

Microplastic Sampling, Processing and Detection in Whole and Dissected Fish

SUPPLIES:

Field Sampling for Fish/Biota

Coastal/shallow water fishes obtain by one or more of the following:

1. Hook and Line
2. Otter trawl
3. Seine Nets

Mesopelagic fishes:

1. I-K midwater trawl

Larval Fishes obtain by one or more of the following:

1. Neuston nets
2. Plankton nets

Additional supplies:

1. MS222, clove oil, or ice bath
2. Gloves for handling fishes
3. Coolers filled with glass bottles for transport.

Field Sampling for Water Supplies

Pumping surface water:

1. Pump (TBD)
2. Adjustable 3-Section Extension Pole (Home Depot)
3. Duct tape/zip ties
4. Transect tape
5. Extra D batteries (pump requires 3 batteries)
6. 20 ft of tubing
7. Clamps for attaching tubing to pump
8. 4L amber bottles (5 per sample, i.e. enough to collect 20L per section at a location of interest)
9. 1.6µm filtered nanopure water

If sampling at depth:

1. Nansen or Niskin Bottles
2. Glass funnel
3. 4L amber bottles

Laboratory Supplies

1. Large glass containers (lidded) for fish transport
2. Dissection tools
3. 70% EtOH
4. Dish soap (commercial)
5. Plant Dryer/Incubator able to hold 50°C
6. Large ceramic funnel to hold sieves
7. Sieves
(Fisherbrand™ U.S. Standard Stainless Steel Sieves, 8 in. dia. x 2 in.D, 500 µm mesh (Cat. No. 04-881-10Q),
Fisherbrand™ U.S. Standard Stainless Steel Sieves, 8 in. dia. x 2 in.D, 125 µm mesh (Cat. No. 04-881-10Y),
Fisherbrand™ U.S. Standard Stainless Steel Sieves, 8 in. dia. x 2 in.D, 63 µm mesh (Cat. No. 04-881-10C),
Gilson 8in diameter Stainless Steel Sieve, #635, 20 µm mesh (Cat. No. V8CF #635 BU)
8. Tubing for filter flask
9. 10% KOH Solution
10. 4.4 M NaI solution; density 1.5g/mL
11. Sonicator (~500Hz)
12. Orbital shaker

Sampling: Water collected with Biota samples – follow water sampling SOP provided

Neutrally Buffered Tricaine Methanesulfonate

1. Fiquel (Argent Chemical Laboratories, Inc) or Tricaine-S (Western Chemical)
1. Sodium Bicarbonate
2. Water (Fresh or Salt) – This will affect the amount of buffering solution needed. For fresh water 1:2 (weight MS222:weight Sodium Bicarbonate powder)
 - a) Fresh stock solution is necessary – discard after 10 days
 - b) Stock solution for euthanasia should be at least 250mg/L ms-222 and significantly higher for large fish and elasmobranchs.

Staining with Nile Red

1. Acetone (Cat. No. A18-4)
2. N-Hexane (Cat. No. AC160780010)
3. Nile Red (Cat. No. AC415711000)
 - a. Create a stock at 1mg/ml in pure acetone
 - b. Create a working solution at 10µg/ml by diluting your stock in n-hexane
 - c. Filter working solution on a 1.6µm filter
4. Foil

Glassware preparation

1. Wash with commercial dishwashing liquid
2. Rinse with HPL-grade distilled water
3. Rinse with 70% ETOH and dried in oven

Note: All surfaces should be cleaned in a similar manner prior to each extraction to prevent contamination.

Detection of unstained and stained particles:

1. Compound microscope with a 4X and 10X Objective
2. External light source (e.g. Dyna Light #DL-150)
3. CMOS mounted camera (e.g. Olympus Lumenera Infinity-3C)
4. Fluorescence Microscope
 - a. Capable of green wavelength excitation (e.g. excitation 460-500, emission 535LP)
 - b. Or- Crime Light (450-510nm, with orange filter at 529nm) with viewing goggles

PPE:

1. Cotton lab coat
2. Nitrile gloves
3. Face mask
4. Perform extractions in a laminar flow cabinet to prevent potential contamination with airborne MPs.

PROTOCOL:

Field Sampling:

1. Prior to field sampling, wash required number of glass holding bottles with 1.6µm filtered nanopure water three times.
2. Fill cooler with ice and embed cleaned holding jar to keep samples cool
3. Collect fish via humane methods listed above (be sure the collection method is IACUC, DFW, AVMA approved)
4. Immerse O2 bubbler in euthanasia solution (Ice bath or MS-222 or Clove oil) – there should be adequate oxygen during this process
5. Once fish are on deck, immediately submerge in ice bath (0-4°C cold shock method) for large number of fish being processed at once or neutrally buffered tricaine methanesulphate MS-222 of at least 98% purity (>300mg/L) or clove oil (>400mg/L) for small batches of fish to euthanize quickly.
6. Keep fish in stock solution for at least 10 min following cessation of opercular movement

Laboratory:

1. Rinse euthanized fish with ultrapure water prior to dissection

2. Dissect alimentary tract, muscle tissue, gills, or other focus tissue from whole body of fish, prep/store separately from body.
3. Grind muscles/other tissues using a blender and store at -20°C until ready for extraction.

Be sure to run samples in parallel with blank sample to assess contamination.

Extraction:

1. Whole fish extraction and/or alimentary canal extraction
2. Rinse fish/AC with nanopure water
3. Add whole fish to 10% KOH solution (10:1 v/w)
4. Incubate at 40°C for 48-72 h until fully digested
5. Set up stacked sieves on the collection apparatus
6. Vacuum filter through filter membrane (22 µm) rinsing with nanopure water as needed
7. Transfer filter membrane to 10-15 mL 4.4 M NaI solution
8. Sonication at 50 Hz for 5 min
9. Agitation on an orbital shaker (200 rpm) 5 min
10. Centrifugation (500 x g for 2 min)
11. Separate pellet from supernatant
 - I. Pellet (x2)
 - II. Resuspend in 10-15 mL 4.4 M NaI solution
 - III. Repeat sonication and agitation process
 - IV. Separate pellet from supernatant – repeat
 - a) Supernatant
 - b) Filter through filter membrane (8µm) – 1 filter membrane per 2L solution/solvent
 - c) Dry filter membrane in glass petri dish in 50°C oven for 5 h
 - d) Optical examination (optional prior to staining with Nile red)
 - e) Nile red staining

Microplastic detection using a light microscope and subsequent staining with Nile Red for detection on a fluorescent microscope

Below procedures are the same as water extraction protocol.

Visual Examination using Light Microscope:

1. Remove lid of petrislide and replace with clean glass slide
2. Turn on and position external light to shine directly under objective lens
3. Use 4x objective lens to count all microplastics sized above 63 µm
4. Use 10x objective lens to count all microplastics sized below 63 µm
5. Take a picture of each particle of interest in using an attached camera
6. Size class each particle using imaging software

Staining debris with Nile Red:

1. Place a drop of nanopure water onto the filter in the PetriSlide to loosen filter

2. Using 2 forceps, remove filter from PetriSlide and place onto filtering unit
3. Plug filtering unit with rubber stopper
4. Place filtering unit into foil covered 600 mL beaker
5. Add 5 mL 10 $\mu\text{g/mL}$ Nile Red working solution onto filter
6. Place lid onto filtering unit and place foil lid onto beaker
7. Incubate at room temperature for 30 min
8. Vacuum filter remaining liquid
9. Rinse filter with 5 mL N-hexane
10. Rinse sides of filtering unit with 20% 2-Propanol
11. Take off the top clear portion of the filtering unit and rinse sides and edges onto filter
12. Using 2 forceps, remove filter from filtering unit and place into PetriSlide

Detection of stained particles on a fluorescent microscope:

1. Remove PetriSlide lid and replace with clean glass slide
2. Set fluorescent microscope to FITC (excitation 460-500, emission 535LP) filter
3. Use 4x objective lens to count fluorescent items sized above 20 μm
4. Use 10x objective lens to fluorescent items sized below 20 μm
5. Take picture of each particle of interest
6. Size class each particle using an imaging software such as Image J or MATLAB