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Proceeding Paper

Standard Operating Procedure for the Analysis of Microplastics in Larval Fish Diets †

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Abstract: Microplastics (MPs) pollution has increasingly exposed the pelagic biota to physical harm. The small size of micro-particles makes them more suitable for passive ingestion by a wide variety of organisms with serious effects on growth rates, respiration and vital functions, bioaccumulation of pollutants, and, ultimately, species survival. Nevertheless, our knowledge of plastic intake in nursery habitats is still very limited. When encounters with MPs occur at the larval stage, it is suggested that fish can develop altered feeding behaviors with cascading effects on the entire food web. This study provides a step-by-step protocol to identify and enumerate polymer particles found in fish diets. The procedure is intended for the analysis of larval and juvenile fish populations with a developed digestive tract. It includes guiding questions for research design, a list of supplies and reagents to extract and mount the fish diets on microscope slides for semi-permanent conservation, the protocol for microscopic and statistical analysis, and the interpretation of the results. We suggest that the gut content could be used to assess (i) the bioavailability of polymers in water systems, (ii) the incidence of an encounter between larval fish and MPs, and (iii) the possible alternations in fish' feeding behaviors as soon as they leave their parental stage.

Keywords: microplastics; larval fish; ingestion; microscopic analysis

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1. Introduction

Plastics have been documented in every marine and freshwater environment. Microplastics (MPs) generate from the breakdown of larger plastic debris into infinitesimal particles that can be ingested by a wide variety of organisms [1]. Increasing attention has also been drawn to MPs that originate from land and make their way into the water as microbeads for abrasive uses, employed in cosmetics or dental hygiene products, and filaments from synthetic clothing [2,3].

Plastic pollution jeopardizes the health and subsistence of the pelagic biota resulting in physical harm, such as entanglement and ingestion or chemical contamination [1].

Encounters between organisms and MPs account for a greater risk of biological incidents. In fact, due to their small size, MPs can promptly enter the food web bearing negative consequences across all trophic levels [4–7]. The most documented effects of MPs ingestion include reduced feeding up to starvation due to saturation of the digestive tract [7], decreased energy reserves [8], hepatic stress, and increased liver toxicity caused by bioaccumulation and translocation of pollutants via polymer particles [9,10].

While passive ingestion of MPs by marine organisms is receiving increasing attention [11–17], there is limited information on plastic intake in freshwater systems [18–21], especially when assessing the feeding behaviors of larval and early-life-stage fish. Previous studies indicate that fish larvae can feed on plastic in their nursery habitats with detrimental consequences for their survival, growth, and reproductive rates. It has been suggested that the bioavailability of polymer particles could alter their ecological functions

(e.g., metabolism) as soon as they leave their parental stage of yolk sacs to develop feeding behaviors [22,23].

The least understood are the mechanisms of translocation of polymers throughout the food web. The occurrence of unanticipated changes in the natural feeding environment could substantiate the incidence of cascade effects across all levels of the food chain, with increased concerns for species survival when such effects fall below the threshold level [24]. It has been demonstrated that MPs can accumulate in prey fields where micro-preys are readily available and abundant, thereby impacting larvae's food supply [23]. When encounters between fish larvae and microdebris occur, altering feeding behaviors, it is suggested that plastic could make its way to the top of the chain transferring from one trophic level to the next via food supply (e.g., the discovery of MPs in higher level trophic organisms) [25,26].

The central aim of this paper is to produce a reproducible, representative, and accurate Standard Operating Procedure (SOP) for MP analysis. The procedure explained hereinafter is intended for the analysis of larval and juvenile fish populations with a developed digestive tract. It includes guiding questions for research design, a list of supplies and reagents to extract and mount the fish diets on the microscope slides for semi-permanent conservation, a protocol for microscopic analysis, and an interpretation of the results. The remained of the paper is structured as follows: Section 2 presents the step-by-step protocol for MP analysis. Following, Section 3 presents an application of the SOP. Finally, conclusions and final remarks are presented in Section 4.

2. Methodology

2.1. Experimental Design

Prior to the MP analysis, it is imperative to define the study's research questions, the methodology to be employed, and related impacts on the results, including limitations, possible uncertainty, and assumptions, in line with the principle of experimental design [27]. In general, an MP's assessment consists of three broad steps: sample pool selection, microscopic analysis, and statistical analysis and interpretation of results. Table 1 identifies a list of questions to guide the design of the analysis in its three sections.

Table 1. Guiding questions for sample selection and analysis (adapted from [27]).

	1. Sample Pools Selection
✓	How many samples are needed?
✓	Which sample type is relevant?
✓	Should samples be collected within or across species? If selection occurs across species, what does this mean for interpretation of the results?
✓	Do sample's natural habitats differ? How does this affect interpretation of the results?
✓	What are the external factors/conditions to be considered when choosing samples?
✓	What are the limitations of the sample pool(s) used?
	2. Microscopic Analysis
✓	Which polymer types are accounted for?
✓	Which finding types (e.g., number of particles, particle color/size/weight, or particle visual coverage) are accounted for?
✓	How are MPs sorted from other organic content in the fish diets? • What are the limitations of the methods used?
	3. Statistical Analysis and Interpretation of Results
✓	Which polymer types are accounted for?
✓	Which statistical tests are needed?
✓	Which graph/chart types are used to explain findings?
✓	Which types of findings are significant to the study?
✓	Which statistical errors are accounted for?
✓	What are the limitations of the analyses used

2.2. Preparation of the Samples and Equipment

One of the processes that the CR aimed to improve was related to the delivery of thousands of histopathology reports. A project about machine learning was a solution implemented in one of the DMAIC cycles performed by the CR. Histopathology reports are primary data. After collection, when samples are returned to the lab, they can be conserved in vials filled with ethanol solution. Vial labels should indicate vial ID, sample species, sample stage, sampling site, sampling depth, sampling date, and storing solution.

For this analysis, the following supplies and reagents have been used:

- Dissecting microscope with 80× magnification
- Microscope slides, 25 mm × 75 mm
- Cover slips, 22 mm × 22 mm
- Microscope slide box
- Vials, 20 mL and 3.7 mL
- Vial box
- Fine tipped probes
- Minuten pins
- Tweezers
- Eyedropper
- Ethanol 99%, 100 mL
- Permount™ Mounting Media, 100 mL
- Glycerin-Alcohol 50:50, 100 mL

2.3. Larvae Fish Dissection

(i) For microscopic examination of individual samples, we place one slide at a time under the dissecting microscope and, using an eyedropper, pour a small amount (2 drops recommended) of the Glycerin–Alcohol solution. (ii) One sample was then gently removed from the selected vial using tweezers and placed on the slide following the direction of the slide. It is recommended to handle the sample from its tail to avoid unintended damage to the digestive tract. (iii) An additional small amount (2 drops recommended) of the Glycerin–Alcohol solution was added to facilitate dissecting operations. (iv) To prepare the sample for diet removal, we focused the microscope at 10× and adjusted the lenses and lights as needed to have a clear view of the entire sample. (v) Using fine-tipped probes, we gently incised the sample to separate the digestive tract from the rest of the body, avoiding tearing the stomach apart during the process and adding Glycerin–Alcohol if needed as the sample dried out. (vi) When the digestive tract had been entirely excised, the body freed of mouth, esophagus, stomach, and intestine was returned to vials filled with an ethanol solution for conservation.

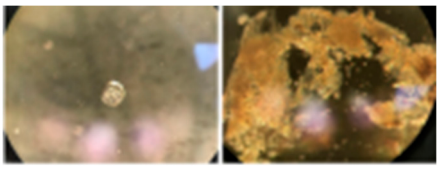
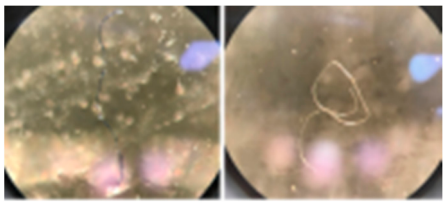
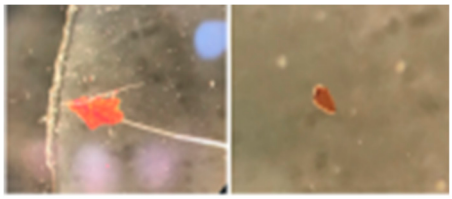
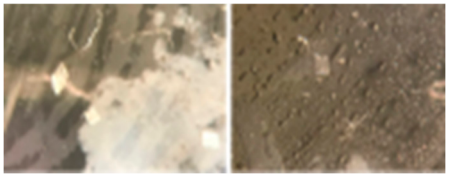
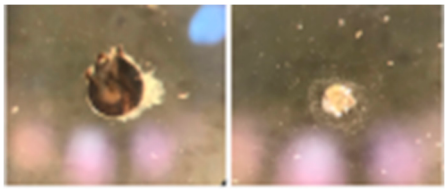
2.4. Diets Removal and Slide Preparation

(i) To remove the fish diets, we focused the microscope at 40× and adjusted the lenses and lights as needed. (ii) Following, we added an additional small amount (2 drops recommended) of the Glycerin–Alcohol solution to facilitate diet removal operations. (iii) Using minuten pins, we carefully opened the stomach and peeled off the content. (iv) For slide preparation, we gently covered the guts and their content with a slipcover making sure that it was evenly distanced from the slide corners and all air bubbles were removed. (v) Using an eyedropper, a small amount of Permount™ was poured on the cover slip, sealing one side at a time, making sure that there was no air infiltration when sealing one side. (vi) Mounted slides should then be stored in a safe space, and 24–48 h (depending on the drying conditions of the lab) are required prior to handling. Each slide should carry a label indicating slide ID, sample species, sample stage, sampling site, sampling depth, and sampling date as indicated on the relative vial.

2.5. Counting Particles

For MPs analysis, the mounted slides were placed under the dissecting microscope and focused at 80× magnification, adjusting lenses and lights as needed. Microscopes with greater magnification capacity can also be used for further analysis. MPs particles were categorized by shape and color. Based on their visual features, we classified MPs as beads, fibers, fragments, films, and foams using the methods described by [3] (Table 2). The dominant color was identified using the methods described by [16], using a 5- color scale (blue, black, red, orange, and transparent). Subsequently, the software Image-Pro Premier 9.3 was used to record the size of identified particles (e.g., length of fibers and area of beads, fragments, films, and foam). After the analysis, mounted slides were conserved in the microscope slide box.

Table 2. Abacus of polymer particles by physical characteristics (adapted from [3]).

Particle	Characteristics	Images
Bead	Hard, Rounded	
Fiber	Thin, fibrous, linear	
Fragment	Hard, jagged	
Film	Thin, flimsy	
Foam	Lightweight, sponge like	

3. SOP Application

3.1. Scope and Objectives

Using the protocol presented above, three sample pools were analyzed to assess the incidence of encounters between MPs and fish larvae. For this study, three freshwater environments with different characteristics were chosen. Sampling site 1 (S1) represents a connecting channel heavily used for tourism purposes and commercial and recreational fishing. The second site (S2) is a distributor channel connecting smaller lakes and wetlands and serves ecological restoration and recreation purposes. Finally, the third site (S3) represents an urban river subjected to anthropogenic influences from adjacent urban areas that

account for the discharge of municipal wastewaters and sewage, runoff of chemical and hazardous materials from industrial activities, fishing, and intensive shipping operations. The choice of these sites serves the purpose of exploring possible effects of the external environment and sampling conditions (e.g., location and site) on the bioavailability of MPs in waterways.

3.2. Sample Selection

For this analysis, we sampled 218 larval fish native to the study area. For S1, we sampled 20 rainbow smelts (*Osmerus mordax*) and 28 burbots (*Lota lota*); for S2, we sampled 30 yellow perches (*Perca flavescens*), 30 rainbow smelts (*Osmerus mordax*), and 28 burbots (*Lota lota*); finally, for S3, we sampled 20 cyprinidae, 30 rainbow smelts (*Osmerus mordax*), and 33 yellow perches (*Perca flavescens*).

3.3. MPs Analysis

MPs were found at all sampling sites, confirming the results of similar studies [28–32]. In our analysis, 117 fish had ingested MPs, of which 24 fish (41.4%) from S1, 47 fish (53.4%) from S2, and 46 fish (55.4%) from S3. A detailed description of our findings is reported in Table 3.

Table 3. Summary of MPs analysis for the three sampling sites.

Site	Species	Samples (#)	Ingestion Rate (%)	Particle Count (#)	Particle Types	Particle Size (Mean)		Particle Color
						Length (Fiber)	Area (Bead, Fragment, Foam)	
S1	Rainbow smelt	20	45%	10	Bead (1), Fragment (1) fiber (8)	912.6 nm	22,855.4 nm^2	Orange, black, blue, red
	Burbot	28	53.6%	29	Fragment (7), fiber (22)	1424.0 nm	15,685.6 nm^2	Red, black, blue, transparent
S2	Yellow perch	30	80%	58	Fragment (3), foam (3), fiber (52)	1208.3 nm	52,942.9 nm^2	Red, orange, black, blue, transparent
	Rainbow smelt	30	56.7%	34	Fiber (34)	1610.2	-	Red, blue, black, orange
	Burbot	28	17.9%	6	Fragment (2), fiber (4)	2106.9 nm	5576.4 nm^2	Red, black, blue
S3	Cyprinidae	20	25%	5	Fiber (5)	691.1 nm	-	Black, blue, red
	Rainbow smelt	30	60%	35	Fragment (2), fiber (33)	1367.0 nm	4100.2 nm^2	Red, blue, black, orange
	Yellow perch	33	72.7%	52	Fragment (1), fiber (51)	1405.5 nm	2092.0 nm^2	Blue, black, red, transparent

Like previous studies [28], we found that fiber was the most prevalent particle type, with an average length of 691.1 nm to 2106.9 nm. Second, we found fragments, followed by foams and beads, with an average area of 2092.0 nm^2 to 52,942.9 nm^2. The most dominant colors for identified particles were blue, black, and red; while orange and transparent were found in at least one species for each sampling pool. A possible explanation for this pattern is provided by [16], that observed that fish are more likely to selectively ingest blue particles due to association with micro-preys (e.g., blue-pigmented copepods) and avoid

orange particles). Concerning species-specific patterns, we observed that yellow perches reported the highest ingestion rates for S2 and S3, confirming the trends observed by [33,34]. No substantial differences were observed among the three sampling pools, and plastic pollution was ubiquitous at all sites. Therefore, a detailed analysis of possible sources and pathways of MP pollution could yield more insightful results on how specific features of the feeding environment affect larval fish's feeding behaviors.

4. Conclusions

The SOP presented in this study provides a reproducible, step-by-step methodology to assess MPs accumulation in larval fish by analyzing the types, size, and color of micro debris found in the fish digestive tract. The findings of this study establish a foundation for further research related to feeding behaviors and the long-term impacts of plastic debris. In particular, future studies could compare the results of this analysis with MPs accumulations in water samples to understand if the likelihood of MPs ingestion is statistically correlated to selective changes in feeding behaviors or depends on the concentration of particles in the fish's feeding environment (higher particle density). Some limitations of this approach can be associated with the depth and accuracy of the microscopic analysis. For example, this MPs analysis can be reinforced using spectroscopic analyses (e.g., Fourier transform infrared (FTIR) and Raman spectroscopy) [28,35] or thermal analyses [36], which provide a further level of verification by detecting the polymer composition of microparticles.

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