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Standardised Methods for Feed Tabs Preparation to Assess Feeding Rate and Exposure to Insoluble Substances in *Parhyale hawaiensis*

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Research Project 1



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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 standardised procedures for preparing feed tabs to assess feeding rates and conduct dietary toxicity testing of insoluble substances in *Parhyale hawaiensis*. By addressing the need for reliable testing methodologies, particularly those for evaluating the effects of insoluble pollutants on aquatic organisms, this protocol offers a robust framework for researchers. It facilitates comprehensive ecotoxicological studies, contributing to a better understanding of the environmental risks of toxicants (soluble or insoluble) using feeding metrics in marine ecosystems. This protocol uses benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH), as a model toxicant because of its ecotoxicological concerns and physicochemical properties (e.g. low water solubility [0.00162 mg/L at 25°C], high lipid–water partition coefficient [$\log K_{Lipw}$ 7.35], and high octanol–water partition coefficient [$\log K_{ow}$ 6.13]). Because BaP has low solubility, toxicity investigations must include assessment via diet as a more realistic and reliable method to assess its effects on aquatic organisms rather than just aqueous exposure.

Protocol status: Working

We use this protocol and it's working

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MATERIALS

These materials are essential for the preparation of feed tabs and for conducting the experiments outlined in the protocol.

1. Acetone as a carrier solvent
2. Agar (Fisher Scientific)
3. Aeration system
4. Analytical Balance (Sartorius digital analytical scale)
5. Artificial seawater
6. Benzo(a)pyrene (BaP) as a model toxicant
7. Cellulose (Sigma-Aldrich)
8. Cylindrical moulds (1.1 cm diameter and 0.6 cm height, custom-built aluminium device)
9. Desiccator (Secador, USA)
10. Fume cupboard (ecoflow)
11. Glass beakers (300 ml, 500 ml, 1 litre)
12. Glass marbles
13. Granulated feed supplement (containing 52% crude protein and 12% lipid)
14. Incubator (Stuart SI500 orbital incubator)
15. Metal tray
16. Milli-Q water
17. Microwave (Micro Chef ST44 microwave)
18. Refrigerator
19. Scraper
20. Spoon
21. Tissue papers

Introduction

1. Dietary exposure and toxicological assessments of insoluble substances represent emerging frontiers in aquatic ecotoxicology, necessitating the development of standardised bioassays that accurately reflect real-world exposure scenarios (Connon et al., 2012). Current toxicity testing methodologies primarily rely on soluble substances introduced into the aqueous phase, which inherently limits their applicability for evaluating the effects of insoluble compounds (Gotz et al., 2021). Unlike their soluble counterparts, insoluble substances exhibit distinct chemical properties that lead to heterogeneous distribution and non-uniform uptake routes within aquatic environments (Hartmann et al., 2015). Therefore, there is a critical need to adapt test scenarios to address these challenges effectively. Dietary uptake has emerged as the predominant route of exposure to insoluble substances in aquatic organisms; however, this pathway is often overlooked in conventional testing protocols (Bundschuh et al., 2019). To address these limitations, we propose a standardised protocol utilising decomposition and consumption tablets (DECOTABs) (Gotz et al.,

2021; Kampfraath et al., 2012) as a reliable food source for *Parhyale hawaiensis*, thereby facilitating systematic and rigorous toxicity testing via the oral pathway.

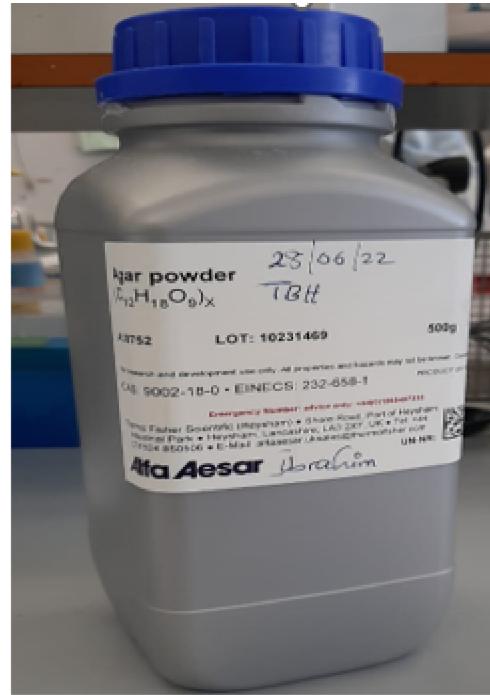
Parhyale hawaiensis, a tropical marine amphipod, has significant ecological importance and is a valuable model organism in various scientific disciplines (Paris et al., 2022). Recognised for its sensitivity to environmental stressors, *P. hawaiensis* has emerged as a promising candidate for tropical marine studies and laboratory investigations. Previous studies have established protocols for laboratory culturing, acute toxicity testing in water and sediment, internal dose determination, gene expression analysis, and assessment of DNA damage and chromosomal alterations in *P. hawaiensis* (Dos Santos et al., 2022). Leveraging these advancements, our objectives are twofold: 1) to develop a protocol for chronic toxicity evaluation with a particular focus on feeding rate by modifying the decomposition and consumption tablets (DECOTABS), and 2) to standardise a protocol for preparing feed tabs tailored for the toxicity testing of insoluble substances, with benzo(a)pyrene (BaP) serving as a model toxicant. The physicochemical properties of BaP, including its low water solubility (0.00162 mg/L at 25°C), high lipid–water partition coefficient ($\log K_{lipw}$ 7.35), and octanol-water partition coefficient ($\log K_{ow}$ 6.13), underscore its profound insolubility and facilitate its bioaccumulation and toxicity in aquatic organisms (Hylland, 2006; Latimer & Zheng, 2003; Pampanin & Sydnes, 2013). In addition to addressing the necessity for reliable testing methodologies to evaluate the effects of insoluble pollutants in aquatic organisms, our protocol is poised to make significant contributions to ecotoxicology, especially when assessing the effects of both soluble and insoluble substances using feeding metrics.

Step I – Preparing Standard Feed Tabs

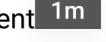
2m 45s

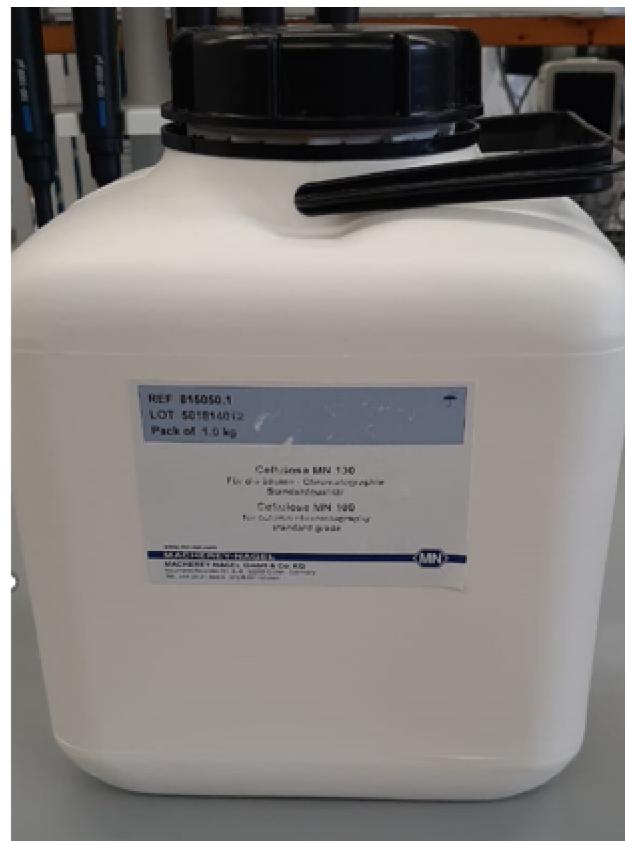
- 2 Prepare feed tabs with a total weight of five (5 g). All measurements below can be scaled up or down based on the target feed weight.

- 2.1 a. Mix 30 mL Milli-Q water and 1.2 g agar (Fisher Scientific) in a **300-ml glass beaker**
(NB: use larger beaker when a higher mass of agar is used as it foams and spills during heating).



Agar powder (Fisher Scientific)

- 2.2** b. Heat the mixture in a microwave to about  100 °C to dissolve the agar for approxim  1m 45s
  00:00:45 to  00:01:00 until it foams.
- 2.3** c. Add  1.08 g cellulose (Sigma-Aldrich) and  2.72 g small granular feed supplement  1m (52% crude protein and 12% lipid) and homogenise for  00:01:00 .



Cellulose (Sigma-Aldrich) as binder, filler, and anti-caking agent



Granular feed supplement (52% crude protein and 12% lipid)

- 2.4** d. Before cooling, pour the mixture into 36 cylindrical moulds of 1.1 cm diameter and 0.6 cm height (custom-built aluminium device).



Aluminium cylindrical moulds (1.1 cm diameter and 0.6 cm height)

(NB: Aquatic animals, such as amphipods and fish, do not utilise cellulose because it is indigestible in their gut (Ighwela et al., 2012). Therefore, cellulose is nutritionally unavailable to these aquatic organisms and is used as a dietary binder, non-nutritive filler, anti-caking agent, bulking agent, and stabiliser. It is soluble in ether but insoluble in water).

Step II – Supplementing Insoluble Substance (BaP) in Feed Tabs

5m

- 3 As described below, the selected insoluble substance, such as **BaP**, can be added to the feed supplement before adding **agar**.
 - 3.1 a. Sum up the weights of the agar, cellulose, and feed supplement to know the total weight and determine the concentration of the insoluble substance that can be added per gram of diet.

3.2 b. Prepare the BaP-supplemented diet in a 300-ml glass beaker by combining the Δ 2.72 g of granulated feed supplements and Δ 10 mL acetone with or without BaP (**NB:** high volume of carrier solvent was used to facilitate even distribution of toxicant).

3.3 c. Prepare diets with 0 (control), acetone-treated (control), and 50, 250, or 1250 μg BaP (nominal)/g diet and mix for approximately Δ 00:02:00 (**NB:** concentrations can be changed based on the insoluble substance used and the determined effective concentration).

3.4 d. Keep the mixture under a fume cupboard to evaporate acetone for Δ 00:03:00 . 3m



Fume cupboard (ecoflow)

3.5 e. Add Δ 1.08 g cellulose and complete the feed preparation as in **Step I above**.

(**NB:** Acetone was chosen as the carrier solvent because it can quickly evaporate under a stream of air and has been shown to cause minimal biological effects in previous studies (Hallare et al., 2006; Shatilina et al., 2020).

Step III – Drying Feed Tabs

1d 0h 45m

- 4 a. Pour the mixture into 36 cylindrical moulds of 1.1 cm diameter and 0.6 cm height (custom-built aluminium device).
- 4.1 b. The tablets may initially have a convex surface that quickly flattens during agar solidification at 6 °C in refrigerator.
- 4.2 c. Scrape the surfaces using an even scraper before removing them from the moulds.



Freshly made feed tabs

- 4.3 d. Remove the feed tabs from the moulds after approximately 00:15:00 in a refrigerator 6 °C .
- 4.4 e. Then, dry them in an incubator at 45 °C for 24:00:00 .

1d

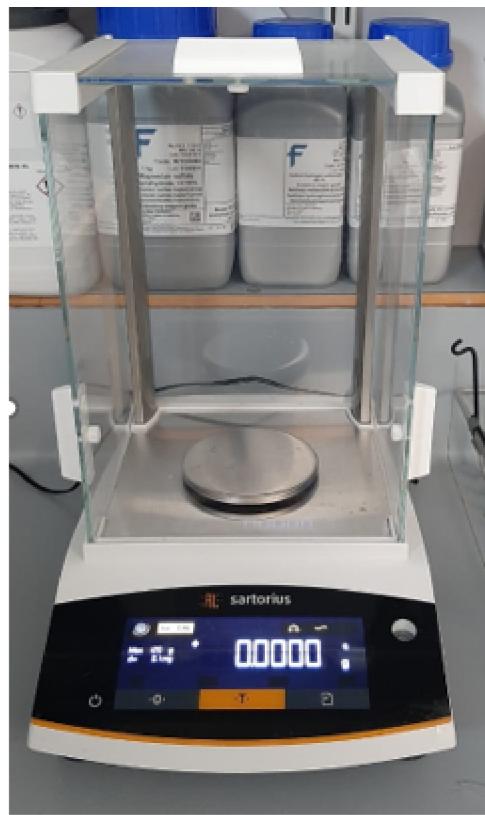


Incubator (Stuart SI500 orbital incubator)

- 4.5 f. After this, place the feed tabs in a desiccator or fume cupboard for  00:30:00 and  30m measure the initial dry weight (**dw1**) with an Analytical Balance.



Desiccator (Secador, USA)



Analytical Balance (Sartorius digital analytical scale)

- 4.6** g. Feed tabs can be stored at room temperature for use within months or at -20 °C for long-term use.

Step IV – Evaluating Feed Tabs Water Stability and Adjustment Factor 1w 0d 0h 30m

- 5** In this step, the effects of drying and prewetting on the weight stability of the feed tabs at different handling steps were evaluated using a fine analytical balance.

Water Stability – The stability of feed tabs in water was evaluated by mimicking their replacement cycles during exposure to *P. hawaiensis* (**NB**: this section evaluates the expected weight loss of the feed tabs without amphipods feeding on them).

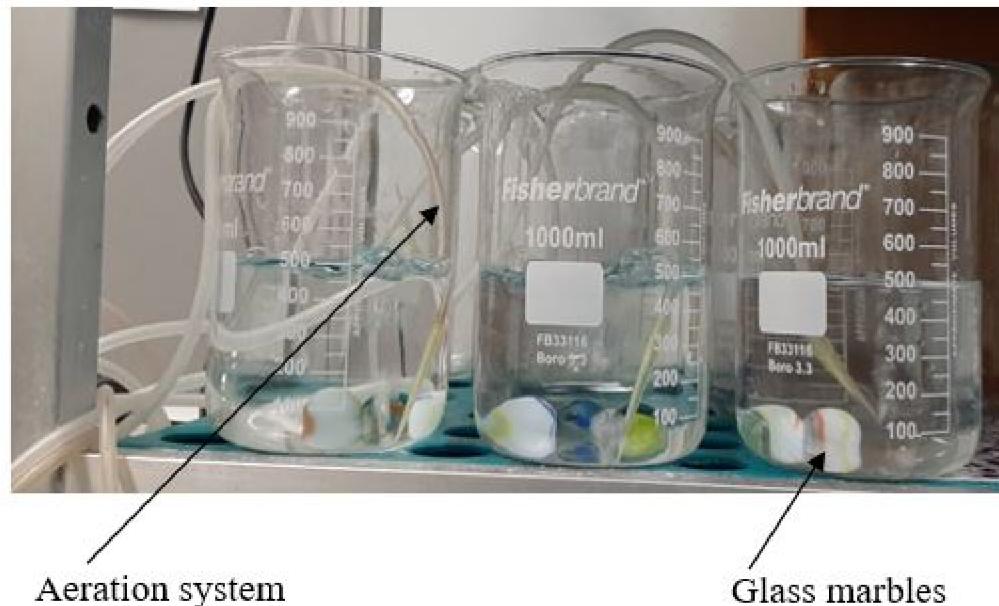
- 5.1** Measure the initial dry weight (**dwi**) of at least four (4) feed tabs, transfer to seawater and prewe 1d them for 24:00:00 before the experiment.

- 5.2 After 24:00:00 of prewetting, transfer them into a mock exposure chamber (a 1 L glass beaker filled with 500 mL seawater with continuous aeration but no test organisms). 1d
- 5.3 Add three (3) glass marbles in the beaker as hiding materials without amphipods and keep in a temperature-controlled room with a **12:12-h light:dark cycle** at approximately 30 °C for 48:00:00 (**NB:** this is to mimic the amphipod exposure scenarios). 2d
- 5.4 After the 48:00:00, remove the feed tabs, gently blot on tissue papers, and dry them for 24:00:00 in the incubator at 45 °C. 3d
- 5.5 Place them in a desiccator or fume cupboard for 00:30:00 and weigh them again using a analytical balance to obtain the final dry weight (**dwF**). 30m
- 5.6 The difference between **dwI** and **dwF** (mean weight loss [**MWL**]) per day provides stability of the feed tabs and allows for comparisons between them.
- 5.7
- $$MWL \left(\frac{mg}{day} \right) = \frac{dwI - dwF}{trial\ days}$$
- (**NB:** trial days = 3 days (**72 h**) in this case)
- 5.8 Use the dry weight difference in milligrams per day as an adjustment factor for the feeding rate calculation of the amphipod. This served as the expected weight loss of a particular feed tabs formulation without any organism feeding on them.

Step V – Feeding Experimental Organisms Feed Tabs and Feeding Rate Ca...

- 6 a. Experiment with a **1 L** glass beaker with an appropriate volume (**500 mL**) of aerated artificial seawater.

- 6.1 b. Add three (3) glass marbles as hiding places in each beaker to reduce the stress on the organisms.



- 6.2 c. Place three pairs of matured male and female *P. hawaiensis* in each prepared beaker and feed them ad libitum with weighed and **24h-prewetted** feed tabs.



Differentiating matured male and female *P. hawaiensis*

- 6.3 d. Weight the feed tab before feeding the organisms to obtain initial dry weight (**dwl**). 1d
- 6.4 e. Prewet the feed tabs in seawater for **⌚ 24:00:00** in a temperature-controlled room (exposure environment) before transferring them to the experimental beakers (**NB**: prewetting facilitates organisms' easy feeding of the feed tabs). ⌚ 1d
- 6.5 f. Replace the feed tabs with new ones in an alternating cycle of three days (**⌚ 72:00:00**) ⌚ 3d throughout the exposure duration.
- 6.6 g. After every removal, dry the used feed tabs for **⌚ 24:00:00** in an incubator at **🌡️ 45 °C**, weigh and record the final dry weight (**dwF**) ⌚ 1d
- 6.7 h. **The feeding rate (FR)** per day of Parhyale with dwl and dwF of the specific feed tabs was adjusted by the number of feeding days and the mean weight loss (**MWL**) of the feed tabs per day calculated from the stability measurement.

Determination of the Feeding Rate

1w 0d 1h

- 6.8** a. As explained above, after feeding the organisms with weighed (**dwI**) and **24h-prewetted feed** **3d** tabs, remove the leftover feed tabs with a spoon every **⌚ 72:00:00** from all the experimental containers and transfer to a small pre-dried glass Petri dishes on a metal tray.
- 6.9** b. Dry the leftover feed tabs for **⌚ 24:00:00** in an incubator at **🌡 45 °C** and then p **1d 0h 30m** desiccator or fume cupboard for **⌚ 00:30:00**.
- 6.10** c. After **⌚ 00:30:00**, weigh the feed tabs with an analytical balance to obtain the feed leftover final dry weight (**dwF**). **30m**
- 6.11** d. Calculate the feeding rate by subtracting leftover final dry weight (**dwF**) from the initial dry weight of feed tabs (**dwI**)
- 6.12** e. Therefore, divide the estimated weight loss by the number of living *P. hawaiensis* at the end **3d** of **⌚ 72:00:00** and calculate per day. Use the formula below:

$$FR \left[\frac{\text{mg}}{\text{Parhyale} \times \text{day}} \right] = \frac{\left(\frac{\text{dwI(mg)} - \text{dwF(mg)}}{\text{feeding days}} \right) - MWL \left[\frac{\text{mg}}{\text{day}} \right]}{n(\text{living Parhyale})}$$

Gotz et al. (2021)

Where:

FR – Feeding rate.

dwI: initial dry weight of the feed tab before **24 h** pre-wetting and **48 h** feeding to the organisms.

dwF – final dry weight of the feed leftover after the organisms feed on it

n = number of living organisms at the end of a particular feeding cycle

MWL: adjustment factor as mean weight loss of the feed tab without organisms feeding on it.

