has more than 200 estuaries and coastal lagoons which could be utilized for aquaculture purposes. However, the country has only two shrimp farms which are found on the eastern coastline, due to the relatively warm waters of Agulhas current. The western and southern sections of the country have cold waters due to the presence of Benguela current. The latter sections are thus not suitable for shrimp aquaculture (Walmsley, 1986). Thus the increased demand for shrimps in foreign markets in general and local markets in particular has generated interest in shrimp farming in South Africa.

2. LITERATURE REVIEW

2.1. Life history of *Peneaus indicus*

Marine shrimps are distributed throughout the oceans of the world, but the commercially important species inhabit the shallower parts. *Peneaus indicus* is one of the most commercially important species. Due to its capability of fast growth its culture is practiced in many countries, including South Africa. It can reach an adult size of 18 – 30 cm in length. The sexes are separate and fertilization of females by the males takes place during their intermolt period. The sperm are stored by the females. The eggs are then released together with the sperm into the sea, where they hatch, after the hardening of the female exoskeleton. Once the eggs are hatched they pass through the nauplei, zoea, mysis and postlarval stages to complete their complicated life cycle. Each stage has a different body form and structure until the postlarval stage which has almost the same structure as the adult. Once they reach the postlarval stage they start migrating into the more shallow inshore brackish waters, where they will feed themselves due to the availability of detritus rich feeding grounds there until they return back to deeper parts of sea, where they will spend their life as adults (Swift, 1988).

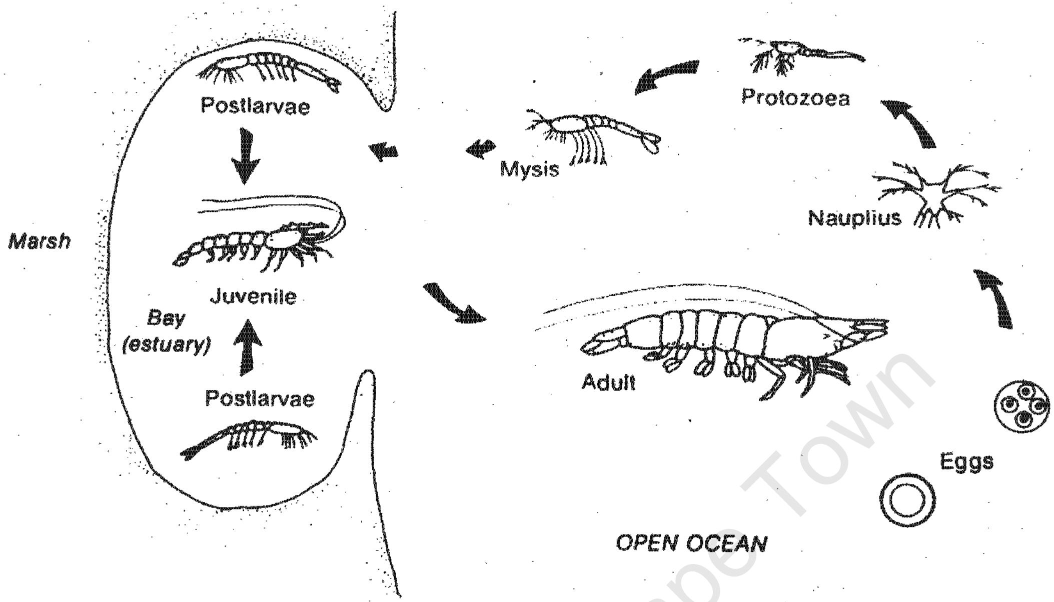

Fig. 1. Typical development stages of Penaeid shrimp. The adult and the juvenile stages have the same body structure (Treece and Yates, 1988).

2.2 Spawning and postlarvae development

Females from the wild environment are fertilized in the sea before capture and are then taken to the spawning ponds by selecting only those which have ripe ovaries. Usually spawning is hastened and ensured by upsetting the hormonal balance by the process known as ablation. Ablation is the process where one eye stalk, or its contents, is removed. Ablation removes an important endocrine organ of the shrimp, which results in a hormonal imbalance in the shrimp, bringing about the spawning of the female. The females are stocked in concrete tanks lined with epoxy resin. Each female spawns about 200 000 eggs and probably only half of these eggs hatch. They hatch in 14 hours at 28 °C.

The eggs hatch into nauplii which carry a yolk sac, which serves them as food, so that they do not take external feed. In the next 36 hours the nauplii undergo six moults before changing into the next stage, the zoea. During this time they are fed on diatoms which are cultured in separate culture tanks. The diatom *Skeletonema costatum* is commonly used as feed at this stage. The zoea change to mysis after moulting three times. This takes about four days. The mysis moult three times before they change into small adult forms, the postlarvae. The mysis commonly feed on the nauplii of brine shrimp, *Artemia salina* and diatoms. Rotifers are also cultured separately and are given to the prawns. The postlarvae, known as PLs, are fed on *Artemia* nauplii for about ten days and then fed with a variety of feed such as egg yolks, minced clam and manufactured dry feed. They are fed two to three times the body weight of the postlarvae a day (Swift, 1988).

In farm ponds all the above mentioned stages of the complicated life cycle do not happen in the growout ponds, as the physical and biological parameters of water in the ponds are different from their natural habitat. Thus an artificial hatchery with similar conditions as their natural habitat is required, so that the females will spawn and the hatched eggs will complete their life cycle. Therefore the seed for commercial farms is obtained either from the wild, where migrating stocks of wild juveniles are collected and taken to the grow out ponds, or it is produced in the hatchery of the farm either from wild fertile females or mature females from the farm (Swift, 1988).

2.3. Sources of pollution (heavy metals)

Based on their sources, the pollution of heavy metals in aquatic environment can be classified into two categories:

A variety of heavy metals, in different forms depending on their specific chemistries, are found as naturally derived components of the estuarine environment. Their presence in the aquatic environment is mainly as a result of natural processes, such as weathering and land drainage. Some of these heavy metals may be acted upon by estuarine microbes to produce alkyl-metallic compounds that can be accumulated by estuarine species and are potent toxicants (Treece and Fox, 1993). Spotte (1970) reported the copper level in normal seawater to be 0.003 ppm. According to Sverdrup *et al.*, (1970), Forstner and Wittman (1979) and Spotte (1970), normal sea water has 0 to 0.01 ppm of zinc in it respectively.

The other category includes the heavy metals that are added to the aquatic environment as a result of human activities. The sources of these heavy metals include agricultural run offs, waste disposal, industrial effluents and many others. They increase the concentration of the naturally-existing heavy metals. The heavy metals and other pollutants from the above sources directly pollute the estuarine environment as they are carried by rivers which flow into the sea via the estuarine environment. Estuarine environments are the nursery grounds for shrimps and other aquatic species. Thus excessive addition of heavy metals and other pollutants to the aquatic environment in general and to the estuarine systems in particular can have an adverse effect both on the animals and on people who eat them (Treece and Fox, 1993).

2.4. Direct effect of heavy metals

Most of the studies done on the effect of heavy metals are done on the direct toxicity of the heavy metals on fish, postlarvae and other species (Boyd, 1990; Szefer *et al.*, 1990). Different studies show that excessive addition of heavy metals to the aquatic environment could have an adverse effect both on animals and on people who eat them (Treece and Fox 1993).

The biological effects of metals are complicated by their synergetic and antagonistic interactions with other metals (Foster and Morel, 1982) and by metal speciation (Zamuda and Sunda, 1982). For example the toxicity of cadmium is influenced by zinc (Dunlop and Chapman, 1981) and chelating agents such as EDTA, NTA and Tris greatly reduce the toxicity of metals by sequestering reactive species (Engel and Sunda, 1979; Muramoto, 1980; Castille and Lawrence, 1981; Lawrence *et al.*, 1981). Heavy metals such as mercury, tin, palladium, platinum, gold and thallium, that can be methylated, should continuously be monitored, as some pose special threats to the aquatic environment. Others such as cadmium, lead, and zinc do not form stable alkyl-metals in aqueous solutions, but may have different modes of toxic action than do the alkyl-metals such as methylmercury, a neurotoxicant. Cadmium, like zinc and lead does not exist as an alkyl-metal in aquatic systems, but does exist as an ion, is a strong cytotoxicant to gill cells of crustaceans (Treece and Fox, 1993).

According to Couch (1979), in an experiment where the nauplii, protozoea and mysis of *P. aztecus* and *P. duorarum* were exposed to copper concentrations in a seawater-brine mixture similar to that derived from desalination plants, a copper concentration of 0.5 μg/lt was lethal to the larval stages. The same larval stages were able to grow normally in seawater (35 ppt) containing 0.025 mg/lt copper.

Reports from Chinnayya (1971) showed that zinc and lead caused a decrease in the rate of oxygen consumption in some fresh water shrimps and Dunlop and Chapman (1981) reported that zinc influences the toxicity of cadmium. The Environmental Agency of Japan (1972) set 0.1 ppm of zinc to be the upper limit of the heavy metal allowed in seawater for fisheries related activities.

The safe level of zinc and lead, and for copper are reported to be 100μg/liter and 25μg/liter, respectively (Boyd, 1990). However, these levels are the concentrations where 50 % of the adult animals under the direct effect of the heavy metals die in 96 hours exposure time. It is quite interesting to know the immediate effect of these heavy metals on spawning and fertilization of cultured species. Thus, it is important to know the lethal and safe concentration of any given heavy metal so that a hatchery would be sustainable in supplying post larvae for grow-out ponds. A variety of chelating agents such as EDTA are commercially used to reduce the toxicity of heavy metals (Mark *et al*., 1965).

A lot of studies have been done on shrimps to identify the specific organs that favour selectively the accumulation of heavy metals (Szefer *et al.*, 1990). Previous investigations on the accumulation of heavy metal concentrations have shown some tendencies where specific organs are selective in accumulating specific metals in their tissues. According to Eisler (1981) highest cadmium concentrations were found in hepatopancreas and digestive glands, and lowest in edible muscle. On the other hand higher zinc concentrations were recorded in viscera and muscle than other tissues (Horowitz and Presely, 1977; and Canali and Furness, 1993; Páez-Osuna *et al.*, 1995). Bryan (1968), Szefer *et al.* (1990), Darmono and Denton (1990), and Canali and Furness (1993) reported that the hepatopancreas exhibited the highest concentrations of copper. However, Páez-Osuna and Tron-mayen (1996) showed that *P. vannamei*, which has a typical life cycle of Penaeid shrimps (Hendrickx, 1995), had the highest concentrations of copper in gills and antennal organs both in wild and farmed population. Hepatopancreas and exoskeleton exhibited intermediate levels, whereas muscle presented the lowest copper levels. In the same study zinc was found in large quantities in the hepatic material and gills, and lowest concentrations in the exoskeleton. In all tissues lead was below the limit of analytical detection.

2.5. Indirect effect of heavy metals

It is unfortunate that most studies include data only on direct toxicity of heavy metals. However, the effect of substances, in this case heavy metals, on reproductive potential and growth is just as important as the direct toxicity of the substances (Boyd, 1990). In an experiment done by Liu and Chen (1987), on the hatching rate of *Artemia* cysts in seawater containing heavy metals, a negative linear relationship between hatching rate of *Artemia* cysts and heavy metal concentration was observed. They also found that heavy metal concentration in *Artemia* nauplii increased linearly with an increase in the heavy metal concentration.

Lorenzo *et al.* (2001) reported that immersion of the shrimp, *Palaemon elegans* (Rathke) in artificial seawater containing mercury, cadmium, copper, cromium, zinc, or lead caused a decrease in the haemocyte count during the first eight hours exposure, although the haemocyte count returned to the initial level over the following 16 hours immersion.

In a study done by Yuan *et al.* (1992), on egg hatching and metamorphosis to protozoea of *Penaeus chinesis* (Osbeck) by removal of heavy metals from rearing systems with polymeric absorbent, the embryogenesis of the species was stopped at concentrations above 52.1 μg/l copper, 112.5 μg/l zinc, 504.8 μg/l lead and 100.8 μg/l cadmium in the absence of the polymeric heavy metal ions absorbent (PHMA), and at concentrations above 1.0 mg/l copper, 1.5 mg/l zinc, 1.5 mg/l lead and 1.0 mg/l cadmium in the presence of PHMA.

2.6. EDTA (Ethylene dinitrotetraacetic acid) Treatment

Chelating agents are synthetic chemicals that are used in aquaculture hatcheries in order to avoid toxic effects of heavy metals to crustaceans of commercial importance during their early stages of life (Treece and Fox, 1993). Chelating agents such as EDTA have been used in penaeid hatcheries for nearly half a century (Cook and Murphy, 1966; 1969). They have been used with an application rate of 10.0 ppm on nauplii and the other larval stages of penaeid shrimps (Cook, 1969).

A study done by Licop (1988) on the effects of sodium-EDTA on the survival and metamorphosis of *Penaeus monodon* larvae show that larval survival was highest at levels of 5 and 10 ppm, both levels being significantly different from the control. This was attributed to the fact that the toxicities of heavy metals were reduced in the presence of Na - EDTA. The same explanation was also given by Lawrence *et al.* (1981) and Castille and Lawrence (1981) for experiments with *P. stylirostris*. The study further explains that the addition of Na-EDTA did not affect the larval growth.

Given the continuous rate of waste and industrial disposals to the sea, application of chelating agents may provide safe water qualities for intensification of culture operations in shrimp hatcheries (Licop, 1988). Other chelating agents such as citrates (Droop, 1961; Fox, 1983) and nitriloacetic acid (NTA) (Fox, 1983) might also be used in hatchery operations. However, care must be taken as overchelation of trace metals, which are very important for the growth and development of larvae, might occur (Johnson, 1964; Davey *et al.*, 1973). This is why only dosages of 5 and 10 ppm are considered to be safe levels to be applied for shrimp larval rearing procedures (Licop, 1988).

A heavy metal, zinc, was used as an anode in water pumps in the Amatikulu Prawn Farm to avoid rusting of the water pumps. In such situations some considerable concentration of the heavy metal might have mixed with the water that was used for the hatchery. If the concentration of the heavy metal is beyond a certain minimum level, then spawning and fertilization may not take place. Such incidents have been observed in the Amatikulu Prawn Farm, KZN, where *Peneaus indicus* stopped spawning and fertilization (Laurence, Personal Communication).

This paper will investigate the effect of zinc and copper on spawning and hatching of *P. indicus* by adding these two heavy metals into two different sets of tanks, where female *P. indicus* spawn. Zinc and lead will also be added into the tanks after a female *P. indicus* has spawned to ascertain the effect of the latter two heavy metals on hatching. After the lowest level of the heavy metals that has adverse effect on spawning and hatching have been identified, EDTA treatment are undertaken to see if it helps to improve the spawning and hatching conditions of *P. indicus*.

3. MATERIALS AND METHODS

3.1. Species and site description

Amatikulu Prawn Farm is located in KwaZulu-Natal, on the eastern coastline of South Africa, near the small town of Ginginhlovu. This farm cultures *Penaeus indicus,* which is one of the most important shrimps in the world market. The farm has about 36 ha of earthen ponds where the postlarvae are grown until they reach marketable size. The hatchery is constructed on an elevated plot along-side the Amatikulu River, where clean seawater of 29-30°/oo, as well as fresh water, are available. The elevation is also very suitable for the drainage system. The hatchery supplies postlarvae to 10 ha of nearby earthen ponds and there is another hatchery for the rest of the earthen ponds. This experiment was conducted from May-July 2003.

3.2. Water supply for the hatchery

The Amatikulu River passes between the hatchery and the Indian Ocean before it joins the ocean (Fig. 2). The pipes from which the hatchery gets seawater are laid across the river into the coast of the Indian Ocean. The river and the ocean are separated by a dune with dense trees. The hatchery gets seawater from a well dug on that beach. The advantage of taking water from a well dug in sand is that the sand plays a great role in purifying the water. It is basically a mechanical filtration system where larger particles are trapped in the sand.

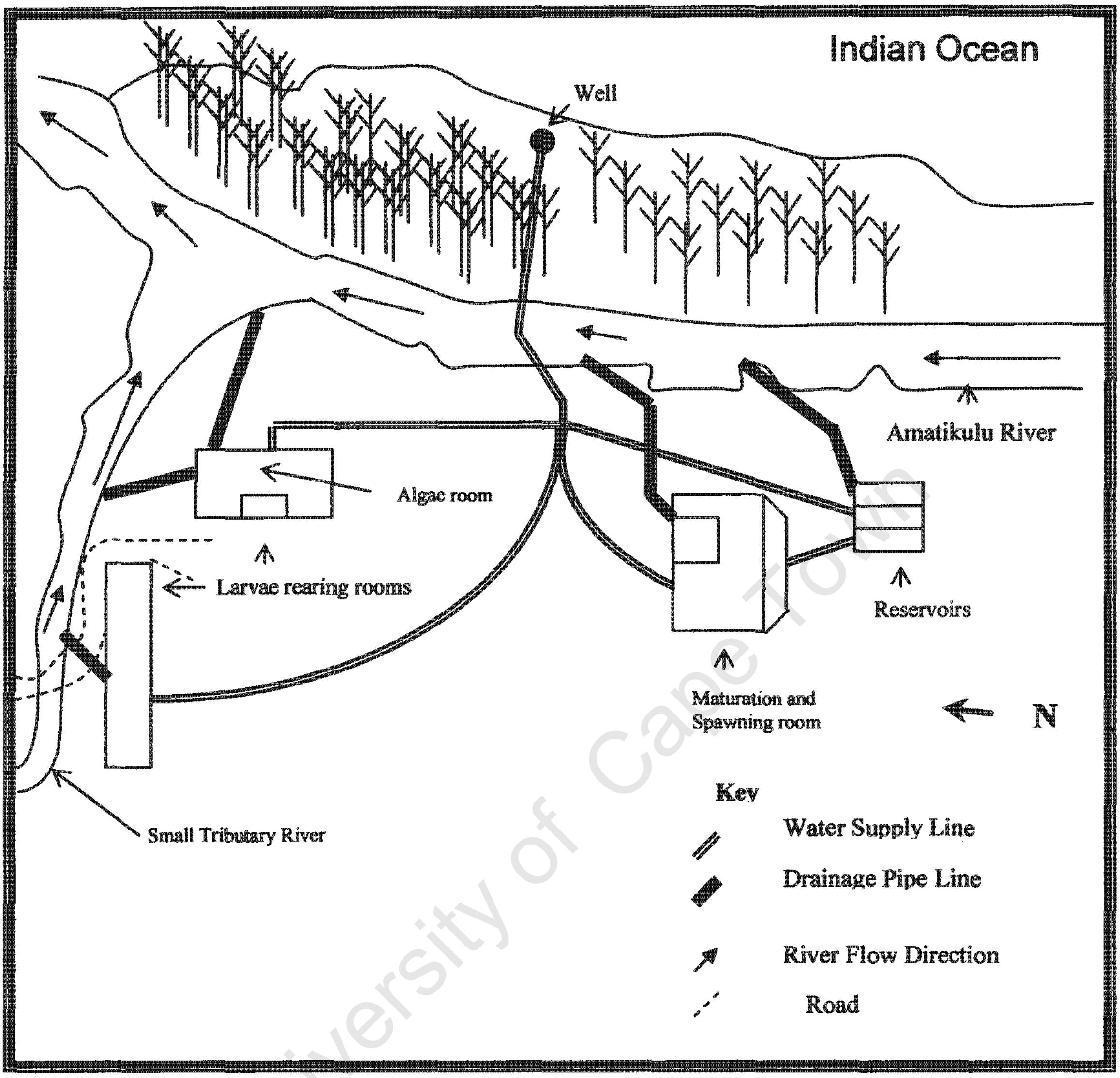

Fig.2. Sketch of the location of Amatikulu Prawn hatchery in KZN. The earthen ponds are located about 3 km away from the hatchery.

The water for the maturation tanks is pumped from the well to large three reservoirs, where further settlement of suspended particles takes place. The reservoirs are continuously washed, after the water is finished, to remove settled particles. The water is then pumped into a series of tanks with oyster shells and fine nets to further block suspended particles from entering the maturation tanks. The water is then pumped into especially designed filter tanks for further filtration. Finally the water passes through a very fine mesh to make its way to the maturation tanks. The water in the maturation tanks is heated to 28 °C by thermostatically controlled heaters to maintain the optimum temperature for the breeders. To avoid the loss of energy, since the heaters consume high voltage; the heated water from the maturation tanks is circulated in the system with little addition of new seawater from the reservoirs. Lime treatment is done once in a week to adjust the PH of the water in the maturation tanks to 7.8 – 8.2. The salinity of the water is 30 $^0/_{00}$.

The water for the spawning tanks is also pumped from the same well, but it does not go to the reservoirs. It is directly UV filtered and poured into the spawning tanks. The seawater passes through a special, wider tube where it has UV light inside. The UV light is switched on whenever seawater is pumped into the spawning tanks killing some micro-organisms that can cause harm during spawning.

3.3. Heavy metals

The heavy metal ions were in solutions of zinc ($ZnSO_4.7H_2O$), copper ($CuSO_4.5H_2O$) and lead ($PbSO_4$).

3.4. Feeding schedule and air supply

There were five maturation tanks (5 x 1.0 x 1.6m) and 90 breeders (60 females and 30 males) were stocked in each tank. They were fed five times a day with squid, sardine and pellets (especially made for breeders at the farm) at regular intervals. Remaining feed was cleaned twice a day. The maturation and spawning rooms were painted in black and the breeders in the maturation room got 14 hours of light and 8 hours of darkness.

An air blower was placed in a room along-side of the spawning room. It supplied air to the spawning tanks, maturation tanks, reservoir tanks and to the tanks where all the mechanical and physical filtration of the water took place.

3.5. EXPERIMENTAL PROTOCOL

Four rectangular spawning tanks of size 5.0 x 1.2 x 0.5 m and four circular tanks of 500 L were prepared for conducting the experiment. Three heavy metals: zinc, copper and lead, were selected to test their effect on spawning of *P. indicus*. Different concentrations, 0.001, 0.1, 1.0, 10 and 100 ppm of these three heavy metals were used in the experiment. The experiments for the three heavy metals were done separately. The four spawning tanks had different concentrations of a given heavy metal at a time. Thus the experiment for a given heavy metal at different level of concentrations was done simultaneously. Each of the rectangular and circular tanks was filled with 960 and 400 L of water respectively. The volume of the experimental tanks was determined by the time required to fill a 20 Lt bucket. The sample was taken several times. Average time was taken and multiplied to make the above mentioned volumes. Marks were made on the tanks for continuous use. Temperature was maintained at 28 °C using thermostatically controlled heaters (Treece and Yates, 1988).

Female breeders were always checked after sunset because it was difficult to see the eggs during day time and they spawn after sunset in darkness. From 6:00 – 6:30 pm the lightly green painted lights of the maturation room were switched off. The water and air supply were cut to calm the continuously stirred and circulated water of the maturation tanks. A water-proof torch tied to an end of a long rod was used to light each shrimp from below. Since light can penetrate through the shrimps the eggs, which appear as black in the contrast of light, were clearly seen from the dorsal side of a female breeder. Projections of the eggs on both sides of the intestine near the head were signs of a ready female breeder. Each of the maturation tanks was searched in the same way for the best ready female breeders. The females were then taken to the spawning tanks. After they had spawned, the length and weight of each breeder was measured by a measuring tape and a digital balance respectively.

3.5.1. Pre-spawning experiment

A pre-spawning experiment was carried out for zinc and copper. The different concentration of the heavy metals were added before a mature and ready-to-spawn female *P. indicus* was brought from the maturation tanks to the spawning tanks. The addition of the heavy metals was done when the tanks were filled with water from 3:00 – 5:00 pm. The heaters were switched on to keep the water temperature in the spawning tanks at 28 °C. From 6:00 – 6:30 pm the ready-to-spawn females were brought from the maturation tanks to the spawning tanks. One female was put in each tank. The spawning room was kept dark so that the females would release their eggs, where external fertilization takes place. Males and females mated while they were in the maturation tanks, which were different from the spawning tanks. The same procedure was followed for the other tanks with different concentration of the heavy metal under experiment. At 6:00 am the next day, the water in the tank was mixed homogeneously and softly, so as not to damage the eggs, but so that the released eggs would be dispersed through-out the tank, and a sample of 250 ml water was taken. Eggs were counted and multiplied by four to make it one liter and then by the volume of the water in the tank to estimate the number of eggs per female. In the afternoon, 1:00 – 3:00 pm, tanks were homogeneously mixed and samples of 250 ml water were taken. Hatched eggs or nauplii were counted and multiplied by the volume of water in the tank to estimate the number of nauplii per female. Five samples from each tank were taken and counted. The same procedure was applied for the rest of the tanks that had different concentration of the heavy metal under experiment. The experiment continued daily for 10-20 days. The same procedure was also applied for the other heavy metals.

There was a control, which was done under zero concentration of the heavy metals. This was used as a reference bottom line for the effect of heavy metals on spawning.

3.5.2. Post-spawning experiment

A post-spawning experiment was carried out for zinc and lead. In the post-spawning test, heavy metal concentrations were added when eggs were released. Usually females spawn throughout the night, so the chemicals were added early in the morning when the eggs were counted. In the afternoon the hatched eggs under different concentration levels were counted.

There was also a control, which was used to know the natural mortality of nauplii under ambient water conditions.

3.5.3. EDTA (Ethylene Diamin Tertraacetic Acid)

From the pre-spawning experiment, a lethal concentration of the heavy metals of zinc and copper was identified. The lowest lethal concentration of the heavy metals was then treated with different concentration of EDTA. The heavy metal under experiment and EDTA were added before the females were brought to the spawning tanks. The eggs and nauplii were counted in the morning and in the afternoon, respectively.

3.6. DATA ANALYSIS

For the pre-spawning experiments of zinc and copper, the effects of the heavy metal ion concentrations on spawning and hatching were analyzed by a non-parametric, Tukey HSD analysis to see if there is a significant difference in spawned eggs, hatch rate and hatched eggs between each concentration of the heavy metals. Where the interactions of the effects of EDTA and the concentrations of the heavy metal ions were significant, differences in e.g. spawning and hatching due to the presence or absence of EDTA were evaluated separately at each concentration.

For the post-spawning experiments of zinc and lead, the effects of the heavy ion concentrations on hatching of the spawned eggs were analyzed by Tukey HSD method of analysis (Zar, 1999).

The non-parametric means of analysis, Tukey HSD, was also used to see if there was a significant difference in the number of eggs spawned and hatched in the EDTA experiment.

Bar charts were plotted for each experiment to observe the trends of spawning and hatching in respect of the different heavy metal concentrations.

4. RESULTS

4.1. Post-spawning for zinc

The posthoc analysis, Tukey HSD analysis, for the effect of different zinc concentrations on the mean hatch rate of *P. indicus* (Table 1) shows that there is no significant difference in the mean hatch rate between the control, 0.1 and 1.0 ppm of zinc, whereas 10.0 and 100.0 ppm show significant difference of mean hatch rate among each other and between the other concentrations of the heavy metal. At 100.0 ppm of zinc concentration hatching completely fails.

Concentrations Of zinc	**0.0 ppm** **M=78.90±13.88**	**0.1 ppm** **M=84.95±9.29**	**1.0 ppm** **M=79.97±15.90**	**10.0 ppm** **M=57.87±28.93**	**100.0 ppm** **M=0.000**
0.0 ppm					
0.1 ppm	0.847126				
1.0 ppm	0.999791	0.902624			
10.0 ppm	**0.006153**	**0.000183**	**0.001872**		
100.0 ppm	**0.000121**	**0.000121**	**0.000121**	**0.000121**	

Table 1. Tukey HSD analysis for the effect of zinc concentrations on the mean hatch rate of *P. indicus*. M is the mean hatch rate at each concentration. Highlighted differences are significant at p < (0.05).

Figure 3 shows the trend of the mean hatch rate under different concentrations of zinc. The mean hatch rate drops drastically from 10.0 ppm and reaches zero when the concentration is 100.0 ppm.

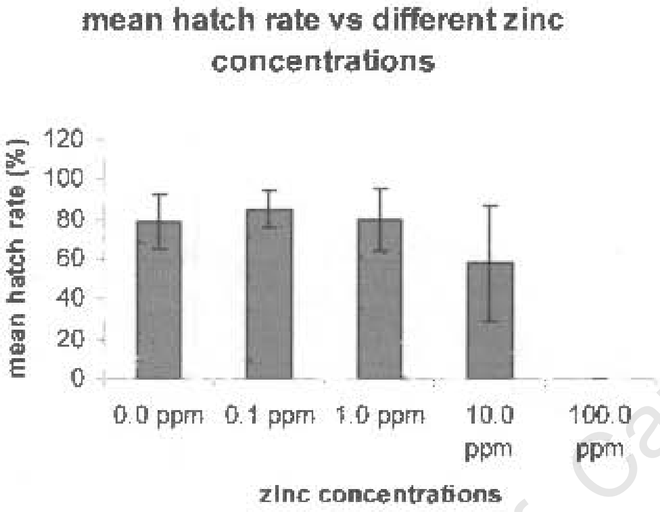

Fig 3. Plot of mean hatch rate at different concentrations of zinc.

4.2. Post-spawning for lead

The Tukey HSD analysis for the effect of different lead concentrations on hatch rate of *P. indicus* (Table 2) shows that only 100.0 ppm of lead brings about a significant decline in hatch rate, where the hatch rate completely fails.

Concentrations Of lead	**0.0 ppm** **M=77.63±13.60**	**0.1 ppm** **M=77.79±18.79**	**1.0 ppm** **M=67.12±17.82**	**10.0 ppm** **M=71.25±27.23**	**100.0 ppm** **M=0.000**
0.0 ppm					
0.1 ppm	1.000000				
1.0 ppm	0.757469	0.747026			
10.0 ppm	0.950127	0.945543	0.989892		
100.0 ppm	**0.000123**	**0.000123**	**0.000123**	**0.000123**	

Table 2. Tukey HSD analysis for the effect of lead treatment on hatch rate of *P. indicus*. M is the mean hatch rate under different lead concentrations. Highlighted differences are significant at p < (0.05).

Figure 4 shows the trend of mean hatch rate under different concentrations of lead. At 100.0 ppm of lead there is no hatching of *P. indicus* eggs

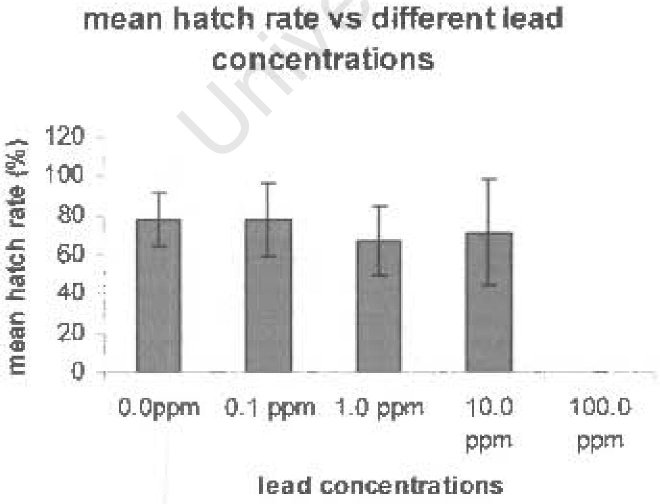

Fig. 4. Plot of mean hatch rate at different concentrations of lead.

4.3. Pre-spawning for zinc

In the pre-spawning experiment of zinc (Table 3) there is no significant difference in the hatch rate between the control (0.00 ppm zinc) and 0.01 ppm of zinc, but there is a significant difference between control and 0.01 ppm, and the higher zinc concentrations, where hatch rate completely fails.

Concentrations Of zinc	**0.0 ppm** **M=78.90±13.88**	**0.01 ppm** **M=65.64±13.38**	**0.1 ppm** **M=0.000**	**1.0 ppm** **M=0.000**	**10.0 ppm** **M0.000**
0.0 ppm					
0.01 ppm	0.228585				
0.1 ppm	**0.000125**	**0.000125**			
1.0 ppm	**0.000125**	**0.000125**	1.000000		
10.0 ppm	**0.000125**	**0.000125**	1.000000	1.000000	

Table 3. Tukey HSD analysis for the effect of zinc treatment on hatch rate of *P. indicus*. M is the mean hatch rate at different zinc concentrations. Highlighted differences are significant at $p < (0.05)$.

There is no significant difference in the hatched eggs between control and 0.01 ppm zinc (Table 4). However, the difference is significant between control and the other zinc concentrations beyond 0.01 ppm zinc, with a complete failure of hatching in the latter concentrations. There is no significant difference of hatched eggs between 0.01 and 0.1, 1.0 and 10.0 ppm of zinc.

Concentrations Of zinc	**0.0 ppm** **M=63780±36408**	**0.01 ppm** **M=28405±27519**	**0.1 ppm** **M=0.000**	**1.0 ppm** **M=0.000**	**10.0 ppm** **M=0.000**
0.0 ppm					
0.01ppm	0.179723				
0.1 ppm	**0.005713**	0.470035			
1.0 ppm	**0.002170**	0.378614	1.000000		
10.0 ppm	**0.002170**	0.378614	1.000000	1.000000	

Table 4. Tukey HSD analysis for the effect of zinc concentrations on hatched eggs of *P. indicus*. M is the mean hatched eggs at different zinc concentrations. Highlighted differences are significant at $p < (0.05)$.

Table 5 shows that there is no significant difference in the spawned eggs between control and 0.01 ppm zinc, whereas the difference is significant between control and the other zinc concentrations beyond 0.01 ppm zinc. There is no significant difference of spawned eggs between 0.01 and, 0.1, 1.0 and 10.0 ppm of zinc.

Concentrations Of zinc	0.0 ppm M=83524±36969	0.01 ppm M=41596±32631	0.1 ppm M=25427±24848	1.0 ppm M=29933±23205	10.0 ppm M=20533±13982
0.0 ppm					
0.01 ppm	0.156256				
0.1 ppm	**0.040745**	0.921690			
1.0 ppm	**0.037839**	0.965463	0.999414		
10.0 ppm	**0.010106**	0.765310	0.999159	0.984349	

Table 5. Tukey HSD analysis for the effect of zinc concentrations on spawned eggs of *P. indicus*. M is the mean spawned eggs at different zinc concentrations. Highlighted differences are significant at p < (0.05).

Figure 5 shows the trend of mean hatch rate under different zinc concentrations. Hatch rate fails starting from 0.1 ppm zinc.

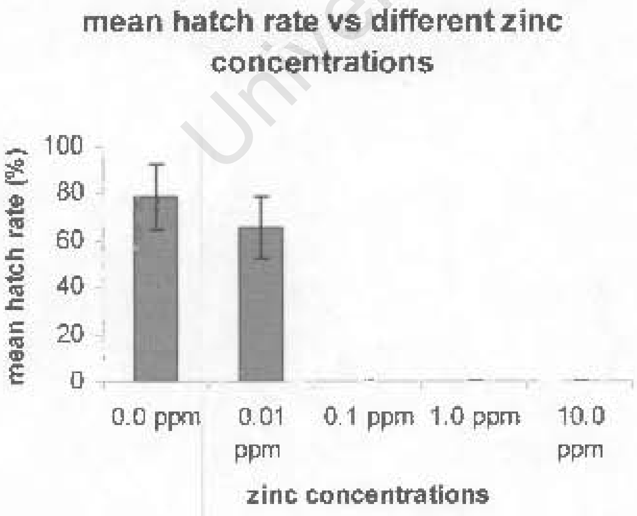

Fig. 5. Plot of mean hatch rate at different concentrations of zinc.

4.4. Pre-spawning for copper

Control and 0.01 ppm copper show (Table 6) no significant difference of hatch rate, whereas the difference is significant between them and the other concentrations. Concentrations of 0.1, 1.0 and 10.0 ppm copper have no significant difference among each other. The hatch rate completely fails in the later concentrations.

Concentrations Of copper	**0.0 ppm** **M=59.78±26**	**0.01 ppm** **M=47.02±37**	**0.1 ppm** **M=0.000**	**1.0 ppm** **M=0.000**	**10.0 ppm** **M=0.000**
0.0 ppm					
0.01 ppm	0.843071				
0.1 ppm	**0.000690**	**0.007480**			
1.0 ppm	**0.001758**	**0.016241**	1.000000		
10.0 ppm	**0.001758**	**0.016241**	1.000000	1.000000	

Table 6. Tukey HSD analysis for the effect of copper concentrations on mean hatch rate of *P. indicus*. M is the mean hatch rate at different copper concentrations. Highlighted differences are significant at $p < (0.05)$.

Table 7 shows that there is significant difference of hatched eggs between control and, 0.1, 1.0 and 10.0 ppm copper concentrations, whereas there is no significant difference among 0.01, 0.1, 1.0 and 10.0 ppm copper. There is also no significant difference between the control and 0.01 ppm copper in the hatched eggs.

Concentration Of copper	**0.0 ppm** **M=34614±20233.67**	**0.01 ppm** **M=19970±22317.40**	**0.1 ppm** **M=0.000**	**1.0 ppm** **M=0.000**	**10.0 ppm** **M=0.000**
0.0 ppm					
0.01 ppm	0.432926				
0.1 ppm	**0.003348**	0.158972			
1.0 ppm	**0.007937**	0.228650	1.000000		
10.0 ppm	**0.007937**	0.228650	1.000000	1.000000	

Table 7. Tukey HSD analysis for the effect of copper concentrations on hatched eggs of *P. indicus*. M is the mean hatched eggs at different copper concentrations. Highlighted differences are significant at $p < (0.05)$.

The different copper concentrations and mean hatch rate plot shows (Figure 6) that the drastic decline of hatch rate as the copper concentration increases. Starting from 0.1 ppm copper the hatch rate completely fails.

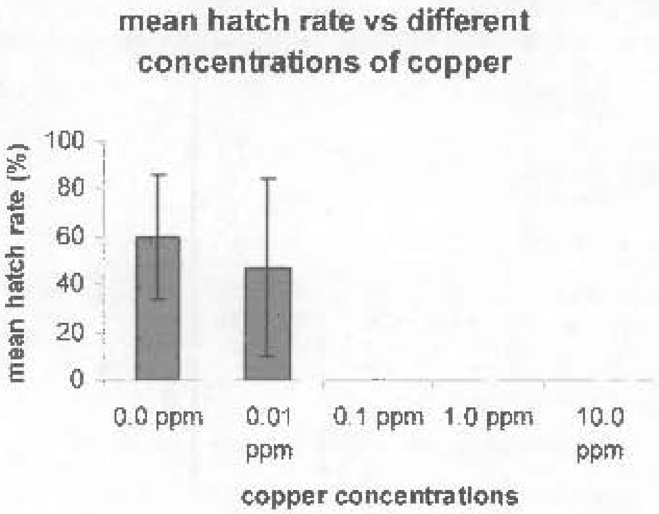

Fig. 6. Plot of mean hatch rate at different concentrations of copper.

4.5. EDTA TREATMENTS

4.5.1. Zinc

Table 8 shows that there is significant difference in the hatch rate between 0.1 ppm zinc and 0.0 ppm EDTA and the other concentrations, whereas there is no significant difference among the remaining treatments. The hatch rate has greatly improved in the later concentrations.

Concentrations of EDTA	**0.0 ppm EDTA** **M=0.000**	**0.1 ppm EDTA** **M=43.92±26.27**	**1.0 ppm EDTA** **M=51.43±10.25**	**10.0 ppm EDTA** **M=55.70±22.62**
0.0 ppm EDTA				
0.1 ppm EDTA	**0.010842**			
1.0 ppm EDTA	**0.007632**	0.945092		
10.0 ppm EDTA	**0.001544**	0.718864	0.989080	

Table 8. Tukey HSD analysis for the effect of EDTA treatment on the hatch rate of deliberately zinc polluted spawning tanks of *P. indicus*. The concentration of zinc in all cases was 0.1 ppm. M is the mean hatch rate at different EDTA concentrations and 0.1 ppm zinc. Highlighted differences are significant at $p < (0.05)$.

There is no significant difference among all the treatments in the spawned eggs (Table 9) but the spawned eggs have improved as the EDTA concentration increased.

Concentrations of EDTA	**0.0 ppm EDTA** **M=25427±24848.94**	**0.1 ppm EDTA** **M=48328±25127.45**	**1.0 ppm EDTA** **M=45955±10416.15**	**10.0 ppm EDTA** **M=55405±36899.22**
0.0 ppm EDTA				
0.1 ppm EDTA	0.562443			
1.0 ppm EDTA	0.717787	0.999378		
10.0 ppm EDTA	0.339530	0.969293	0.960824	

Table 9. Tukey HSD analysis for the effect of EDTA treatment on the spawned eggs of deliberately zinc polluted spawning tanks of *P. indicus*. The concentration of zinc, in all cases, was 0.01 ppm. M is the mean spawned eggs at different EDTA concentrations and 0.1 ppm zinc. Highlighted differences are significant at $p < (0.05)$.

Figure 7 shows that the improved hatch rate of *P. indicus* as the EDTA concentration increased. In the case where there is no addition of EDTA (0.0 ppm), the hatch rate completely fails.

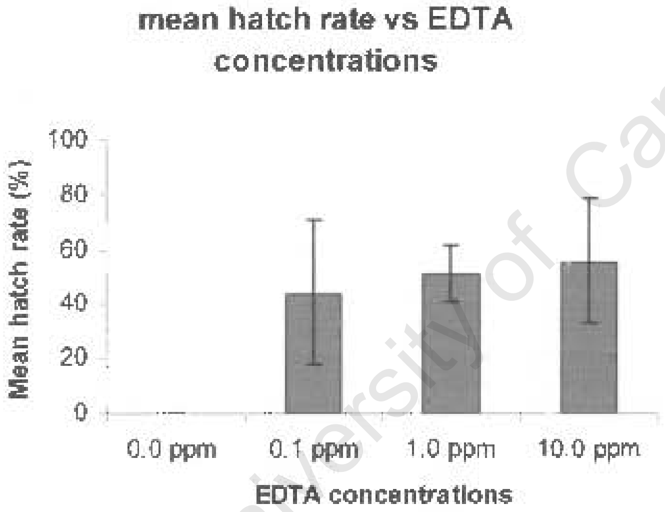

Fig. 7. Plot of mean hatch rate at different concentrations of EDTA treatments on zinc polluted spawning tanks.

4.5.2. Copper

There is significant difference in the hatch rate between the treatment (0.1 ppm copper and 0.0 ppm EDTA) and the other treatments, whereas there is no significant difference between the remaining treatments.

Concentrations of EDTA	**0.0 ppm EDTA** **M=0.000**	**0.1 ppm EDTA** **M=47.99±22.39**	**10.0 ppm EDTA** **M=64.10±11.24**
0.0 ppm EDTA			
0.1 ppm EDTA	**0.000296**		
10.0 ppm EDTA	**0.000178**	0.179571	

Table 10. Tukey HSD analysis for the effect of EDTA treatment on the hatch rate of deliberately copper polluted spawning tanks of *P. indicus*. The copper concentration was 0.1 ppm in all cases. M is the mean hatch rate at different EDTA concentrations and 0.1 ppm copper. Highlighted differences are significant at $p < (0.05)$.

Table 11 shows that there is no significant difference of spawned eggs among all the EDTA treatments of copper.

Concentrations of EDTA	0.0 ppm EDTA M=55656±33082	0.1 ppm EDTA M=52378±28732	10.0 ppm EDTA M=45669±25996
0.0 ppm EDTA			
0.1 ppm EDTA	0.982933		
10.0 ppm EDTA	0.826792	0.930465	

Table 11. Tukey HSD analysis for the effect of EDTA treatment on the spawned eggs of deliberately copper polluted spawning tanks of *P. indicus*. The copper concentration was 0.1 ppm in all cases. M is the mean spawned eggs at different EDTA concentrations and 0.1 ppm copper. Highlighted differences are significant at $p < (0.05)$.

The plot of mean hatch rate vs EDTA treatment of copper polluted spawning tanks (Figure 8) shows the dramatic increase of hatch rate as EDTA concentration increases.

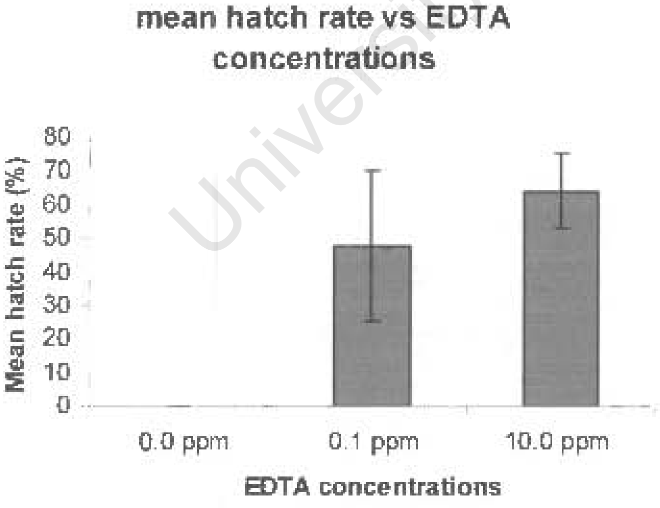

Fig. 8. Plot of mean hatch rate at different concentrations of EDTA treatments on copper polluted spawning tanks.

5. DISCUSSION

Environmental pollution by heavy metals can be either anthropogenic or natural. However, metals from all sources are threat to many species of aquatic animals worldwide (Lorenzon *et al.*, 2000). Estuaries and brackish waters, the breeding and nursery grounds for many marine species, are especially subject to heavy metal pollution through industrial effluents and domestic disposals, as they are subjected to the continuous and seasonal running rivers that carry different forms of pollutants (Chinni *et al.*, 2000). The physical, chemical and biological parameters are continuously altered as there is addition and dilution of fresh water from the rivers. Therefore estuaries are prime examples of multivariate and complex ecosystems where no single parameter is changed without affecting other parameters (Treece and Fox, 1993). Thus Penaeid shrimp have to face all the ever changing factors as they have to migrate from the open ocean to the tidal estuary at early stages of their life cycle.

5.1. Direct effect of heavy metals

Unfortunately most of the previous studies done on the effect of heavy metals have been done on their direct toxicity to the prawns and other organisms (Boyd, 1990). Lorenzon (2000) investigated the effect of short-term (96 hr) exposure of *Palaemon elegans* (Rathke) to dissolved heavy metals on the number of circulating haemocytes in the shrimp. It was found that immersion of the shrimp in artificial seawater containing copper, zinc, lead, cadmium, mercury or cromium caused a significant decrease in the haemocyte count during the first eight hr exposure, although over the following 16 hrs it returned to the initial levels. Zinc and lead induced the greatest decrease.

Paez-Osuna *et al.* (1996) studied the concentration and distribution of heavy metals in tissues of wild and farmed shrimp *Penaeus vannamei* from the North West coast of Mexico and showed that copper and zinc were the most abundant elements in the different biological materials analysed; whereas lead was the least abundant element detected. Chinnayya (1971) also showed that copper, lead, zinc and mercury ions affect the rate of oxygen consumption of the shrimp *Caridina rajadhardi* (Bouvier).

5.2. Effect of zinc and lead on hatching (post-spawning experiment)

In this study results from the post-spawning experiments of zinc showed that hatching rate was not significantly affected by 0.1 and 1.0 ppm zinc, whereas 10.0 and 100.0 ppm zinc showed significant difference among each other and with the other concentrations. At 100.0 ppm zinc hatch rate failed completely (Table 1 and Fig. 3).

The post-spawning experiments of lead showed a different result. With only 100.0 ppm lead resulting in significant difference of hatch rate (Table 2 and Fig. 4). Similarly for zinc, hatch rate failed completely at 100.0 ppm lead.

Concentrations of 100.0 ppm of zinc and lead were lethal to the hatching process. Either it had caused a significant damage to the egg or killed the nauplii at their very early stage, assuming that fertilization took place before the addition of heavy metals.

5.3. Pre-spawning experiments for zinc and copper

In the pre-spawning experiment of zinc the control and 0.01 ppm zinc trials did not show significant difference in hatch rate, whereas 0.1, 1.0 and 10.0 ppm of zinc showed significant differences. Hatch rate in the later concentrations completely failed thus showing no significant difference among each other (Table 3 and Fig. 5).

Spawned and hatched eggs were significantly different at 0.1, 1.0 and 10.0 ppm zinc, as compared with the control, but were not significantly different among each other. Neither did 0.01 ppm zinc show any significant difference with the control nor with the other concentrations of zinc. At 0.01 ppm zinc spawned and hatched eggs decreased by less than half when compared with the control. Eggs failed to hatch starting from 0.1 ppm zinc, but spawning persisted even at 10.0 ppm (Tables 4 and 5).

Hatch rate in the pre-spawning experiment for copper did not show significant difference between the control and 0.01 ppm copper, whereas significant differences were observed in 0.1, 1.0 and 10.0 ppm copper. Hatch rate completely failed in the later concentrations (Table 6 and Fig.6). Control showed significant difference with 0.1, 1.0 and 10.0 ppm copper in case of hatched eggs, but not with 0.01 ppm copper though the number was reduced almost by 50 % (Table 7).

Sperm are stored by the female and released into the water upon spawning. The sperm are therefore free swimming until they fertilize the egg (Swift, 1988). In the situation where the heavy metals were added before spawning took place small concentration of the heavy metals might have killed the sperm, or greatly reduced their motility, thus inhibiting hatching (Laurence, personal comm.). Another possibility is also the heavy metal concentration could have affected the quality of the egg. That was why, maybe, hatching could not take place beyond 0.01 ppm of zinc and copper.

Reports from Paez-Osuna *et al.* (1996) on concentration and distribution of heavy metals in tissues of wild and farmed *Penaeus vannamei* from the north-west coast of Mexico showed that the species from both wild and farmed areas accumulated high concentrations of heavy metals in their tissues. Thus this accumulation of heavy metals in different parts of their body could have put the species under stress, causing the females to spawn significantly less beyond 0.1 ppm concentrations of the heavy metals.

5.4. EDTA treatments

EDTA treatment for zinc-polluted spawning tanks showed that there was significant difference in hatch rate between the EDTA treated and non-EDTA treated tanks, though there was no significant difference between the different EDTA treated tanks. The hatch rate in the tank treated with no EDTA was a complete failure, whereas in the other tanks with EDTA the hatch rate increased by greater than 40% (Table 8 and Fig.7). The spawned eggs did not show significant difference in all cases, though it was doubled in the EDTA treated tanks (Table 9).

There was also a significant difference in hatch rate between the EDTA treated and non-EDTA treated copper polluted spawning tanks. The non-EDTA treated tank showed no sign of hatch, whereas the EDTA treated tanks gave almost 50 % hatch higher. There was no significant difference among the EDTA treated tanks (Table 10). There was also no significant difference in spawned eggs in all the EDTA and non-EDTA treated tanks (Table 11).

EDTA has been extensively used in larvae rearing ponds, culture media of unicellular algae and others. Johnson (1964) indicated that both EDTA and EDTA-chelated trace metals intensify the growth of phytoplankton in seawater. Two mechanisms have been proposed for the enhancement of algae growth by EDTA. Johnson (1964) proposed that EDTA increased the solubility of trace metals, thus making them available for growth. Sunda and Guillard (1976) suggested that the algal growth enhancement was due to the reduced toxicity of the heavy metals by EDTA. Several authors have backed up the latter suggestion. Nugegoda and Rainbow (1988) reported that *Palaemon elegans* was able to regulate body zinc at higher external concentrations in the presence of EDTA. Thus the improved hatch rate in the presence of EDTA was most probably due to the reduced toxicity of the heavy metals by the chelating effect of EDTA. EDTA also probably avoids the stress imposed by heavy metal pollution by chelating free ions and thus reducing their concentration. In that way it might help breeders improve the quality and quantity of the eggs they spawn.

With the continuous disposals of waste and industrial effluent to seashores, application of chelating agents may provide safe water qualities for intensification of culture operations in prawn hatcheries (Licop, 1988). EDTA has definitely removed heavy metal ions from the spawning tanks thus allowing hatching to be very successful as compared to the same concentration of heavy metals where hatching failed completely in the absence of EDTA. However, Batchelder *et al.*, (1980) and Laurence *et al.*, (1981) have demonstrated the detrimental effects of EDTA to shrimp larvae at concentrations of 0.67 mM and lethal effects at 1.34 mM. Thus caution must be taken when such chelating agents are used in shrimp hatcheries due to their adverse effects to shrimp larvae. Very narrow range exists between the concentrations needed to chelate heavy metal ions and the concentrations that produce adverse effects to shrimp larvae (Treece and Fox, 1993). The adverse effect of EDTA at high concentrations may come either from overchelation of trace metals that are important for the growth and development of larvae (Johnson, 1964), or from their direct toxicity to the larvae. That is why only dosages of 5 and 10 ppm are recommended to be safe levels for prawn larval rearing procedures (Licop, 1988).

6. CONCLUSION AND RECOMMENDATIONS

The site selected for a prawn hatchery, hopefully, does not involve pollution from heavy metals, but if heavy metal pollution is a problem then there are several ways, which have been used around the world, to deal with this. Charcoal filtration and chelation of ionic metals are the commonly used methods. Charcoal filtration is probably the best treatment. Complexation or chelation of ionic metals can be done either by natural substances, such as humic acids, or by synthetic compounds like EDTA. Those natural and synthetic substances decrease the toxicity of the metals through a diminished rate of uptake as compared with the free ions of the heavy metals (Holwerda *et al.*, 1988).

Since the concentration of heavy metals in natural waters varies over time due to natural and human activities, it is advisable to treat these waters routinely by adding chelating agents. This could help in reducing the risks of toxicity from heavy metals to a certain extent (Treece and Fox, 1993). Some chelating agents remove heavy metal ions completely from natural sea waters and since the chelating efficiency is proportionate to the time of exposure to the chelating solution, the removal efficiency of the heavy metal ions is increased with absorption time. Thus treatments with chelating agents should be routine and for a longer time (You-Xian Yuan *et al.*, 1992).

The Amatikulu Prawn Hatchery is located near the mouth where the Amatikulu River joins the Indian Ocean. The Amatkulu River crosses many commercial farming areas in the vicinity where sugar cane is produced for commercial purposes. A lot of pesticides and insecticides might be used to enhance the production of these farms. During rainy seasons, the chemicals sprayed over the sugar cane farm to eradicate disease causing agents might be washed and carried by the river down to the ocean. This will increase the concentration of heavy metals and other pollutants. Thus a basic assessment of the natural seawater might help to identify the possible potential toxicants in the area. This might help to take measures to enhance the production of the hatchery accordingly.

Reports from You-Xian Yuan *et al.* (1992) showed that the chelating efficiency of chelating agents is proportional to the time of exposure to the solution. Hence, the removal efficiency of heavy metal ions is increased with absorption time. Therefore EDTA or any other chelating agent might be added to the tanks where shrimp larvae are kept days ahead before the larvae are put into the tanks. They might also be used in maturation tanks to avoid the up take of heavy metal ions by the breeders, which eventually will cause mortality of the breeders, or will make the availability of eggs very scarce or of bad quality. This might be done by adding the chelating agent into the reservoir tanks, where water from pumps is kept for removal of suspended particles before distributed to the maturation tanks. This will increase the removal efficiency by increasing the absorption time.

The time taken to carry out this experiment was very short. Hence, it did not give any chance to carry out preliminary, range-testing experiments as the most interesting concentrations of the heavy metals used; those that showed major changes on spawning and hatching, are between 10 ppm and 100 ppm. Further studies, if there are any, should think about taking preliminary, range-testing experiments first or can use this paper as a reference indicating which concentration ranges should be taken into consideration to base their studies.

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