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Developmental abnormalities of *Bettasplendens* embryos reared in oil and grease contaminated water samples of Vavuniya reservoir, Sri Lanka

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Abstract

In Vavuniya urban area the wash-outs from petroleum automobile service stations contain Kerosene oil, lubricating engine oil, grease as major compounds that contributing oil and grease pollution in Vavuniya reservoir, Sri Lanka. To investigate the potential oil and grease toxicity on fish embryo development, the Bettasplendens (Siamese fighting fish) embryos were reared in water samples from Vavuniya reservoir. Average value of oil and grease content from polluted location was determined for the preparation of artificial aliquots of oil and grease (20 mg of Engine oil: Kerosene oil: Grease =1:1:1 w/w). These aliquots mixed with the 1000 ml of water samples obtained from non-polluted location of this reservoir. Prepared medium was shaken vigorously for 5 minutes and 25ml was added in each Petridish to prepare the larval growth medium. Meantime, 25ml of non-polluted water was taken in another set of Petri dishes as a control setup. Treatments and control were allowed under natural diffuse photo periods. Frequent microscopic examinations were done until 72hpf (hours post-fertilization) for embryological abnormalities and compared with control group from March to December2016. Significant embryo developmental abnormalities such as delayed and reduced outgrowth of the caudal fin-fold (p= .008), reduction in melanin pigment formation (p= .04) and higher larval mortality (75 %) were observed. Hence, much higher potential of oil and grease impacts on fish embryo development observed in this study, suggests the threats for survival of local fish species in Vavuniya reservoir. Assessment of oil and grease impact on the reproductive potential of the local fish species is necessary for its conservation in their natural habitats.

Keywords: Developmental abnormalities, Oil and grease pollution.

Introduction

Most of the studies on phototoxicity of polycyclic aromatic compounds (PAC) in water bodies have done on zooplanktons[1]. However, only a few researches have focused on phototoxicity of PAC preparations in fish embryo development and among this, majority of research works examining the mechanism of this toxicity in fish early life stages, especially on undefined crude oil[2]. Though, minority of studies have only addressed the oil and grease toxicity[3]. Tricyclic fluorenes, dibenzothiophenes and phenanthrenes are the most abundant PAC that acts as cardiotoxicagents and causes pericardial and yolk sac edema on fish larvae[5,6]. Although, researchers found some toxic effects like intracranial bleeding and blister-like abnormalities of the caudal fin tissues in Zebra fish embryos resulting from oil exposure, the exact pathological origin is unknown[6]. These oil and grease (O&G) compounds are poly-aromatic hydrocarbons (PAHs) and containing toxic impurities which could cause the harmful effects to the aquatic organisms, such as in Vavuniya reservoir, Sri Lanka. As this reservoir is situated in the urban area, experiencing frequent oil and grease pollution that caused by petroleum automobile service stations. Kerosene like odour in *Oreochromisniloticus* due to oil and grease pollution in Vavuniya reservoir causes negative impacts on inland fisheries in recent years[4]. In order to study the effects of O&G on fishembryo development and larval growth performance, Siamese fighting fish (*Bettasplendens*) was selected. Indeed, *Bettasplendens* have showed a viable growth performance and higher percentage of survival in the laboratorial conditions in the northern region of Sri Lanka[7]. Hence, *Bettasplendens* is an ideal oviparaous fish species with aerial respiratory adaptations for laboratory oriented studies.

Materials and methods

Sample collections: Water samples were collected fortnightly from polluted location of the Vavuniya reservoir using water sampler (grab sampling methods was adapted) and average content of oil and grease was determined by using gravimetric method.

Determination of oil and grease: The samples collected from the polluted location of the Vavuniya reservoir were extracted with three aliqoutes of Hexane in a separator funnel. Samples were shaken vigorously for 2 minutes per extraction. The first Hexane aliquot was used to rinse the sample container so that its entire contents were transferred to the extraction vessel. The ratio of solvent to sample was 1:10, i.e. 20 ml of Hexane (per extraction) per 200ml of sample. The solvent extracts were passed through a drying funnel containing anhydrous sodium sulfate to absorb the additional moisture. Then the oil and grease combined with Hexane was separated using rotary evaporator then the weight of the oil and grease was determined after being kept at desiccators overnight.

Artificial preparation of oil and grease: According to the average value of O&G content of Vavuniya reservoir water sample (polluted location), artificial aliquots of O&G were prepared (set to resemble the sample water from Vavuniya reservoir polluted location in terms of weight in parts per million). Artificially prepared oil and grease of 20mg (Engine oil: Kerosene oil: Grease=1:1:1 w/w) was mixed with the 1000 ml of water samples obtained from non-polluted location of this reservoir where, embryos to be reared as treatment. The prepared water medium was shaken vigorously for 5 minutes and 25ml was placed in each Petridish to prepare the larval growth medium. As a control setup, 25ml of non-polluted water (without O&G contaminations) was taken in another set of Petri dishes. Three replicates were made for treatments and control and allowed under natural diffuse photo periods. Frequent microscopic examinations were done until 72 hpf (hours post-fertilization) for embryological abnormalities and analyzed with control group from March to December 2016.

***Bettasplendens* embryo exposure:** Onset of spawning of *Bettasplendens*, embryos were transferred to the Petridishes at a rate of 10 embryos per Petridish by using delicate droppers. Mild aeration was initiated which resembles the semi-artificial incubators at room temperature under natural diffuse photo periods (Figure-1). Assumptions were made that all the eggs were fertilized and viable onset of spawning.

Microscopic imaging: Frequent microscopic observations were doneuntil 72hpf (hours post-fertilization) from early embryos (zygote) via fry to early larval stages[3]. Abnormalities among tail morphometry (caudal fin outgrowth) and pigmentation intensity were analyzed by using XENON Student's Microscopes. Microscopic images of embryo development from control and experimental setup were imaged using digital camera (Sony DSC-W350 14.1 Mega Pixel, 72dpi). Mortality rates were recorded by direct observations from 6 – 120 hpf.

Statistical analysis: To compare the morphometric differences in control and in treatment, 2t –Proportion test was performed for the ratios of Normal: Reduced fin-folds in both control and treatment. This analysis were performed (at 95% confident interval, α=0.05) using Minitab 16.0) to find-out the significant differences.

Results and discussion

The fish embryo developmental abnormalities such as delayed and reduced outgrowth of the caudal fin-fold, reduction in melanin pigment formation were significantly (p= .04) greater in the oil and grease contaminated water sample (20 *ppm*) than the control. Onset of fertilization, active cell division started in the blasto-disc of fish eggs (Figure-2: B_1, B_2) in all setups. Though cytolysis occurred in the oil and grease contaminated water sample (treatment) (Figure-2: A_1, A_2). Percentage of larval mortality was higher in the treatment than control.

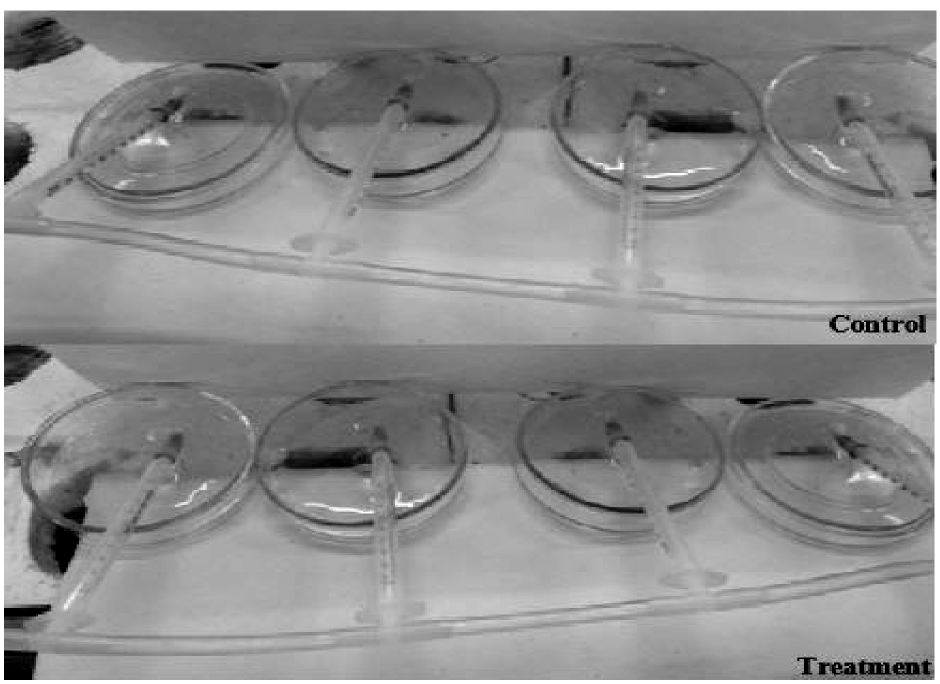

Figure-1: Artificially prepared incubator units for fish embryo development.
[Control setup with non-polluted water samples, Treatment with O&G constituents]

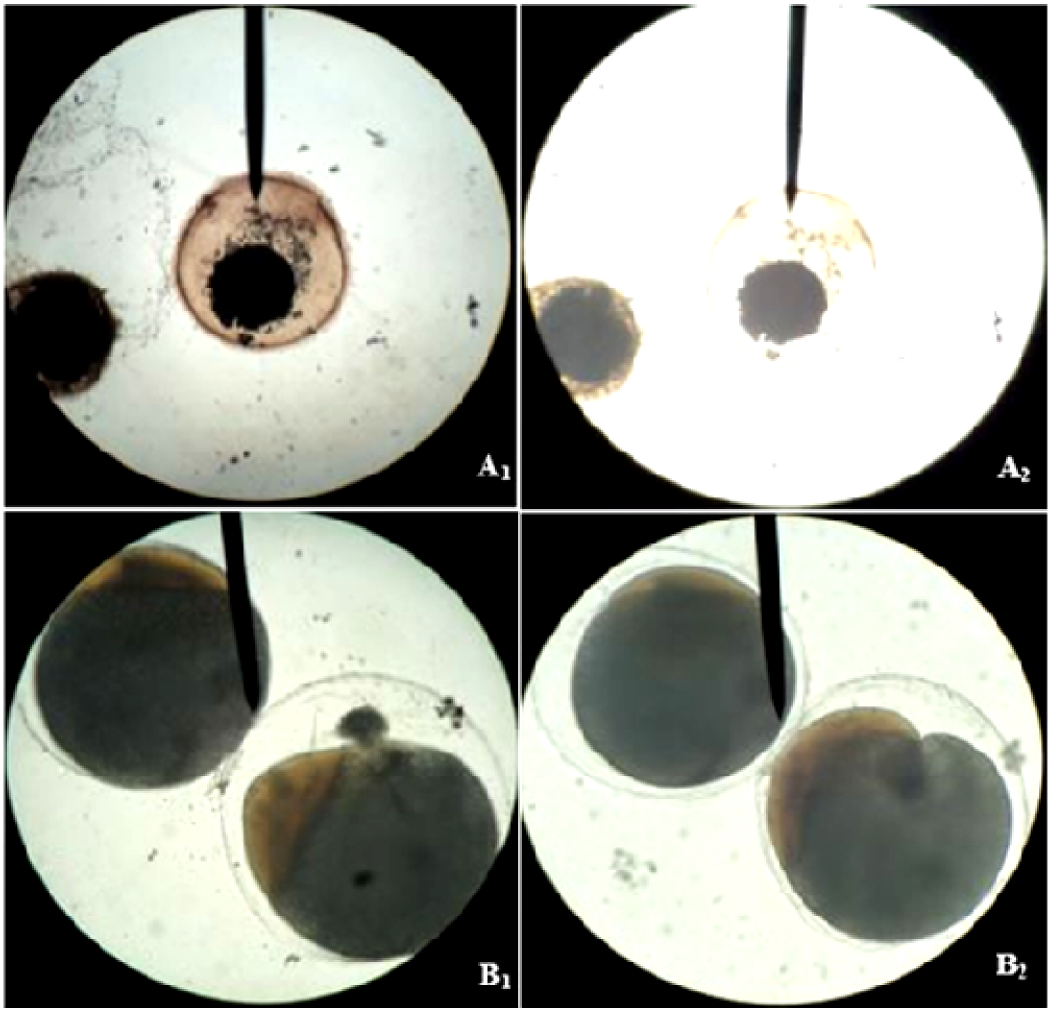

Figure-2: Dead eggs were opaque (A_1, A_2). Viable eggs are clear with prominent previtteline space and active cell division visible in the blasto-disc in the animal pole (B_1, B_2).

The embryo development in the control setup was found as the ratio of normal fin-fold: reduced fin-fold growth is 9:1 and the percentage of larval mortality is 20% (Table-1). This reduced fin-fold growth phenomena of few individuals may be due to the genetic influences (in-born). However, majority of the embryos showed viable growth performance in the experimental setup.

In treatment, higher larval mortalities (75%), reduced fin-fold ratio and less pigmentation were observed. This was suggested, mainly due to the acute toxicity of oil and grease constituents. There was a significantly strong difference ($p = .008$) observed in normal: reduced caudal fin-folds (Figure-3) between control and treatment.

Pigment intensity (Figure-3) was also higher in control than treatment in this analysis (Table-2).

There was not any remarkable pericardial edema, yolk sac edema and intra cranial hemorrhaged observed in *Bettasplendens* (Figure-4).

Table-1: Summary of embryo development of fish (*Bettasplendens*).

Developmental variables	Control	Treatment
Number of embryos	120	120
Survival rate (%)	80	25
Mortality rate (%)	20	75
Normal fin-fold	86	20
Reduced fin-fold	10	10
Ratio; Normal: Reduced	9:1	2:1
High pigmentation	84	21
Low pigmentation	12	9
Ratio : high: low	7:1	7:3

Table-2: Summery of statistical analysis of 2t-Propotion test for caudal fin & pigmentation.

Factors	Treatment (Observed values)	Control (Expected values)	p-values
Caudal fin:			0.008
Normal outgrowth	13	58	
Reduced outgrowth	07	06	
Melanin pigmentation:			0.042
High pigment intensity	21	84	
Low pigment intensity	09	12	

Comparatively lesser melanin pigment formation and reduced caudal fin outgrowth of *Bettasplendens* larvae was clearly visible in the oil and grease contaminated water sample than the control (Figure-3 and 4). This kind of effects observed in the Zebra fish larvae kept in crude oil aliquots with natural sunlight exposure[3].

Poly-aromatic hydrocarbons (PAHs) and toxic impurities from petroleum automobile service stations caused harmful effects to the fish early life histories. Indeed, the embryo development of *Bettasplendens* highly affected by these compounds under indoor laboratorial conditions with diffuse natural photo periods. Especially, embryo development of the fish is highly affected by thesecompounds[6]. Reduced caudal fin outgrowth of Zebra fish embryos reared in the crude oil treatment in the presence of solar radiation has been proved in other studies[3].

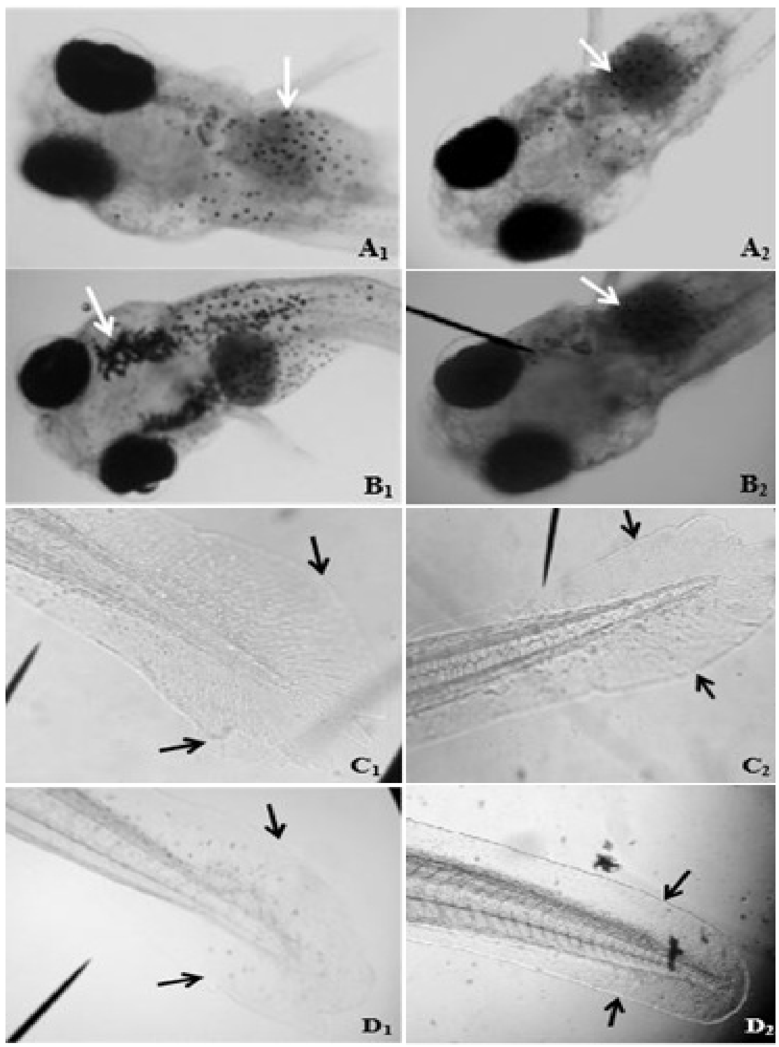

Figure-3: Morphological abnormalities of *Bettasplendens* larvae (x 100) in control (1) and treatment (2): Variation in pigment intensity (dorsal view), higher pigmentation in A_1 and B_1 comparing with A_2 and B_2 (white arrows indicates the location of pigmentations). C & D: Morphology of caudal-fin outgrowth (lateral view); higher caudal-fin outgrowth in C_1 and D_1 comparing with C_2 and D_2 (black arrows indicates the outline of the caudal-fin).

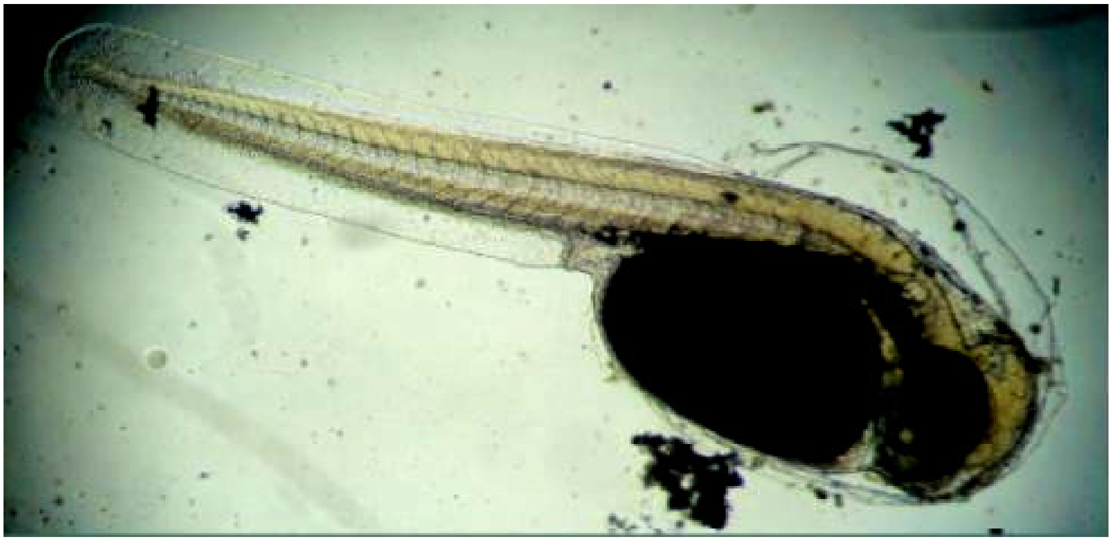

Figure-4: Newly hatched fry (x 100) of *Bettasplendens* in the treatment set up where the caudal fin out growth is reduced but pericardial and yolk sac edema were not clearly visible.

Conclusion

Reduced caudal fin outgrowth, reduction in melanin pigment formation and higher larval mortality rates justifies the acute toxicity of oil and grease on *Bettasplendens* embryo development. Hence, much higher phototoxic potential of oil and grease impacts on fish embryo development observed in this study, suggests the threats for survival of local fish species in Vavuniya reservoir. Thus, understanding the consequences of the consequences of varying oil and grease discharges from petroleum automobile service stations on abnormalities of local fish species in inland water bodies are necessary for safeguard the rich fish species composition and abundance.

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