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# Toxic Effects of Flucloxacillin on the Early Development of the Polychaete *Hydroides elegans*

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## Abstract:

***Hydroides elegans* is a gregarious tube building polychaete which occurs in tropical and sub-tropical waters. Adults were found to have good tolerance in laboratory assays using. The effect of flucloxacillin on fertilization and early development was explained through three experiments. Flucloxacillin is a antibiotics to treat bacterial infections. In normal fertilization the percentage of successful development at FM stage was 100±0.00 upto larval release stage was 80.11±0.68. In Expt. I higher concentration 50% of the embryos showed abnormal development and deformities. Expt. II in lower concentrations upto 800ppm the embryo showed high deformities. Expt. III, in 400ppm nearly 60% of embryos showed abnormal development in Blastula stage. Finally, more than 100ppm concentrations did not reach the release stage. The percentage of successful development was decreased in Expt. I to Expt. III. The present paper deals with the toxic effects of flucloxacillin on fertilization and early development of *H. elegans* and the percentage of successful development of embryos were studied.**

**Keywords:** developmental stages, flucloxacillin, *Hydroides elegans*, polychaete, toxic effects

## 1.0 Introduction:

*Hydroides elegans* (Haswell) is a tube-building polychaete species conspicuous in tropical and subtropical coastal fouling communities. In most places, settlement of H. elegans peaks in summer or autumn (Qiu and Qian, 1997; Gopalakrishnan et al., 2005). *H. elegans* is a sedentary fouling tubiculous polychaete available in plenty on the hulls of the ships, fishing boats and floating material on the sea water (Udhayakumar and Karande, 1996). The *H. elegans* on fishing boat reduces the speed of the boat and increasing the fuel coast (Raja, 1999; Gopalakrishnan et al., 2005; Rani, 2005). *H. elegans* a sedentary polychaete common in temperate intertidal waters produces viable gametes throughout the year. The organism is widespread forming dense layers within the intertidal zone (Marsden and Anderson, 1981; Gopalakrishnan et al., 2005).

In the past several decades, laboratory studies on *Hydroides sp*. have mainly focused on systematic (Fauchald, 1977), oogenesis and fertilization (Nordbaek, 1956), maturation (Leone, 1970), salinity tolerance in adults (Mohan and Aruna, 1994), larval settlement (Hurlbut, 1991) and description of early life history (Miura and Kajihara, 1981; Gopalakrishnan et al., 2005). Marine invertebrate larvae are usually more sensitive to stress than adults and juveniles of the same species and such sensitivity may help us explain seasonal or annual variations in recruitment success in the field (Qiu and Qian, 1997). *H. elegans* adults would retain their gametes where external salinity dropped to levels too to support fertilization and development. Whether failure of fertilization or a failure of fertilized eggs to cleave (Pechanic et al., 2007). Toxic effects on aquatic and sediment dwelling organisms caused by antibiotics uptake inhibitor were recently summarized (Brooks, 2003). Antibiotic is a chemical substance produced by a micro organism that suppresses the growth or directly kills another micro organism (Levine, 1978). Antibiotics are used to assist the immune system and inhibits the growth of pathogenic microorganisms (Timmreck, 1998). Flucloxacillin is an antibiotic used for the treatment of infections caused by susceptible gram-positive bacteria. It is commonly used when a patient is suffering from various infections such as bone and joint, skin and wound, organs involved in breathing and the inflammation of the lining of the heart cavity and valves due to the bacteria Staphylococcus (Harrison, 2005). Flucloxacillin is a white or almost white powder. Slightly soluble in water and in

chloroform; freely soluble in methyl alcohol (Sweetman, 2005). The toxic effects of antibiotics on fertilization and early development of *H. elegans* were studied by Rani (2005), Hemalatha (2006) and Senthil (2006) and suitability of *H. elegans* to assess the toxicity of pollutants were established. Flucloxacillin may not cause any damage to fertilization in human beings at normal concentration. Increased concentrations of flucloxacillin may cause damage to fertilization in human beings and other animals. The present study is designed to study the toxic effect of flucloxacillin on fertilization and early development of *H. elegans*.

## 2.0 Materials and Methods:

### 2.1. Source of Animals:

Adult *Hydroides elegans* were collected from the hulls of fishing boats berthed at Rayapuram fish landing center, Chennai, India. Other sedentary organisms like lepas, bernacles, neries, mytilus, ascidians, algae, few crustacean, arthropods and some mollusk were also seen. Other fouling organisms were carefully removed from the collection before *H. elegans* were placed in the collection chambers. The specimens were transported to the laboratory (Department of Zoology, Presidency college, Chennai), within two or three hours after collection. Adults were found to have good tolerance in laboratory assays using.

### 2.2. Obtaining gametes for fertilization studies:

Gametes were obtained using standard procedures (Raja and Sellappan, 1993; Gopalakrishnan et al., 2005). *H. elegans* has separated sexes. Tubes of adult *H. elegans* were gently broken and observed for the release of gametes, which occurred after few minutes. Eggs were pink in colour and sperm milky white. Gametes were then generally released by mature individuals within several minutes and were collected by pipette. Eggs were immediately transferred into about 30 ml of seawater at a salinity of 35‰ while sperm were kept undiluted in small beakers until use. Sperm were then diluted with about 10-20 ml of filtered seawater. For each experiment, eggs were obtained from 5-8 females, and sperm were obtained from 4-6 males (Fig.1 and 2).

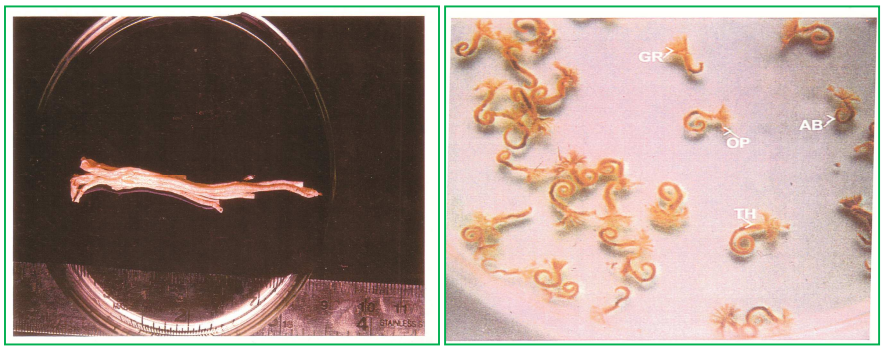



**Fig. 1: Alive *Hydroides elegans* inside the tube and removed from the tube**

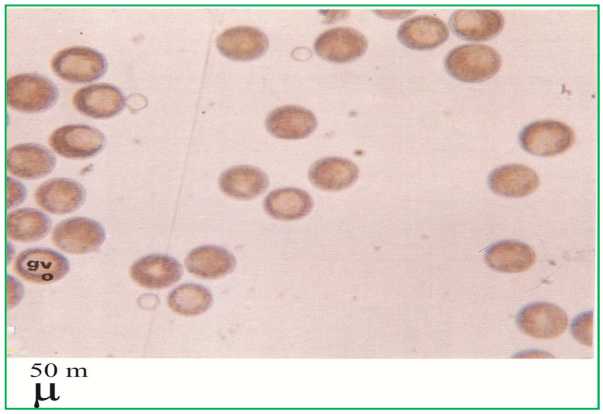



**Fig. 2: After mixing of gametes**

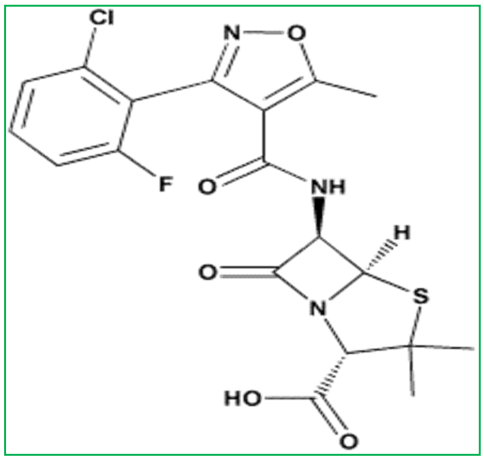
## 2.3. Properties of Flucloxacillin:

Flucloxacillin is a white or almost white, crystalline hygroscopic, powder. Freely soluble in water and in methyl alcohol; soluble in alcohol. A 10% solution in water has a pH of 5.0 to 7.0. store at a temperature non exceeding 25°C in air tight containers (Sweetman, 2005). A systematic (IUPAC) name 6-((s)-3-(2-chloro-6-fluourophenyl)-5-methylisoxazole-4-carboxamido)-3, 3-dimthyl-7-oxo-4-thia-l azabicyclo [3.2.0] heptane-2-carboxylic acid. The molecular formula of flucloxacillin $C_{19}H_{17}ClFN_3O_5S$ with a molecular weight 453.87 (Anonymous, 2005). Fluxloxacillin is an isoxazolyl penicillin used primarily for the treatment of infections due to staphylococci resistant to benzylpenicillin.

## 2.4. Preparation of Working Stock Solution:

200 mg of flucloxacillin was dissolved and made up to 250 ml of filtered sea water in a volumetric flask to prepare 800ppm flucloxacillin solution. This stock solution was stored in amber colored bottle.

From the stock solution the following concentrations of flucloxacillin were prepared as 10ppm, 50ppm, 100ppm, 200ppm, 400ppm and 800ppm (Table 1).




**Structure of Flucloxacillin**

**Table 1: Various concentrations of flucloxacillin prepared from stock solution**

| 200 mg of flucloxacillin + 250 ml of filtered sea water = 800 ppm of stock solution | | | |
|---|---|---|---|
| **Sr. No.** | **Stock solution (ml)** | **Sea water (ml)** | **Concentrations of flucloxacillin solution (ppm)** |
| 1. | 100 ml | --- | 800ppm |
| 2. | 50 ml | 50 ml | 400ppm |
| 3. | 25 ml | 75 ml | 200ppm |
| 4. | 12.5 ml | 87.5 ml | 100ppm |
| 5. | 6.25 ml | 93.75 ml | 50ppm |
| 6. | 1.25 ml | 98.75 ml | 10ppm |

## 2.5. Experiment:

The present work, the time of mixing gametes was taken as time of fertilization and the percentage of successful development of eggs in each developmental stage were recorded. The developmental stages were divided into three experiments as Expt. I (FM stage to 4 cell stage), Expt. II (8 cell stage to 64 cell stage) and Expt. III (Blastula stage to Larval release stage).

### Expt. I: Fertilization membrane stage to 4 cell stage

Expt. I was designed to examine flucloxacillin affect in developmental stages of FM stage to 4 cell stage. The experiment conducted on various concentrations (10ppm, 50ppm, 100ppm, 200ppm, 400ppm and 800ppm) of flucloxacillin. These concentrations are prepared from fresh stock solution. Developmental stages were checked often, and the cumulative time and the percentage of successful egg development was recorded.

### Expt. II: 8 cell stage to 64 cell stage

Expt. II was designed to examine the effects of flucloxacillin on survival and duration of developmental stages of 8 cell stage to 64 cell stage. The experiment was the same as in Expt. I.

### Expt. III: Blastula stage to Larval release stage

Expt.III was designed to examine the effects of fluxloxacillin on survival and duration of development of Blastula stage to Larval release stage. The experiment was the same as in Expt.I.

## 2.6. Data analysis:

ANOVA was done by transforming values to ranks and then applying parametric statistics on the data, as described in Zar (1984). A 2-way ANOVA (analysis of variance) was used to detect flucloxacillin effects on duration of developmental stages in Expt. I, II and III. The difference in the mean was statistically significant between different concentrations of flucloxacillin for all developmental stages at 0.05% level. $EC_{50}$ or median effective concentration value of flucloxacillin for different early embryonic stages of H. elegans were calculated by probit analysis followed by Finney (1971). $EC_{50}$ values were calculated for each experiment and all developmental stages.

## 3.0 Results and Discussion:

## 3.1. Normal Fertilization and Early Development:

After fertilization, the negatively buoyant eggs sink to the bottom where they undergo cleavage upto the larval release stage. The first cleavage occurs after 30 minutes of fertilization, at optimal conditions such as 27˚C, pH 8.1 and salinity 35‰. The elevation of fertilization membrane was initiated 3 to 5 minutes after fertilization. The meiotic division is not completed before spawning. The first cleavage plane was meridional and complete, both the blastomeres were almost of equal in size. Completion of first cleavage occurred at 30 minutes. The percentage of successful development at FM stage was 100±0.00. The second cleavage plane was also meridional but right angle to the first one. The completion of 4 cell stage occurred 52 minutes after fertilization. The successful development of this stage was 94.54±1.41. The third plane of cleavage was horizontal, dividing the four blastomeres into 8 cells. The time required for the completion of 8 cell stage was 1hour 2 minutes and the percentage of successful development of this stage was 93.05±1.88. The embryo reached 64 cell stage at 1 hour 39 minutes after fertilization. The successful egg development at this stage was 88.06±2.83. The blastula stage reached at 1 hour 58 minutes after fertilization. The percentage of successful egg development was 86.09±1.78. Finally the larval release stage occurred 4 hours 50 minutes after fertilization. At the end of the stage 80.11±0.68 percentage egg was successfully developed.

## 3.2. Toxic Effect of Flucloxacillin on Fertilization and Early Development:

### Expt. I: Fertilization membrane stage to 4 cell stage

Expt. I was explained FM stage to 4 cell stage (FM stage, 2 cell stage, 3 cell stage and 4 cell stage) at various concentrations of flucloxacillin. In FM stage, after fertilization the time showed a steady increase from 6 minutes at 10ppm concentration to 7.5 minutes at 800ppm concentrations of flucloxacillin. The percentage of successful development of FM stage showed variation at various concentrations of flucloxacillin. The percentage of success was 97.41±0.41 up to 10ppm and then declined steadily to 82.16±0.05 in 800ppm. After formation of fertilization membrane reached 4 cell stage with a steady increase from 54 minutes at 10ppm to 1 hour 15 minutes at 800ppm of solution. The percentage of successful development of 4 cell stage declined steadily from 84.18±5.92 at 10ppm to 16.25±0.40 at 800ppm (Table 2) of flucloxacillin solution. In higher concentration 50% of the embryos showed abnormal development, retarded growth and deformities (Fig.3).

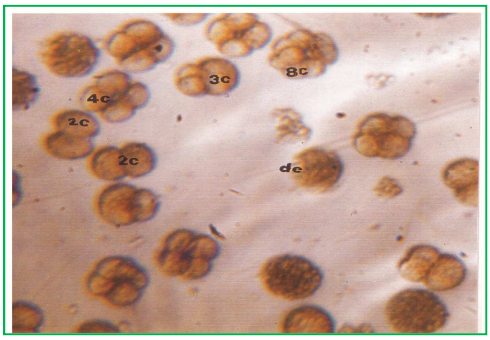



**Fig. 3: Expt. I: Fertilization membrane stage to 4 cell stage**

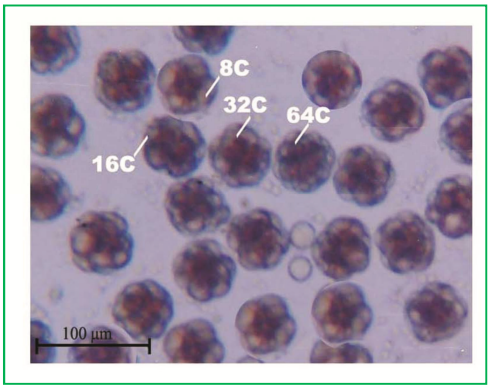



**Fig.4: Expt. II: 8 cell stage to 64 cell stage**

**Table 2: Percentage of success * of various embryonic stages of *H. elegans* in normal and in different concentrations of flucloxacillinin sea water**

| Devpt. Stages | Control | Percentage of successful development | | | | | |
|---|---|---|---|---|---|---|---|
| | | Concentrations of Flucloxacillin expressed in ppm | | | | | |
| | | 10ppm | 50ppm | 100ppm | 200ppm | 400ppm | 800ppm |
| **Expt. I** | | | | | | | |
| FM-Stage | 100 ±0.00 | 97.41 ±0.41 | 96.35 ±0.48 | 93.73 ±1.58 | 92.41 ±0.91 | 86.36 ±0.84 | 82.16 ±0.05 |
| 2- Cell Stage | 97.52 ±1.45 | 92.85 ±1.55 | 88.52 ±1.16 | 85.06 ±2.11 | 78.81 ±0.06 | 53.19 ±13.95 | 28.86 ±4.47 |
| 3- Cell Stage | 95.03 ±0.92 | 89.09 ±3.20 | 81.23 ±1.23 | 75.39 ±1.53 | 68.33 ±1.67 | 42.79 ±8.62 | 20.86 ±1.35 |
| 4- Cell Stage | 94.54 ±1.41 | 84.18 ±5.92 | 74.97 ±2.34 | 67.71 ±4.08 | 57.43 ±6.32 | 33.60 ±1.96 | 16.25 ±0.40 |
| **Expt. II** | | | | | | | |
| 8- Cell Stage | 93.05 ±1.88 | 79.57 ±8.92 | 69.2 ±6.05 | 60.67 ±7.27 | 48.26 ±10.49 | 26.86 ±0.28 | 12.26 ±1.15 |
| 16- Cell Stage | 91.52 ±1.39 | 72.69 ±12.9 | 62.39 ±9.76 | 55.98 ±9.39 | 43.05 ±11.94 | 20.83 ±0.58 | 9.92 ±1.04 |
| 32- Cell Stage | 90.55 ±2.36 | 66.35 ±16.35 | 54.55 ±12.45 | 48.87 ±11.37 | 38.47 ±14.03 | 14.1 ±0.18 | 6.98 ±0.32 |
| 64- Cell Stage | 88.06 ±2.83 | 62.13 ±18.66 | 48.28 ±14.6 | 41.05 ±11.51 | 30.59 ±12.41 | 10.29 ±0.29 | 4.04 ±0.39 |
| **Expt. III** | | | | | | | |
| Blastula Stage | 86.09 ±1.78 | 56.44 ±21.66 | 44.15 ±14.64 | 35.07 ±12.35 | 22.98 ±10.76 | 8.00 ±0.86 | 2.96 ±0.63 |
| Blastula Start Rotation | 85.09 ±2.78 | 55.49 ±20.71 | 41.51 ±14.15 | 31.37 ±10.92 | 16.94 ±8.06 | 6.01 ±0.30 | 2.86 ±0.64 |
| Blastula Stop Rotation | 83.11 ±1.72 | 52.86 ±22.43 | 36.33 ±12.12 | 23.48 ±9.85 | 11.52 ±5.97 | 3.32 ±0.47 | 1.15 ±0.05 |
| Release Stage | 80.11 ±0.68 | 50.83 ±23.66 | 23.41 ±2.36 | 12.73 ±1.37 | ND | ND | ND |

ND= No Development, n= number of experiments, ± = Standard Deviation, * = number of eggs observed in each concentration = 10

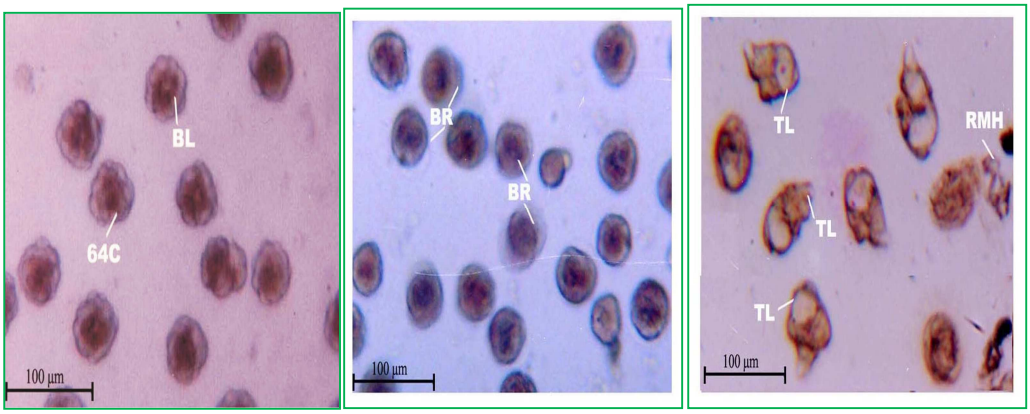
## Expt. II: 8 cell stage to 64 cell stage

Various concentrations of flucloxacillin affected embryos during development from 8 cell stage to 64 cell stage (8 cell stage, 16 cell stage, 32 cell stage and 64 cell stage). Flucloxacillin strongly affected embryos and duration of development at 10ppm to 800ppm concentrations of flucloxacillin solution. The cumulative time of 8 cell stage increased steadily from 1 hour 7 minutes at 10ppm to 1 hour 33 minutes at 800ppm concentrations. The successful development of 8 cell stage declined from 79.57±8.92 at 10ppm to 12.26±1.15 at 800ppm. In 400ppm nearly 60% of the embryos showed abnormal development and deformities where as abnormal development increased in 16 cell stage. At higher concentration to reach 64 cell stage time was taken from 2 hours 22 minutes. The percentage of successful development of 64 cell stage decreased 4.04±0.39 at 800ppm (Table 2). In lower concentrations upto 800ppm the embryo showed high deformities (Fig.4).

## Expt. III: Blastula stage to Larval release stage

Cleavage of embryo during development from Blastula to Larval release stage (Blastula stage, Blastula start rotation, Blastula stop rotation and Larval release stage) was affected by various concentrations of flucloxacillin solution. However, duration of embryo development was highly affected with increasing flucloxacillin solution. The cumulative time of Blastula stage increased

steadily from 1 hour 27 minutes at 10ppm to 3 hours 12 minutes at 800ppm. In 400ppm nearly 60% of embryos showed abnormal development in this stage. In lower concentrations also most of the embryos showed abnormal development and deformed embryos did not reach rotation stage. The percentage of successful development of Blastula start rotation and stop rotation stage in 2.86±0.64 and 1.15±0.05 at 800ppm respectively. It has taken long period reach release stage after fertilization from 5 hours 17 minutes at 10ppm to 5 hours 37 minutes at 100ppm of flucloxacillin solution. More than 100ppm concentrations did not reach the release stage (Table 2 and Fig.5).




BL - Blastula stage BR - Blastula Rotation stage TL - Trochopore larva

**Fig.5. Expt. III: Blastula stage to larval release stage**

## 3.3. Relative Sensitivity of Flucloxacillin:

A careful perusal of the embryonic stages were more sensitive than the FM stage and the sensitivity decreased steadily as the developing embryo enter into next stage of development. The FM stage (9248.62ppm) was the least sensitive stage of flucloxacillin. The larval release stage (52.66ppm) was the highest sensitive stage of flucloxacillin solution (Table 3).

**Table 3: Relative sensitivity ($EC_{50}$ values) of Flucloxacillin for different embryonic stages of *H. elegans* ($EC_{50}$ values are expressed in ppm)**

| DEVELOPMENTAL STAGES | $EC_{50}$ VALUES |
|---|---|
| FM-STAGE | 9248.62 |
| 2-CELL STAGE | 508.58 |
| 3-CELL STAGE | 394.77 |
| 4-CELL STAGE | 309.39 |
| 8-CELL STAGE | 249.89 |
| 16-CELL STAGE | 236.05 |
| 32-CELL STAGE | 211.11 |
| 64-CELL STAGE | 165.49 |
| BLASTULA STAGE | 139.69 |
| BLASTULA START ROTATION STAGE | 109.22 |
| BLASTULA STOP ROTATION STAGE | 73.71 |
| RELEASE STAGE | 52.66 |

Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of microorganisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of human, animals and plants. They have long been present in the environment and have played a crucial role in the battle between man and animals (Serrano, 2005). The wide use of antibiotics in animal nutrition and disease has resulted in the sensitization of a relatively large number of the susceptible people, many of whom react violent in contact with these drugs (Harvey, 1975). In recent years environmental researchers have drawn attention to reports on measure contents of drugs in waste water, surface water, ground water and drinking water all over the world (Canton, 1976). Concentrations of the antibiotic, not necessarily pure are then examined for toxicity in mice. As well as measured contents of drugs in the environmental information have also begun to be available in the literature on drugs toxic effects on aquatic organisms (Addison, 1984). The discharge of antibiotics and their metabolites in farm waste could create a reservoir of resistant micro organisms in the environment. Several antibiotics enter aquatic and terrestrial ecosystems through the discharge of effluents from farms (Bates et al., 1994). Flucloxacillin is an antibiotic, commonly used when a patient is suffering from various

infections such as bone and joint, skin and wound; organs involved in breathing and the inflammation of the lining of the heart cavity and valves due to the bacteria Staphylococcus (Harrison, 2005; Anonymous, 2006). During fertilization and early development the biological activity of eggs, sperm and blastomeres are very complex and involved in active synthesis of various macromolecules (Schuel, 1978). Hence, the effect of antibiotic may be reflected quickly during the process of fertilization and cleavage. The rate of activity of various embryonic stages studied in the present work clearly suggest that flucloxacillin affect the biological activities of various embryonic stages even at 10ppm concentration. At higher concentrations of flucloxacillin in seawater the embryo did not develop normally and showed deformities and stopped further development. In Expt. I (FM stage to 4 cell stage) the percentage of success gradually decreased from lower concentration to higher concentration of flucloxacillin sea water. The percentage of successful development of FM stage to 4 cell stage varied from 97.41% to 16.25% respectively at various concentrations. In higher concentration 50% of the embryos showed abnormal development. Expt. II (8 cell stage to 64 cell stage) the successful development gradually decreased from lower concentration to higher concentration. In higher concentration 60% of the embryos showed abnormal development and retarded growth. At various concentrations of flucloxacillin was affected highly in 64 cell stage. The percentage varied from 78.09% at 10ppm to 800ppm of flucloxacillin solution. In most, 400 and 800ppm of flucloxacillin solution, the embryo showed highly deformities in Expt. II. Expt. III (Blastula stage to Larval release stage) In lower concentrations also most of the embryos showed abnormal development and the abnormal embryos did not reach rotation stage. The percentage of successful development of Blastula start and stop rotation varied at 54.34% in various concentrations of solution. In larval release stage occurred only in 10ppm, 50ppm and 100ppm. More than 100ppm concentrations did not reach the release stage. Expt. I was moderate tolerance of lower and higher concentration of developed normal embryonic stages but Expt. II embryos showed high deformities. The relative sensitivity decreased steadily from 9248.62ppm in FM stage to 52.66ppm in larval release stage. It clearly suggests that the later stages are more sensitive to flucloxacillin than the earlier stages or it may be due to the higher exposure to time. This suggests that the impact of toxicity may be additive as the development progress and the later stage are exposed for longer duration in the test solution.

## 4.0 Conclusions:

1. *Hydroides elegans* is a sedentary fouling tubiculous polychaete available in plenty on the hulls of the ships and adults were found to have good tolerance in a laboratory assays using.
2. Flucloxacillin is an antibiotic used for the treatment of infections caused by susceptible gram-positive bacteria.
3. The toxic effects of antibiotics on fertilization and early development of *H. elegans* was studied.
4. The effect of flucloxacillin on fertilization and early developmental stages of *H. elegans* was directly proportional to the concentrations as well as the exposure time to flucloxacillin in sea water.
5. Expt. I and II in higher concentrations 50% and 60% of the embryos showed abnormal development and retarded growth respectively.
6. Expt. III, in lower concentrations most of the embryos showed abnormal development and the abnormal embryos did not reach the release stage.

## 5.0 Acknowledgement:

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