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# Preparation of mollusc larval shells for individual geochemical analysis V.2

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**We use this protocol and it's working**

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## Abstract

This protocol describes the digestion process of mollusc larvae and is modified from the procedure found in Becker et al. (2005). Here, we indicate the entire process to obtain the digestion solution, as well as cleaning glassware, actual larval digestion and extraction of shells until storage for subsequent geochemical analysis or observation. This protocol is applicable to shelly larvae of any species, once they are collected and brought to the laboratory.

The goal of this preparation is to isolate each larval shell to perform individual geochemical analyses, instead of dissolving several specimens for bulk analysis. To do this, great care needs to be taken to the handling of the shells using ultra-pure solutions and acid-washed glassware to avoid chemical contamination of all kind.

Reference:
Becker, B.J., Fodrie, F.J., McMillan, P.A., Levin, L.A., 2005. Spatial and temporal variation in trace elemental fingerprints of mytilid mussel shells: a precursor to invertebrate larval tracking. *Limnology and Oceanography*, 50, 48-61. doi: 10.4319/lo.2005.50.1.0048.

## Guidelines

The protocol that follows was used to study the elemental fingerprints of *Shinkailepas tollmanni* larval shells. This is a small hydrothermal vent limpet that is particularly abundant on the shells of larger provannid gastropods. We collected nearly ready-to-hatch veliger larvae (approximately 100 µm) by opening egg capsules deposited by females in ridges at the surface of *Ifremeria nautileii* shells. Larvae were collected from *Ifremeria* shells aboard the research vessel *L'Atalante* during an oceanographic cruise in the southwest Pacific in spring 2019 (CHUBACARC, Hourdez & Jollivet, 2019). Upon collection, larvae were stored dry at -20°C in groups of 10 to 100 in 2mL microtubes until processed according to the protocol described here.

Reference:
Hourdez, S. & Jollivet, D. 2019. CHUBACARC cruise, L'Atalante R/V. doi: 10.17600/18001111. Sismer.

## Materials

Equipment:
-Horizontal laminar flow cabinet (ISO5; equivalent to class 100)
-Fume hood
-Polarized-light stereomicroscope (under the laminar-flow cabinet)
-Cold light source (under the laminar-flow cabinet)
-pH meter, calibrated between 4 and 10
-Magnetic stirrer
-Ultrapure water system
-Sink

Consumables:
-White coat
-Nitrile gloves
-Low lint wipes (Kimberly-Clark Kimtech Science Delicate Task Wipes, 15402680)
-Beaker (50 mL)
-Beaker (2L) for storage of HNO3 solution.
-Pipette (1 mL)
-Low-retention tips (1 mL)
-Two PTFE beakers with cover (2 L)
-Microscope glass slides
-Standard Carbon Adhesive Tabs, Extra Pure, Diameter: 12mm (Science Services, MN77825-12)
-Pasteur pipettes (150 mm)
-Short sable brush
-Glass salt dips (2 mL capacity) and lids
-Petri dishes
-Dust particles should be avoided as possible. For this purpose, it is recommended to place an adhesive mat (BioClean SupaTac White STW, 15278318) at the doorstep of the lab where digestion will be performed
-Parafilm
-Dedicated chemical waste container
-Permanent marker

Reagents:
-Suprapur® hydrogen peroxide ($H_2O_2$) 30% (Merck KGaA: 107298)
-Primar Plus – Trace analysis grade nitric acid ($HNO_3$) (>68%) (Fisher Chemicals, 10098862)
-sodium hydroxide (NaOH) 0.5 M (15604900)
-absolute ethanol

## Troubleshooting

## Safety warnings

Wear labcoat and nitrile gloves at all times. Work under a clean laminar-flow cabinet.

## Preparation of the digestion solution (for 20 mL final volume of approximately 15% H2O2)

1d

1 In a glass beaker
Add 10 mL Suprapur® hydrogen peroxide ($H_2O_2$) 30%

2 Place the beaker with hydrogen peroxide on a magnetic stirrer and control pH. This step is made to prevent the calcified shells from dissolving in the $H_2O_2$ solution.

2.1 Activate shaker and place pH meter electrode in the $H_2O_2$ solution.

2.2 Using a 1 mL cone with pipette, slowly introduce sodium hydroxide (NaOH) 0.15 M in the beaker until pH reaches 8.5.

**Expected result**

The value of pH usually reaches 8.5 when the added volume of NaOH is approximately similar to the initial volume of $H_2O_2$.

2.3 Remove pH meter electrode and deactivate shaker.

3 Cover the beaker with parafilm and leave it at least 24h, for the solution to release gas (may cause the shells to “explode” when filled with gas otherwise). This solution is active for approximately a week. For multiple series of successive sample digestions (*i.e.*, over more than a week), prepare this solution every Monday to use it from Tuesday to Friday.

## Cleaning tools and containers

2d

4 Put 500 mL of Primar Plus$^{TM}$ – Trace analysis grade nitric acid ($HNO_3$) (>68%) in each of two 2-L PTFE beakers (subsequently referred to as beakers A and B, respectively).

5 Complete each beaker with 1.5 L ultra-pure water (resistivity 18.2 MΩcm).

6 Place in the PTFE beaker A: Pasteur pipettes (at least one per sample to prepare, plus backup), Petri dishes, zip plastic bags for storage and transport of all materials.

7 Cover with a PTFE lid both beakers and leave Overnight under a fume hood.

8 On the next morning, transfer all materials from the PTFE beaker A to the PTFE beaker B (the one that contains the clean $HNO_3$ solution) for rinsing for 00:10:00 .

9 Empty and discard the $HNO_3$ solution from the PTFE beaker A in a dedicated chemical waste container.

10 Rinse the PTFE beaker A (now empty).

10.1 In the PTFE beaker A: add 1 L ultrapure water and discard in the sink.

10.2 Repeat step 10.1 twice.

10.3 In the PTFE beaker A: add 2 L ultrapure water.

11 Transfer all materials from the PTFE beaker B (containing $HNO_3$) to the beaker A (now containing ultrapure water).

12 Transfer the $HNO_3$ solution from the PTFE beaker B to another beaker for storage. This "used" $HNO_3$ solution can be used as a first bath (equivalent of beaker A at Step 6) for additional tools to clean.

13 Rinse the PTFE beaker B (now empty) following Step 10.1 and Step 10.2.

14 Place both PTFE beakers (A: containing ultrapure water and all materials; B: empty) Overnight under the laminar flow cabinet with PTFE lids.

15 On the following morning, add 2 L ultrapure water to the PTFE beaker B.

16 Have each material from the PTFE beaker A rinsed a few seconds in the ultrapure water from the PTFE beaker B, then placed to dry under the laminar flow cabinet. Pasteur pipettes can hold some water at their tips even under the laminar flow. Try and dry those by shaking the pipettes by hand with a few strong strokes.

17 Discard used ultrapure water from both PTFE beakers in the sink.

18 Once dried, place the tools in the cleaned plastic bags and zip. Leave those under the laminar flow cabinet until use.

## Cleaning laboratory room

1h

19 At the beginning of each sample preparation day, activate the laminar flow cabinet and wait for at least 01:00:00 prior starting with the sample preparation. Wear lab coat and nitrile gloves at all times in the room.

20 In the meantime, clean all surfaces of the laboratory with low lint wipes and absolute ethanol, including the inside of the laminar flow cabinet.

21 Sweep the room.

## Digestion of larvae

1d

22 Clean glass salt dips with absolute ethanol using a squeeze bottle (no wipes to avoid lint) over sink. Leave to dry under the laminar flow cabinet.
All subsequent steps must be performed under the laminar flow cabinet.

23 Label the glass salt dip with sample name using a permanent marker.

24 Let thaw the samples stored in microtubes (approximately 15 minutes).

25 Collect some (approximately 50) larvae from their storage container (*e.g.*, microtubes) using an acid-washed Pasteur pipette. Place those in the glass salt dip under the stereomicroscope, drop by drop, spacing the drops on the glass salt dip surface to avoid previously released larvae to stick on the pipette. Check that no remaining larvae are

stuck inside the pipette by capillarity. If so, attempt extracting them with ultrapure water. Discard the Pasteur pipette.

26 Remove most of the water (that was collected with the larvae) from the glass salt dip using a pipette with a low-retention tip. Avoid contact between the tip and the shells as those can attach on the tip by capillarity. Discard tip.

27 Slowly add 2 mL $H_2O_2$ to fill the glass salt dip with pipette and a low-retention tip. Cover with a lid and leave Overnight .

28 Repeat steps 24 to 27 for each sample to a maximum of 200 specimens total. Expect to process approximately 200 larval shells on the next day. Switch off the laminar flow cabinet before leaving the room (leaving the cabinet on overnight has proven to induce substantial evaporation of $H_2O_2$ where samples were being digested in, even if the containers were covered with a lid).

29 On the following morning, check that the digestion is over. Shells should be translucent under natural light under the stereomicroscope and should exhibit Maltese cross pattern under polarised light. If digestion is still ongoing, remove most of the solution using pipette and low-retention tip without extracting larval shells, fill the glass salt dip (2 mL) with clean $H_2O_2$ solution with pipette and clean low-retention tip and leave until digestion is complete. Discard tip. If digestion is not complete by mid-day, the $H_2O_2$ solution may not be sufficiently active anymore. Our experience is that at this point, larval shells tend to start exhibiting abnormal flexibility and dissolution.

## Extract cleaned larval shells

3h

30 When digestion is over, remove most of the solution using pipette and low-retention tip without extracting larval shells and discard tip.

31 Fill the glass salt dip with ultrapure water with pipette and low-retention tip. Discard tip.

32 Leave 01:00:00 with Petri dish as cover.

33 Clean a microscope slide with a low-lint wipe with absolute ethanol. Place an Extra Pure adhesive tab on the slide and write the name of the sample on the slide.

34 Clean a sable brush with absolute ethanol. Dip the brush in ultrapure water for re-hydration and avoiding hydrophobic interactions.

35 Carefully collect larval shells with the brush (1-5 specimens at a time) by capillarity under the stereomicroscope (use polarized light for easy visualization of the larval shells).

36 Carefully place the larval shells on the Extra Pure adhesive tab on the slide (under the stereomicroscope in natural light using the cold light source directed on the tab's surface) by slowly sweeping the brush on the tab's surface.

37 Clean the brush with absolute ethanol and re-hydrate with ultrapure water.

38 Repeat steps 35 to 37 as necessary to place all specimens on the tab.

39 Let the surface of the tab dry under the laminar-flow cabinet.

40 Surround the group of deposited larval shells on the tab using a permanent marker to facilitate their location during geochemical acquisition.

41 Clean the glass salt dip with ultrapure water and absolute ethanol, check cleanliness under the stereomicroscope.

42 Let the glass salt dip dry under the laminar-flow cabinet.

43 Store the slide in a clean slide box and cover the box with parafilm for storage until geochemical analyses.

44 At the end of the day, wipe all surfaces in the room with low lint wipes and absolute ethanol.

45 Switch off the laminar flow cabinet before leaving the room.