EXTRACTION AND QUANTIFICATION OF MICROPLASTICS IN THE GASTROINTESTINAL TRACT OF FISH

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1. Introduction

This protocol describes how microplastics (MPs), down to 100 µm (Feret diameter), can be extracted from the gastrointestinal tract (GIT) of fish, processed and identified. The extraction methodology is based on the digestion of the GIT matrix by potassium hydroxide (KOH), followed by a 2-step density separation to remove sediment or other denser particles (Dehaut et al., 2016; Bessa et al., 2019). Microplastics identification is done visually by stereomicroscopy (Nile red identification) (Maes et al., 2017), and confirmed by micro-fourier transform infrared spectroscopy, µ-FTIR (Catarino et al., 2018; Bessa et al., 2019).

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2. Materials and reagents

Materials

- Glass petri dishes
- Glass beakers
- Glass Pasteur pipette
- Filtration kit including vacuum pump
- Temperature controlled magnetic stirrer plate
- Glass coated stirring magnet
- µFTIR compatible filter (e.g. PFTE, 10 µm pore size, 47 mm diameter or equivalent)
- Custom-made stainless-steel filter (pore size 20 µm or smaller).
- Sonication bath

Reagents

- 10% KOH solution (CAS no. 1310-58-3)
- 15% H_2O_2 solution (CAS no. 7722-84-1)
- $Na_2WO_4.2H_2O$ (CAS no. 10213-10-2) or an equivalent salt
- Distilled or MilliQ water
- Nile Red (CAS no. 7385-67-3)
- Acetone (analytical grade) (CAS no. 67-64-1)

3. Procedures to mitigate background contamination

- Working in a clean room is strongly advisable. If not available, work in a precleaned laminar flow or fume hood positioned in a separate laboratory compartment.
- Clean the working area with fibre-free tissue or paper.
- Avoid wearing clothes made out of plastic fibres, such as a fleece, or that easily shed fibres. Uniform clothing is advisable (lab coat, white cotton trousers, …).
- Filter (retention of 20 µm or lower) all solvents and solutions that will be used during the analysis or for glassware rinsing.
- Rinse all glassware thoroughly with filtered water. Cover all glassware after rinsing to avoid airborne particles contamination.
- Include positive and negative control samples (see section on quality control).

4. Preliminary actions

- Prepare a 10% KOH solution for the digestion step. For the density separation step, prepare a salt solution with a density of 1.35 $g.cm^{-3}$ or higher: several salts are suitable, e.g. NaI, NaBr, $Na_2WO_4.2H_2O$ or $ZnCl_2$.
- For filtration, use a filter with a particle retention of 20 µm or lower.

5. Sample digestion and filtration

1. Weigh each individual gastrointestinal tract (GIT), or a pooled sample with several GIT (e.g. 3), of fish of the same species and from the same location, in a beaker (beaker 1). Cover the beaker before and after the weighing task to avoid airborne fibre contamination.

2. Add 10 ml of filtered potassium hydroxide solution per gram of sample. Positive and negative control samples are analysed in parallel (see section on quality control).
3. Stir samples at 150 rpm, for 48 h and at 60 °C.
4. Allow the sample to settle for at least 1 h at room temperature.
5. Filter the supernatant liquid layer over a stainless-steel filter.
6. Dissolve the remaining layer on the stainless-steel filter in 300 ml 15% H_2O_2 solution for 10 min in a sonication bath and pour back this liquid in beaker 1.
7. Stir sample at 150 rpm, for 48 h and at 60 °C.
8. Allow the sample to settle for at least 1 h at room temperature.
9. Filter the supernatant liquid layer over a stainless-steel filter.
10. Dissolve the remaining layer on the stainless-steel filter in 150 mL of a saturated sodium tungstate solution for 10 min in the sonication bath and pour back this liquid in beaker 1.
11. Allow the sample to settle for 24 h. Decant the supernatant liquid layer in a new beaker (beaker 2).
12. Add 100 ml salt solution to the beaker 1 and let it stir for 2 min at 150 rpm.
13. Allow once more the sample to settle for 24 h. Then, decant the supernatant liquid layer in beaker 2.
14. Place a filter in the filtration unit.
15. Pour the liquid of beaker 2 over the filter, followed by active (vacuum pumped) filtration. Cover the filter.
16. Place the filter into a covered petri dish for microplastic determination.
17. The microplastics can be stained with Nile red for identification by using active (vacuum pumped) filtration.
    - In the final filtration, keep the filter in place in the filtration system.
    - Add 1 mL of Nile red dye (10 µg / ml in acetone) to the filter using a glass Pasteur pipette, with the vacuum pump turned off.
    - After allowing the microplastic particles to stain for 15 min, turn on the vacuum pump and rinse the filter thoroughly with MilliQ/filtered water. Turn off the pump, transfer the filter into a petri dish, cover the petri dish, and allow it to dry in the dark before MP identification by fluorescence stereomicroscopy.

6. Microplastic identification and enumeration

- Particles are initially observed by stereomicroscope with identification of colour, shape (fibre, granule, film) and size (µm) (Kovač Viršek et al., 2016).
- For microplastic identification, use Nile red staining followed by fluorescence stereomicroscopy observations (Maes et al., 2017; Catarino et al., 2018).
- A hot needle test, in which the point of a hot needle is pointed to the plastic, can assist in distinguishing plastic from non-plastic, but the procedure is not considered to have a high accuracy (Marine & Environmental Research Institute, 2015)
- For microplastic confirmation and polymer identification, µ-FTIR analysis is advised (Catarino et al., 2018; Bessa et al., 2019). Filters must be completely dry before performing the µFTIR analysis.
- Report the number of MP particles observed per wet weight (ww) of sample (single GIT): MPs / g (ww); or the number of MP particles observed per single GIT (i.e. per fish): MPs / GIT.

7. Quality control

- Sample analysis is accompanied by the analysis of positive controls and procedural blanks.

- The use of additional negative control samples, e.g. by the analysis of fish fillet, is strongly recommended. The analysis of a fish fillet sample provides an overview of the blank contamination throughout whole procedure, including the sampling step.
- To assess the rate of recovery of microplastic particles of the extraction methodology, it is recommended to spike positive control samples. To do so, microplastics of a fixed size and colour are spiked into a positive control sample, preferably microplastic free, sample. Positive control samples are analysed according to the analysis procedure and the spiked microplastics recovery efficiency is determined (Catarino et al 2017).
- To analyse procedural blank samples, the entire procedure should be followed without adding the actual biota sample, in order to determine potential contamination during the laboratory procedure.

8. Contamination assessment and reporting

- To determine the mean background contamination (and standard deviation) for each plastic shape (fibre, granule, film) the analysis of a series of blank samples should be done, at least one per analysis day.
- The use of a quantification limit (Limit of quantification, LOQ) is strongly recommended. The LOQ can be determined based on the negative control samples as the mean background contamination plus 3 times the standard deviation.

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