Effect of water-accommodated fractions of No.0 diesel oil on embryos and larvae development of sea urchin, *Hemicentrotus pulcherrimus*

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Abstract—The paper aims at present the acute toxic effect of water-accommodated fraction (WAFs) of No.0 diesel oil on embryos and larval development of sea urchin, *Hemicentrotus pulcherrimus* by adopting embryonic development technology. The experiment was carried out with six concentrations. The results indicate that the water-accommodated fractions have evident toxic effect on two–arm and four–arm larvae. Compared with the control group, the larvae in the oil groups showed shorter body lengths and higher deformity and mortality rate by the increasing concentrations. The 48h, 72h EC50 is 3.39, 1.87mg/l respectively.

Key words- water-accommodated fractions; No.0 diesel oil; embryos development; larvae; toxic effect.

I. INTRODUCTION

*Hemicentrotus pulcherrimus*, which belongs to Echinodermata, Echinoidea, Strongylocentrotidae, is mainly distributed in China and Japan coastal area. Generally there are two breeding seasons in a year (i.e. from May to June and from September to November), with the breeding water temperature ranging from 10 to 20 °C. Its whole life may be classified into 4 phases, such as embryo, plateus larva, juvenile sea urchin and mature sea urchin. Each development phase can be subdivided into several stages, such as 2-cell, 4-cell, 8-cell, 16-cell..., blastula, prism, two arm larvae, four arm larvae and so on. It is easy to obtain cells dividing synchronously and a lot of information is available about the cell cycle, which is a succession of events resembling those of mammalian cells in culture. As we all know, embryos and larvae are usually assumed to be the most sensitive life stages of these echinoderm species. The need for sensitive, short-term tests for aquatic hazard assessment and bio-monitoring has led to the increasing use of gametes in applied toxicology; for this, sea urchin eggs and sperm present an attractive bioassay. They are widely used in sea water toxicity assessment (Diane et al, 2000; Larrain et al, 1999), due to the ease of obtaining gametes, the small test volumes and the rapid and well-characterized development. In the US, early life stage bioassays have been applied to assess sea water monitoring programs (Dinnel et al,1988; Amro et al., 2002; Phillips et al.,2003; Julie et al., 2002; Beiras,2002). These bioassays have been adapted to test for contaminant effects caused by heavy metals, TBT, detergent and so on (Agnello et al, 2007; Shim et al, 2006; Lera et al, 2006; Ward et al,2006; Kobayashin et al, 2006; Iuan et al, 2005). However, they have been employed less frequently for investigations of petroleum hydrocarbons toxicity effects (Fernández et al, 2006).

Oil spill are a common occurrence throughout the world, with many countries involved in oil and petroleum exploration, production, and transport. Increasing demands for and utilization of petrochemicals has resulted in an increase in levels of petroleum hydrocarbons in marine, coastal and estuarine environments. Tanker accidents release approximately one third of the 1.18 million metric tones of petroleum hydrocarbons that enter the aquatic environment worldwide each year. The purpose of this study was to evaluate the sensitivity of sea urchins to the water-accommodated fraction (WAFs) of No.0 diesel. We present here an investigation of the lethal toxicity on the embryos and larvae of sea urchin, *Hemicentrotus pulcherrimus*. The effects of the WAFs on eggs dividing time and larvae body length were studied. The results of these acute tests are often considered to be a good indication of the acceptability of pollutant concentrations to saltwater species in general and are often assumed to be an important consideration when assessing the hazard of materials to other saltwater organisms or when deriving water quality criteria for saltwater organisms.

II. MATERIALS AND METHODS

Test materials

All testing was conducted using the water-accommodated fractions (WAFs) of No.0 diesel oil. The term “WAFs”, as opposed to “water-soluble fractions”(WSFs) is used because the media did not undergo steps to ensure that all possible particulate in the oil had been removed. Untreated oil WAFs were prepared using a standardized low-energy mixing method. They were obtained by adding one part oil to nine parts 0.45μm-filtered material sea water (.temperature is 24±2 °C, salinity 31.35‰, electric conductivity rate 47.3, pH 8.13). Mixing energy was provided by a magnetic stirrer at a rate of 200±10 rpm. Mixing lasted 24h, followed by 4h of settling time to allow the largest oil droplets to resurface. The
aqueous phase was separated as mother solution and preserved in refrigerator at 4°C.

**Toxicity Test Procedures**

Spiked-exposure toxicity tests were completed with the early life stages of the *Hemicentrotus pulcherrimus* sea urchin. The experiment was designed according to the relevant literatures and ASTM “Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos”. The mature sea urchin was collected from culture areas of Dalian Bilong Seafood Limited Company. The undamaged bigger ones were chosen for experiments. Spawning of gametes was induced by injection of 1.0 mL 0.5M potassium chloride. Eggs were collected in sea water and rinsed twice. For fertilization, eggs were suspended in Millipore-filtered sea water(20-50 eggs per milliliter). The sperm suspension was passed through a 37-μm screen to remove feces and other extraneous material. Dilute sperm was added to the eggs and carefully stirred with a plunger to allow fertilization. Four samples of 100μl were taken to record fertilization success (assessed by the percentage of eggs showing a fertilization membrane) and egg density. Experiments were only performed on batches exhibiting greater than 90% fertilization. Embryos were introduced to test containers after developing for 10min following fertilization. Each container was inoculated with approximately 1000 embryos. Selection of nominal toxicant concentrations was based on range-finding test. The concentrations for lethal bioassays are as follows: 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg/l. Controls receive the dilution water without toxicant. Four replicates per treatment were assayed. After a 48h and 72h exposure at 17°C, larvae were fixed with a few drops of 5% buffered formalin. About 100 larvae in each container were examined under an inverted light microscope at 100×. The endpoints recorded in each vial were the percentage of fully developed 2-arm and 4-arm pluteus larvae and the mean larval length.

**Oil Analysis**

The concentration of the stock solution were determined by UV spectrophotometry. The HCs in the WAFs were extracted by dichloromethane. All HCs were conducted with a GC6890 equipped with a 5973MS gas chromatography-mass spectrometry. Conditions and temperatures were as follows: The carrier gas was helium at a flow of 0.8ml/min. The chromatography was conducted on HP-5MS column(30m×0.25mm×0.25μm). The detector were held at 280°C and were held at 250°C. The column temperature was as follows: initial temperature 50°C, held for 1min, then increased at a rate of 8°C/min; final temperature 280°C, held for 10min. At last heated to 300°C at a rate of 10°C/min, and held for 10min. The composition were analyzed by selected ion method.

**Calculation and statistical methods**

For each test chamber in each treatment, including the control treatment(s), A (the percentage of the embryos that did not result in normal pluteus larvae) should be calculated as follows:

\[ A = \frac{100(N-B)}{N} \]

where:
- \( N \) is the number of eggs in the sample taken from that test chamber at the beginning of the test, and
- \( B \) is the number of normal pluteus larvae in the sample taken from that test chamber at the end of the test

\( M \) (the average percentage of the embryos that did not result in normal pluteus larvae in the control treatment(s)) should be calculated as the average of the As for the test chambers in the control treatment(s). The test is unacceptable if \( M \) is greater than 30%.

For each test chamber in each treatment other than the control treatment(s), \( E \) (the percentage of introduced embryos that did not result in normal pluteus larvae adjusted for the controls) should be calculated as follows:

\[ E = \frac{100(A - M)}{(100 - M)} \]

The median effective concentrations( EC₅₀) can be calculated by log-probit method. The EC₅₀ value means the WAFs concentrations that reducing embryogenesis success to 50% of the control values. They were obtained by liner regression of the larval percentage data. (normalized by using angular transformation) against the logarithm of concentration . The 95% confidence intervals were obtained by the Litchfield —wilcoxon method.

**III. RESULTS AND DISCUSSION**

The effect of WAFs of No.0 diesel oil on the development of echinoid embryos

Sea urchin embryos were immersed into seawater containing WAFs at different concentrations ranging from 0.5mg/L to 16mg/L, and were scored for early development progression by microscopy observation. In order to bypass any effect on the fertilization processes, embryo embryos were allowed to develop for 10min following fertilization before transfer into the test solution. The effect of WAFs was further analyzed at the timing of the first cell division, 4-cell, 8-cell and blastula stages. As illustrated in Fig.1, WAFs inhibited embryogenesis success. The results showed that embryos in control could grow normally and the malformation rate was low, as reported in the literature. Low concentration and short time exposure have less toxic effect on embryos. Increasing WAFs concentrations arrested embryo development caused a delay in the progressive shift from 2-cell to the larvae stage. Control and 0.5mg/l WAFS-treated embryos underwent almost identical development at the initial development, reaching 2-cell stage after 100min. At concentrations equal to or greater than 1.0mg/L, WAFs exposure provoked an arrest in the first cell division. With the developing of the embryos, hysteresis effect became more and more obvious. Fig.1 showed that 0.5 mg/l WAFs-treated embryos reached blastula stages after 780min, 60min longer than the control. For the high concentration groups such as 8mg/L, the first cell divided after 120min and more than 50% fertilized eggs couldn’t divide or divide abnormally. Embryos exposure to 8mg/L WAFs reached the blastula stage after 930min, 210min longer than control. The blastula was prevented in almost 70% of the embryos treated with 16mg/L.
WAFs, whereas almost 80% of the embryos treated with 0.5 mg/L WAFs.

The effect of WAFs on the length of larvae

After blastula, gastrula and prism stage, echinoid embryos developed to two-arm larvae about 36h later and four-arm larvae 52h later. The larvae in the exposure group always with less or shorter arm. Concentration dependence in growth inhibition also found in the test as showed in Fig.2. Concentration of 2, 4 and 8mg/l of WAFs caused a two-arm larvae length reduction of 15.6, 20.4 and 30% respectively. For four-arm larvae, the above concentrations caused reductions of 9, 16.3 and 20% in mean larvae lengths. Early larval growth is therefore also a sensitive response in Hemicentrotus pulcherrimus development period.

The effect of No.0 diesel oil WAFs on the development of echinoid embryos

During echinoid embryos were reared in the WAFS, 48h and 72h development were observed as shown in Fig.3-4. The control reached the 2-arm larvae in 48h, and 4-arm larvae in 72h. The observation of larvae were evaluated as following: (1) retarded(R) plutei[1/3 size vs normal plutei];(2) pathologic(P1)malformed pluteus; (3)pathologic embryos(P2) that were unable to differentiate up to the pluteus larvae stage; and (4) dead (D) embryo/larvae . The percentage of the embryos that did not result in normal pluteus larvae(A) can be calculated as following : A = R+P1 + P2+ D. All observations were carried out double-blind by trained readers, each evaluating a complete set of readings. The results indicated that the toxicity effects on embryo and larvae increased significantly with the development and increasing of the concentration. This maybe caused by the accumulation of petroleum hydrocarbon in the larvae body. For the embryos exposed in low concentration, Only 2/3 of them could develop to 4-arm larvae, whereas the number in high concentration exposure group was less than 1/4. Most embryos treated with 16mg/L WAFs did not develop over 20h period but remained arrested in the primary stage. The 48h and 72h EC50 value was calculated and shown in Table1.
Petroleum hydrocarbon can be divided into straight and branch-chain alkanes, cycloalkane, polycyclic aromatic hydrocarbons and unsaturated hydrocarbons according to its structure. The toxicity of petroleum hydrocarbon was decided by its components, especially closely related with the composition and content of the alkane and aromatic hydrocarbons with low molecular weight. Generally, in the petroleum hydrocarbon polluted waters, highly volatile light constituents enter into the atmosphere by sunshine and storm, however, polycyclic aromatic hydrocarbons and some light component can either solve into water or be absorbed by suspended particulates into water. Though the toxicity mechanisms has not been known clearly, the possible mechanisms of soluble fractions such as polycyclic aromatic hydrocarbons are always thought as following which need to be proven by further experiments: (1) cell death or cell proliferation slowing down; (2) embryo cell metabolic disturbance; (3) abnormal embryonic development caused by chromosome mutation; (4) incoordination in the embryo development process.

IV. CONCLUSIONS

The embryo and larva are the most sensitive stage in the life cycle of animal. This study is the first to quantify the toxicity of No.0 diesel oil WAFs to embryos and larval development of Hemicentrotus pulcherrimus sea urchin. Exposure of embryos (20-30embryos/mL) occurred throughout development from zygote(10min after fertilization)up to the pluteus larval stage(72h after fertilization). Results from this study show that No.0 diesel oil WAFS have an obvious effect on embryonic development. The higher the concentration of oil dispersion, the longer the embryonic development retarded. The results also indicate that the WAFS can make the larvae length shorter than the control. Considering that fish embryo and juvenile can be made deforms by poisonous, they have been successfully used for confirming with north Atlantic pollutants and evaluating the quality of coastal marine. The echinoid embryo has been regarded as the typical organism for research. The research widely used in this paper suggest that morphological variation during embryonic development can be in biological monitoring as indicator organism.

REFERENCES