

Toxicity Evaluation of Treated Refinery Effluent using brine Shrimp (*Artemia salina*) Egg and Larval Bioassay

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A bioassay test using *Artemia Salina* egg and larvae is conducted to evaluate the aquatic toxicity treated refinery effluents. The brine shrimp (*Artemia salina*) has gained popularity as test organism for short-term toxicity testing because of its ease of culture, short generation time, cosmopolitan distribution and its commercial availability as dormant eggs. One year study has been carried out to assess toxicity of effluent samples collected from an oil refinery at Mangalore, (India) on the egg hatching and larval survival rate of *A. salina*. The chemical quality of the refinery effluent collected during Oct 2002 - Sep 2003 was found to be within the prescribed permissible limits. The mean EC50 for egg hatching rate using the effluent collected during the dry and wet seasons was 40.6% and 73.4% effluent (% by volume) respectively. The mean LC50 value for larval mortality using the effluent samples collected during the dry and wet seasons was 37.2% and 48% effluent respectively. The mean No Observed Effect Concentration (NOEC) for the effluent in the present study was 6.3%. The reported natural dilution available in the Arabian Sea near the marine outfall of the refinery is sufficient enough to dilute the effluent below the observed NOEC.

Key words : Toxicity, refinery effluent, *Artemia salina*, EC50, LC50, larvae, egg hatching

In many regions of the world, aquatic toxicity test data are routinely used to evaluate risks associated with the discharge of industrial effluents into marine environment and it is a legal obligation in many countries (Wells *et. al.*, 1998). Such tests can provide an indication of the potential toxicity of an effluent to biological communities inhabiting the receiving waters. With regards to the marine environment, a variety of invertebrate test species is currently used in the framework of internationally

approved methods for toxicity studies (Wells, 1999). To be of practical use in such bioassays, a candidate species, or at least one of its life-history stages, should not only be sensitive to potential contaminants, but should also be relatively easy to collect from the field (*i.e.* abundant), amenable to routine maintenance, culture and rearing in the laboratory (Wells, 1999).

The brine shrimp (*Artemia salina*) has gained popularity as a test organism for short term toxicity testing because of ease of culture,

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short generation time, cosmopolitan distribution and its commercial availability as dormant eggs (cysts). These cysts were suggested as an attractive alternative to standard invertebrate stock cultures, since test animals can be hatched synchronously (Persoone *et al.*, 1989). The toxicological studies of different insecticides and chemicals on various crustaceans have been reported in the literature (Anyachukwo & Akintonwaosa, 1992; Buikema *et al.*, 1976; Ferrando & Andreu- Moliner, 1991; Rodrigues & Amin, 1991; Schimmel & Forester, 1977) but few have selected *Artemia* larvae as test organisms for acute toxicity studies (Goh & Chou, 1998; Sanchez & Barahona, 1995,1996).

The type and concentration of pollutants in refinery's effluent depend on the chemical make-up of the crude oil and the processes used to make the final products. Many of these processes require heating the oil to high temperatures or pressures, or the use of chemicals called catalysts. Other chemicals are used to help create and purify the final products. Refineries use large amounts of water in the refining process as a cooling agent. This water picks up waste oil and impurities. Some impurities such as heavy metals, sulfide, and phenols are in the crude oil itself, while others are formed during the refinement process (eg. cyanide, dioxins, and furans). All of these chemicals can be toxic to aquatic life if the refinery effluent is discharged into the aquatic environment (Sherry *et al.*, 1994). There are a number of studies on the biological impact of concentration of pollutants in refinery effluent in the past. (Sherry *et al.*, 1994, Gaur & Kumar, 1986; Lee *et al.*, 1990; Paine & Chapman, 1992; Rowe *et al.*, 1983a, 1983 b; Sprague, *et al.*, 1978; Westlake *et al.*, 1983a, 1983b)

The most frequently applied procedure for assessing the toxicity of industrial effluents discharged into coastal waters in India involves the use of acute toxicity tests using fishes.

Industries are expected to comply with a set of effluent regulations and guidelines, including fish bioassay tests (CPCB, 1986). It requires that a 48/96 h fish bioassay to test the acute toxicity of treated effluent be regularly undertaken by the industry. There is a growing awareness that acute toxicity tests that usually measure lethality to the test organism do not alone provide a sufficiently sensitive or accurate estimate of the effects of long-term exposure to effluents. Hence, we have conducted a one year study to assess effects of effluent collected from an oil refinery at Mangalore, (India) on egg hatching and larval survival rates of brine shrimp (*A. salina*).

Materials and methods

Mangalore Refinery and Petrochemicals Limited, with a total refining capacity of 9 million metric tonnes is located at Mangalore on the west coast of India. The treated effluent from the refinery is discharged into the Arabian Sea off Mangalore through a submarine effluent pipeline. The rate of effluent discharge during the wet season (Jun – Sep) is 900 m³/h while during the dry season (Oct – May) it is 400 m³/h. Treated effluent samples were collected every month for one year (Oct 2002 – Sep 2003) from the guard pond of the refinery. Chemical parameters of the effluent samples were analysed following standard procedures (APHA, 1995). Six concentrations of the treated effluent such as 6.25%, 12.5%, 25%, 50%, 75% and 100% (by volume) were employed for the toxicity study. Different dilutions of the effluent were made by using clean, filtered seawater having 35 ppt salinity. The final salinity of all test solutions was adjusted to 35 ppt.

Artemia salina, the brine shrimp prefers habitats similar to a marine environment, and lives in hyper saline lakes with very high algal production. Like other zooplankton, it forms a significant food source for many fish and aquatic invertebrates. In *Artemia* after fertilization, the

eggs either develop into free-swimming nauplius larvae (termed ovoviviparous reproduction), or they are surrounded by a shell that forms a cyst. Under normal conditions, by 24 h the outer membrane of the cyst bursts and the embryo, still surrounded by a hatching membrane will emerge. After another short period of time, the embryo breaks through the membrane to emerge as nauplius.

Commercially available *Artemia salina* cysts purchased from San Francisco Bay Brand Inc., USA were used for the study. One gram of *Artemia* cysts was hydrated in distilled water at 4 °C for 12 h. After 12 h, the hydrated cysts were collected and washed in cooled ultrapure grade water. The washed cysts were again washed with chilled and filtered seawater and finally with filtered seawater at room temperature. The hydrated and washed cysts were kept for hatching in filtered seawater at 25° C, pH 8.6 and light intensity of 1000 lux. The cysts hatched out

completely by 24 h. The freshly decapsulated cyst was used for the egg hatching test while the 24 h old larvae were used for the mortality study.

The EC50 test, inhibition of egg hatching was conducted under static conditions in 96 well polystyrene tissue culture plates. In each well, 3.0 ml each of different concentrations of the effluent were added. Five decapsulated cysts were introduced in each well and left for hatching in a light intensity of 1000 lux. For each concentration ten replicates were maintained. The hatching of the cysts in each well was examined under a dissection microscope after 18, 24 and 48 h intervals. The hatching was considered complete only when the larvae come out after breaking the egg membrane (Fig. 1A-D). Hatching rates in each well under different effluent concentrations were recorded and the mean percentage hatching rates were estimated for 18, 24 and 48 h exposure.

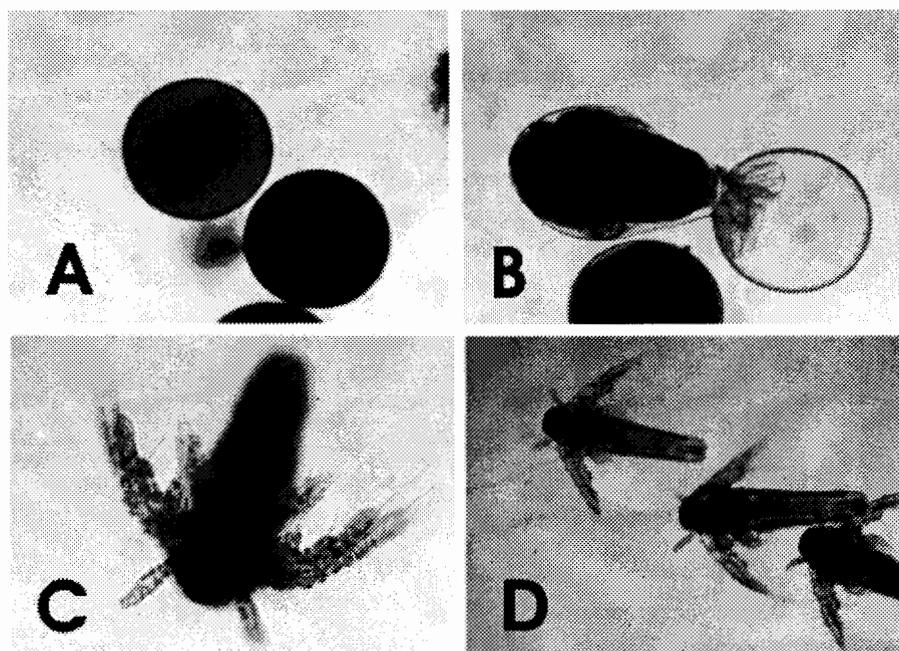


Fig. 1. Photomicrographs showing the *Artemia salina* eggs and larvae (100X) A. Normal eggs with thick capsule (dark coloured) and the decapsulated egg (light coloured) used for the EC50 tests. B. Embryo coming out of the membrane immediately after hatching. C. *Artemia* larva after 24 h used for LC50 tests. D. *Artemia* larvae after 48 h

The LC50 test, larval mortality, was conducted in 96-well polystyrene tissue culture plates under static conditions using 24 h old larvae. In each well five *Artemia* larvae were introduced and the mortality of the larvae were examined under a dissection microscope after 18, 24, 40, 48 and 65 h exposure time. Larvae were considered dead if they did not exhibit any internal or external movement during 10 seconds of observation. Mortality rates in each well under different effluent concentrations were recorded

and the mean % mortality rates were estimated for 18, 24 and 48 h exposure.

The EPA Probit Analysis software (version 1.5) was used for calculating EC/LC50 values and 95% confidence limits. The results of the toxicity tests were statistically analyzed to determine if effluent concentration was significantly different ($p=0.05$) from the clean seawater control with respect to egg hatching and larval survival rates. The short-term chronic

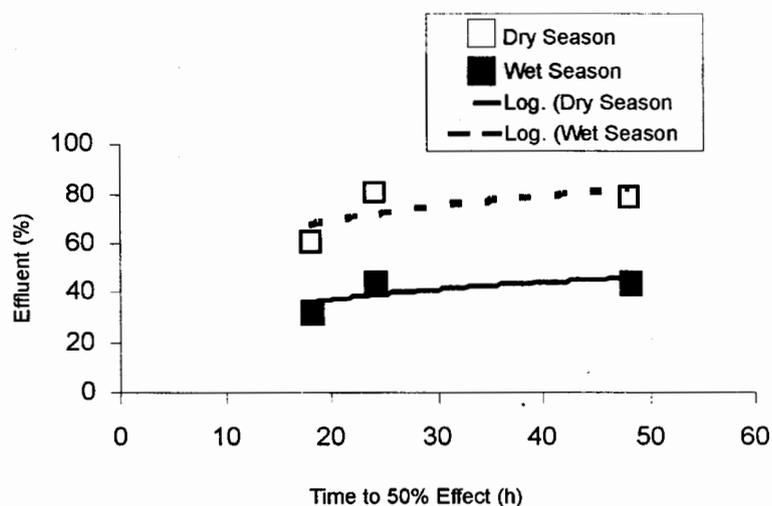


Fig. 2. Toxicity curve showing the effect (EC50) on *Artemia* egg hatching with time to refinery effluent sampled during wet and dry seasons.

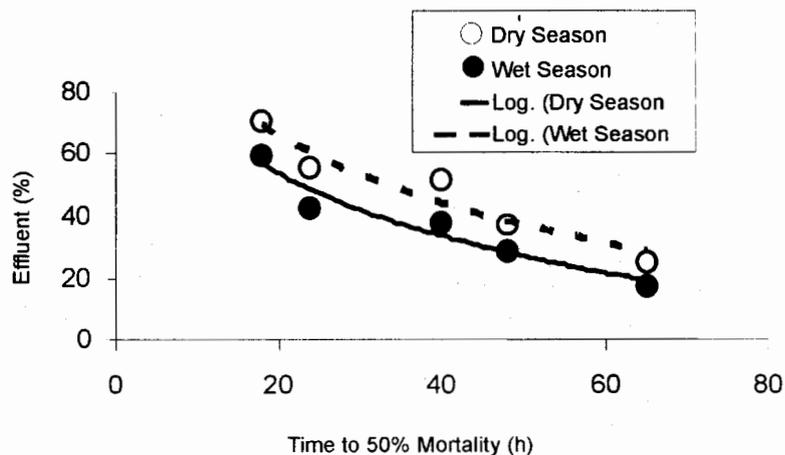


Fig. 3. Toxicity curve showing the effect (LC50) on *Artemia* larvae with time to refinery effluent sampled during wet and dry seasons.

Table 1. Major chemical parameters (mean \pm SD) of the treated effluent sampled during the dry and wet seasons

Chemical Parameters	Dry Season (Oct – May)	Wet Season (Jun – Sep)
Total Dissolved Solids (mg/L)	813 \pm 430.6	272 \pm 124.9
pH	7.38 \pm 0.54	7.37 \pm 0.46
Total Suspended Solids (mg/L)	18.3 \pm 16.7	8.06 \pm 6.9
Biochemical Oxygen Demand (mg/l)- 5 days at 20 °C	7.48 \pm 4.4	6.09 \pm 4.3
Chemical Oxygen Demand (mg/L)	36.41 \pm 17.4	46.13 \pm 52.82
Ammonical nitrogen (mg/L)	1.99 \pm 2.03	1.94 \pm 2.44
Cyanide (mg/L)	0.003 \pm 0.001	0.002 \pm 0.001
Sulphide (mg/L)	0.013 \pm 0.005	0.007 \pm 0.005
Oil and grease (mg/L)	3.16 \pm 2.66	2.75 \pm 2.25
Phenol (mg/L)	0.14 \pm 0.05	0.17 \pm 0.05

Table 2. Toxicity of refinery effluent sampled during dry (Oct – May) and wet (Jun – Sep) seasons to *Artemia salina* egg hatching.

Toxicity parameters	Exposure period	Dry Season (% by volume)	Wet Season (% by volume)
EC50 (95% confidence limits)	18 h	32.8 (22.4 – 44.4)	61.0 (42.5 – 83.6)
	24 h	44.2 (15.7 – 89.4)	80.5 (58.1 – 115)
	48 h	44.8 (32.9 – 58.1)	78.9 (58.0 – 111.3)
	Mean	40.6	73.4
	NOEC	6.3	6.3
LOEC	12.5	12.5	
ChV	9.4	9.4	

Calculated mean EC50 (with 95% confidence limits), No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Chronic Value (ChV)

toxicity test endpoints expressed as the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) and the Chronic Value (ChV) were calculated using EPA Dunnett's test software.

Results and Discussion

The *Artemia* egg and larval bioassay were found to be a very effective tool for evaluating the toxicity of refinery effluent as reported by earlier workers (Persoone *et. al.*, 1989). The mean chemical parameters of the treated effluent samples collected during the wet season and dry seasons are shown in Table 1. The chemical

Table 3. Toxicity of refinery effluent sampled during dry (Oct – May) and wet (Jun – Sep) seasons to *Artemia salina* larvae.

Toxicity parameters	Exposure period	Dry Season (% by volume)	Wet Season (% by volume)
EC50 (95% confidence limits)	18 h	59.7 (47.7 – 73.8)	70.6 (58.1-86.4)
	24 h	42.5 (33.2-53.2)	55.5 (43.9-69.6)
	40 h	37.9 (29.3-47.1)	51.7 (40.4-64.7)
	48 h	28.6 (10.5-51.5)	36.8 (27.6-47.5)
	65 h	17.5 (5.2-30.2)	25.4 (10.0-41.5)
	Mean	37.24	48.0
NOEC		6.3	6.3
LOEC		12.5	12.5
ChV		9.4	9.4

Calculated mean EC50 (with 95% confidence limits), No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Chronic Value (ChV).

parameters of the effluent samples were found to be within the prescribed permissible limits (CPCB, 1986). Generally the chemical quality of the effluent samples collected during the wet season was superior when compared to the samples collected during the dry season. During the wet season the treated effluent stored in the guard pond was diluted by heavy rain.

The mean median effective concentrations (EC50) for egg hatching rates are shown in Table 2. The normal and decapsulated eggs and hatching process are shown in Fig 1. The EC50 value for the effluent collected during the dry season varied from 32.8% to 44.8% and varied from 61 to 78.9% during the wet season. The mean EC50 value (after 18, 24 and 48 h exposure) for the dry season was 40.6% and for the wet season was 73.4% (Tables 2). The EC50 for egg hatching rate was found to be increasing with time as shown in Fig. 2. The mean median lethal concentrations (LC50) are shown in Table 3. The 24 - 48 h *Artemia* larvae are shown in Fig 1. The LC50 value for the effluent collected during the dry season was varying from 17.5% to 59.7% and from 25.4 to 70.6% during the wet season. The

mean LC50 value (after 18, 24, 40, 48 and 65 h exposure) for the dry season was 37.2% and for the wet season was 48% (Tables 3-4). The EC50 for egg hatching rate was found to be increasing with time, while the LC50 for larval mortality was found to be decreasing with time (Figs. 2 and 3). During the dry season, mean 48 h EC50 for egg hatching was 44.8%, while mean 48 h LC50 for larval mortality was 28.6%. Similarly during wet season mean 48 h EC50 for egg hatching was 78.9%, while mean 48 h LC50 for larval mortality was 36.8%.

In the present study, the larval bioassay test was found to be more sensitive than the egg bioassay (Tables 2 - 3). The 72 h old *A. salina* larvae were reported as more sensitive when compared to 24 h old larvae exposed to selected insecticides (Sanchez & Barahona. 1996). The toxicity values obtained using refinery effluent in the present study (Tables 3 - 4) was comparable with similar studies reported earlier (Anyachukwo & Akintonwaosa, 1992; Buikema *et al.*, 1976; Ferrando & Andreu- Moliner, 1991; Rodrigues & Amin, 1991; Schimmel & Forester 1977).

Various short-term toxicity test endpoints such as the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC), and the Chronic Value (ChV) were calculated and given in Tables 2 & 3. The NOEC for both egg hatching and larval survival rate was 6.3% for both the dry and wet seasons. Similarly, LOEC for both egg hatching and larval survival rate was 12.5% for both the dry and wet seasons. The ChV for both egg hatching and larval survival rate was 9.4%. Rowe *et al.* (1983a, 1983 b) studied the sub-lethal effects of effluents from a Canadian oil refinery on rainbow trout and observed severe effect on the growth of young fishes after exposure to 30% effluent. In the present study, the NOEC was found to be 6.3%, while a NOEC of 6% was reported for rainbow trout to the effluent from

a Canadian oil refinery. Effluent from the same refinery had a mean LC50 of 76% toward *Daphnia pulux* in 48 h test (Westlake *et al.*, 1983). The 14-day LC50 was 6.4% effluent and EC50 for the reproductive failure was 3.1%. Reproduction of *D. pulux* was reportedly more sensitive (NOEC, 0.92%) than several bioassay using fishes to the refinery effluent. Effluent from an oil refinery in India was reported to be toxic to *S. capricornutum* at 5% (Gaur & Kumar, 1986). Toxicity of effluent samples collected from two refineries in Canada was at lower level compared to the effluent samples collected from the Finnish and Indian refineries (Sherry *et al.*, 1994). In order to reduce the environmental impact of effluent discharge, the effluent should be diluted to NOEC levels at refineries in western countries (Sherry *et al.*, 1994). In the present study, the observed NOEC for the effluent during wet and dry season was 6.3% (Tables 2 - 3). The reported natural dilution of the effluent at the marine outfall of the refinery in the Arabian Sea off Mangalore varied from 20 to 625 times for a distance of 1 to 1,000 m with a coefficient of diffusion of 5 m²/second (NIO, 1998). Therefore, the final concentration of refinery effluent in the sea near the marine outfall will be 0.16% - 5%. Hence, the highest possible concentration of the effluent in the Arabian Sea near the marine outfall of the refinery will be less than the NOEC observed in the present study and can be considered safe for the health of marine organisms thriving in the area.

Based on the egg bioassay test, mean EC50 value for the effluent collected during the dry and wet season was 40.6% and 73.4% effluent (% by volume) respectively. Based on the larval bioassay test, mean LC50 value for the effluent collected during the dry and wet season was 37.2% and 48% respectively. The mean No Observed Effect Concentration (NOEC) for the effluent in the present study was 6.3% effluent.

The reported natural dilution available in the Arabian Sea near the marine outfall of the refinery is sufficient enough to dilute the effluent below the observed NOEC. The *Artemia* egg and larval bioassay was found to be a very effective tool for evaluating the toxicity of refinery effluent. *Artemia* larval bioassay test was found to be more sensitive than the egg bioassay.

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