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Determination of Lethal and Effective Concentrations (LC_x and EC_x) of Hydrophobic Organic Contaminants in *Parhyale hawaiensis*

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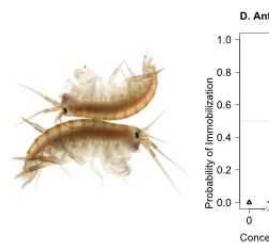
Ibrahim Lawan¹

¹Heriot-Watt University



Ibrahim Lawan

Heriot-Watt University



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We use this protocol and it's working

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Disclaimer

The protocol provided here is intended for educational purposes only and should be conducted in appropriate laboratory settings. The developers of this protocol are not liable for any damages or consequences arising from its use. Users should ensure compliance with relevant safety regulations and ethical guidelines when conducting experiments involving live organisms.

Abstract

This protocol outlines a robust methodology for assessing the acute toxicity of selected Polycyclic Aromatic Hydrocarbons (PAHs) in the tropical marine amphipod *P. hawaiensis*. The procedure integrates standard OECD guidelines with adaptations specific to marine species and includes sex-specific 96-hour static-renewal tests to determine LC_x and EC_x values. The protocol covers all aspects, from initial range-finding tests to the final selection of PAH concentrations, addressing potential sex-based differences in PAH susceptibility, an area often underexplored in ecotoxicological studies. Advanced statistical analyses and stringent quality control measures are incorporated to ensure high reproducibility and reliability. This protocol is designed to enhance our understanding of PAH toxicity in marine environments, thereby contributing to evidence-based environmental management strategies.

Materials

- Test Organisms: Parhyale hawaiensis (adults, 8-12 months old, separated by sex)
- Selected PAHs: Naphthalene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]pyrene, Dibenzo[a,h]anthracene, and Benzo[ghi]perylene.
- Solvents: Dimethyl sulfoxide (DMSO) or Acetone
- Test Medium: Reconstituted seawater
- Containers: 15-mL amber glass bottles (Fisherbrand)
- Analytical Equipment: ATAGO Handheld refractometer, API 5-in-1 test strips, Metrohm pH/Conductometer, Digital thermometer, Stereoscopic microscope

INTRODUCTION

- 1 Polycyclic Aromatic Hydrocarbons (PAHs) are a critical class of hydrophobic organic contaminants known for their persistence, bioaccumulation potential, and toxicity within marine ecosystems (Honda & Suzuki, 2020). With the increasing prevalence of PAHs due to anthropogenic activities, it is essential to understand their impact on marine organisms to inform effective environmental management and conservation strategies.

This study focuses on assessing the acute toxicity of priority PAHs using *Parhyale hawaiensis*, a tropical marine amphipod that serves as an excellent model organism due to its ecological relevance and sensitivity to environmental stressors (Dos Santos et al., 2022; Paris et al., 2022). The key objectives are:

1. To establish a robust procedure for determining the acute toxicity of hydrophobic organic pollutants in *P. hawaiensis*.
2. To assess sex-based differences in PAH toxicity, providing critical insights into the potential variability in susceptibility.

The protocol employs a two-step approach comprising initial range-finding tests followed by definitive acute toxicity tests, aligning with standard ecotoxicological practices (OECD, 2004, 2019, 2024). This methodology ensures that results are reproducible, reliable, and comparable with existing literature.

Objective

To determine the acute toxicity profiles of selected priority PAHs in male and female *P. hawaiensis* by conducting 96-hour static-renewal tests to establish LC_x (Lethal Concentration x%) and EC_x (Effective Concentration x%) values.

1. Procedure: Preparation of Stock and Working Solutions

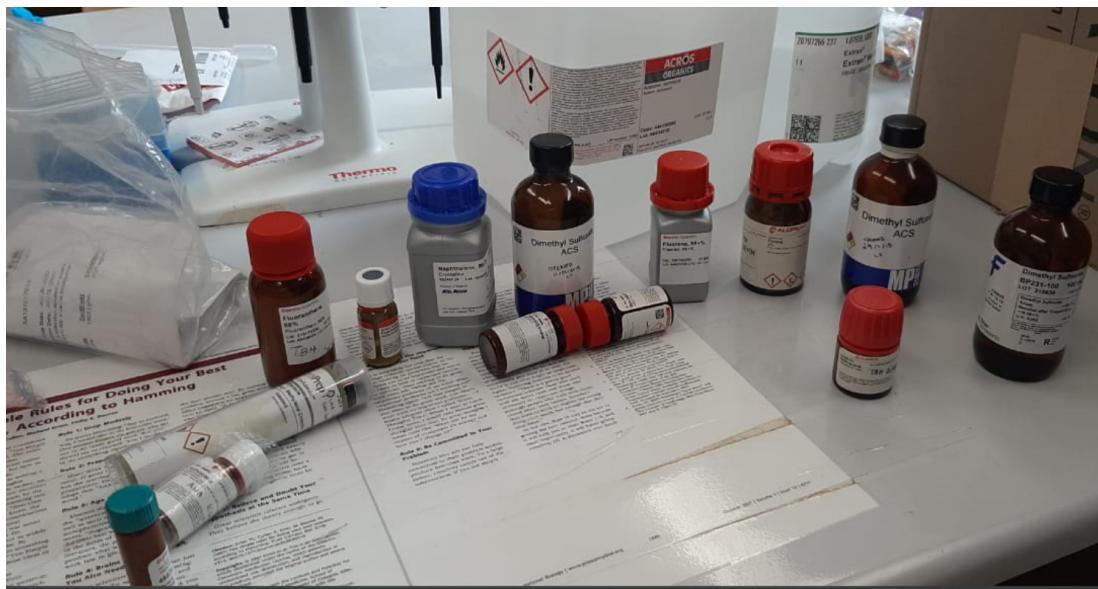
- 2
 - 1.1 Stock Solutions:** Dissolve each PAH in DMSO or acetone to create stock solutions.
 - 1.2 Working Solutions:** Prepare working solutions by serially diluting stock solutions in reconstituted seawater.
 - 1.3 Solvent Concentration:** Ensure the final solvent concentration in the test solutions does not exceed 0.01% (v/v) to 0.03% (v/v), based on PAH hydrophobicity.
 - 1.4 PAH Concentration Verification:** Measure actual PAH concentrations in working solutions using GC-MS (optional but recommended for accuracy).

2. Procedure: Range-Finding Test

- 3 **2.1 Objective:** To determine the appropriate concentration range for the definitive acute toxicity test and to identify the lowest concentration causing 100% mortality and the highest concentration causing no observable effect for both sexes.

2.2 Test Design:

- Utilize a logarithmic series of concentrations (e.g., 0.001, 0.01, 0.1, 1.0, 10 mg/L) for each PAH (Blaise & Férard, 2005).



Some selected PAHs and Carrier Solvents

- Include at least five concentrations plus controls.
- Use three replicates per concentration for each sex, with five organisms per replicate.



Parhyale hawaiensis

2.3 Controls:

- Set up negative controls with reconstituted seawater.
- Include solvent controls with the highest concentration of DMSO or acetone used.

2.4 Exposure Conditions:

- Conduct the test under semi-static conditions for 48 hours, maintaining a temperature of $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a 14:10h light cycle.
- Do not feed the organisms during the test period.

2.5 Test Medium Renewal:

- Replace 100% of the test medium every 24 hours to maintain water quality and PAH concentrations.

2.6 Observations:

- Record mortality and immobility at 24 and 48 hours for each sex separately.
- Consider organisms dead if they show no movement and their body colour has completely changed.
- Consider organisms immobile if they cannot swim within 10 seconds after gentle prodding.

2.7 Water Quality Monitoring:

- Measure temperature, pH, dissolved oxygen, and salinity at the beginning and end of the test and after each renewal.
- Ensure all parameters remain within acceptable ranges for *P. hawaiensis*.

2.8 Chemical Analysis:

- Measure actual PAH concentrations in freshly prepared and used solutions using GC-MS (optional but recommended).

2.9 Data Analysis:

- Calculate the percentage of mortality and immobility for each concentration and sex.
- Plot concentration-response curves for males and females separately.
- Identify the concentration range between the lowest causing 100% effect and the highest causing no observable effect for each sex.

2.10 Result Interpretation:

- Use range-finding results to select appropriate concentrations for the definitive test.

2.11 Quality Control:

- Ensure control mortality does not exceed 10% for each sex.
- Verify that solvent control results do not differ significantly from negative controls.

2.12 Reporting:

- Document all conditions, observations, and results.
- Report the concentration range identified for definitive testing for each PAH, separately for males and females.

3. Procedure: Definitive Acute Toxicity Test

- 4 **3.1 Test Concentrations:** Select at least five concentrations for each PAH, bracketing the expected LC50/EC50 for males and females separately.

3.2 Replicates: Set up 30 replicates (based on the availability of organisms) per concentration for each sex, with one organism per replicate.

Note: Ensure the implementation of the 3Rs principle in animal experimentation (Replacement, Reduction, and Refinement) to enhance the ethical treatment of animals in research.

3.3 Test Containers: Use 15-mL amber glass bottles with 10-12 mL of test solution per replicate.



15-mL amber glass bottles

3.4 Control Groups: Include blank control (reconstituted seawater) and solvent control groups for each sex.

3.5 Exposure Conditions:

- Conduct the test for 96 hours under static-renewal conditions.
- Maintain a temperature of $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a 14:10h light cycle.
- Do not feed the organisms during the test period.

4. Procedure: Observations and Data Collection

5 4.1 EC50 Determination:

- Check for immobility at 24, 48, 72, and 96 hours for each sex.

4.2 LC50 Determination:

- Record mortality at 24, 48, 72, and 96 hours for each sex.
- Remove and record dead organisms at each observation time.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Acute Toxicity Test Data Recording Sheet															
Test Organism: Parthyale hawaiensis															
Test Substance: Anthracene															
Test Duration: 96 hours															
Researcher: Ibrahim Lawan. Date: 05/08/2023															
Commencing Experiment Day-0 (0hr)	Day-1 (24hr)			Day-2 (48hr)			Day-3 (72hr)			Day-4 (96hr)					
S/N	Concentration (μ g/L)	Total Number of Parhyale alive	Number of Parhyale Alive	Immobility (%)	Mortality (%)	Number of Parhyale Alive	Immobility (%)	Mortality (%)	Number of Parhyale Alive	Immobility (%)	Mortality (%)	Number of Parhyale Alive	Immobility (%)	Mortality (%)	
1	312.5	30	30	5	0	30	10	0	19	45	37	0	100	100	
2	625	30	30	2	0	30	8	0	22	33	27	15	83	50	
3	125	30	30	0	0	30	3	0	25	20	17	27	27	10	
4	25	30	30	0	0	30	0	0	30	8	0	29	17	3	
5	5	30	30	0	0	30	0	0	30	0	0	30	0	0	
6	0	30	30	0	0	30	0	0	30	0	0	30	0	0	
7	0	30	30	0	0	30	0	0	30	0	0	30	0	0	

Acute Toxicity Test Data Recording Sheet

Test Conditions:

- Temperature: 27 °C
- pH: 8.0
- Dissolved Oxygen: 6 mg/L
- Salinity (if applicable): 30 ppt
- Photoperiod: 14 hours light / 10 hours dark

Notes:

- Record any observations of abnormal behaviour)(or appearance
- Calculate Immobility (%) = (Number Immobile /Total Number) × 100
- Calculate Mortality (%) = (Number Dead / Total Number) × 100

5. Procedure: Water Quality Monitoring

6 5.1 Daily Monitoring:

- Monitor and record temperature, pH, dissolved oxygen, and salinity daily.
- Ensure all parameters remain within acceptable ranges for *P. hawaiensis*.

6. Procedure: Data Analysis

7 6.1 Analysis of Mortality and Immobility:

- Calculate percentage mortality and immobility for each concentration and time point, separately for males and females.
- Use the drc package in R (with the drm function) to determine LC_x and EC_x values for each sex.

Example R code: 1. model1 <- drm(Immobilized/Exposed ~ Conc, Sex, weights = Exposed, data = Anthracene,

```
type = "binomial", fct = LL.2())
summary(model1)
ED(model1, c(50), interval = "delta") # for EC50 and confidence intervals
```

2. model2 <- drm(Dead/Exposed ~ Conc, Sex, weights = Exposed, data = Anthracene,

```
type = "binomial", fct = LL.2())
summary(model2)
ED(model1, c(50), interval = "delta") # for determining the LC50 and 95% confidence intervals
```

6.2 Dose-Response Curves:

- Generate dose-response curves for each PAH, separately for males and females.

Example R code: plot(model1, broken = TRUE, ylim = c(0, 1), xlim = c(0,5000))

6.3 Statistical Analysis:

- Use generalised linear models (GLM) to investigate interactions between more variables.

```
Example R code: model3 <- glm(Dead/Exposed ~ Concentration + PAH + Sex +  
Concentration*PAH + Concentration*Sex, family = binomial,  
data = PAH_Data, weights = Exposed)  
summary(model3)  
Anova(model3)
```

7. Procedure: Quality Control

8 7.1 Control Mortality:

- Ensure control mortality does not exceed 10% for each sex.

7.2 Validation of Solvent Controls:

- Verify that solvent control results do not significantly differ from blank controls.

7.3 Reference Toxicant Tests:

- Conduct reference toxicant tests to validate test sensitivity and reproducibility.

8. Procedure: Reporting

9 8.1 Documentation:

- Document all experimental conditions, observations, and results.

8.2 LCx and ECx Reporting:

- Report LCx and ECx values with 95% confidence intervals for each PAH, separately for males and females.

8.3 Comparative Toxicity Analysis:

- Discuss the relative toxicity of the tested PAHs, comparing the responses of males and females.

9. Procedure: Safety Considerations

- 10
- Handle PAHs with caution, as many are known carcinogens.
 - Use appropriate personal protective equipment (PPE), including gloves, lab coat, and safety goggles.

- Conduct all experiments in a well-ventilated area or fume hood.
- Dispose of PAH-contaminated materials as hazardous waste according to institutional guidelines.

Conclusion

11 This protocol provides a standardised methodology for assessing the acute toxicity of selected PAHs (as reference hydrophobic organic pollutants) in *P. hawaiensis*. Researchers can follow these procedures to generate high-quality, reproducible data on the LC_x and EC_x values of the hydrophobic organic pollutants. Moreover, this protocol can serve as a template for future toxicity studies on other contaminants or species, contributing to the broader field of marine ecotoxicology.

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