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Accumulation and Distribution of Fluorescent Microplastics in the Early Life Stages of Zebrafish

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TITLE:

Accumulation and Distribution of Fluorescent Microplastics in the Early Life Stages of Zebrafish

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Keywords:

Danio rerio, embryo, larvae, pollutant, waterborne exposure, aquatic toxicology

Summary:

Zebrafish embryos/larvae develop externally and are optically transparent. The bioaccumulation of microplastics in fish at early life stages is readily assessed with fluorescently labeled microbeads.

Abstract:

As a new type of environmental pollutant, microplastic has been widely found in the aquatic environment and poses a high threat to aquatic organisms. The bioaccumulation of microplastics plays a key role in their toxic effects; however, as a particulate, their bioaccumulations are different from many other pollutants. Described here is a feasible method to visually determine the accumulation and distribution of microplastics in zebrafish embryos or larvae using fluorescent microplastics. Embryos are exposed to different concentrations (0.1, 1, and 10 mg/L) of fluorescent microplastics with a diameter of 500 nm for 120 h. It is shown in the results that microplastics can bioaccumulate in zebrafish embryos/larvae in a concentration-dependent manner. Before hatching, strong fluorescence is found around the embryonic chorion; while in zebrafish larvae, the yolk sac, pericardium, and gastrointestinal tract are the main accumulated sites of microplastics. The results demonstrate the uptake and internalization of microplastics in zebrafish at early life stages, which will provide basis for better understanding the impact of microplastics on aquatic animals.

Introduction

Since first synthesized in the 1900s, plastics are widely used in various fields, resulting in rapid growth of global production[1]. In 2018, approximately 360 million tons of plastics were produced worldwide[2]. The plastics in the natural environment will degrade to fine particles due to chemical, physical or biological processes[3]. Generally, fine plastic particles <5 mm in size are defined as microplastics[4]. Microplastics are also engineered for specific applications, such as microbeads from cosmetic products[5]. As near-permanent contaminants, microplastics are accumulated in the environment, and have attracted increasing attention from scientists, policymakers and the public[1,6]. Previous studies documented that microplastics could cause adverse effects in fish, such as gastrointestinal damage[7], neurotoxicity[8], endocrine disruption[9], oxidative stress[10] and DNA damage[11]. However, the toxicity of microplastics has not been fully revealed so far[12,13].

Zebrafish embryos offer a lot of experimental advantages, including small size, external fertilization, optical transparency and large clutches, and is considered as an ideal model organism for in vivo studying the effects of pollutants on fish at early life stages. In addition, only limited amounts of test substances are needed for the evaluation of biological responses. Here, zebrafish embryos are exposed to different concentrations of microplastics (0.1, 1, 10 mg/L) for 5 days, and the bioaccumulation and distribution of microplastics in zebrafish embryos/larvae are evaluated. This result will advance our understanding about the toxicity of microplastics to fish, and the method described here can potentially be generalized to determine the accumulation and distribution of other types of fluorescent materials in the early life stages of zebrafish.

Protocol

Adult zebrafish are originated from the China Zebrafish Resource Center (Wuhan, China). The experiments were conducted in compliance with the national guide “Laboratory Animal Guideline for Ethical Review of Animal Welfare (GB/T35892-2018).

1. Embryo collection

1.1. Maintain fish in 20 L glass tanks with recirculating charcoal-filtered tap water system (pH 7.0 ± 0.2) at a constant temperature (28 ± 0.5 °C) on a photoperiod of 14:10 h light: dark.

1.2. Feed fish twice daily with *Artemia nauplii*. It is recommended that the food is given at max. 3% fish weight per day and should be eaten within 5 min every time[14].

1.3. Transfer well-developed adult zebrafish (with body length of 3-4 cm) into the spawning tank at a ratio of one male to two females the night before the breeding.

NOTE: The following morning, the fish start to spawn after the onset of the light cycle.

1.4. Collect eggs using a Pasteur pipette. Rinse with 10% Hank's solution several times, and then

check for fertilization using a microscope. Fertilized eggs undergo the cleavage period after approximately 2 h post fertilization (hpf) and can be clearly identified[15].

1.5. Incubate the fertilized embryos in a 500 mL beaker containing 200 mL of 10% Hank's solution with 1% methylene blue for disinfection at 28 °C. Do not exceed a loading rate of 1 embryo/2 mL solution.

NOTE: 10% Hank's solution is made up of 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$.

2. Preparation of microplastic suspensions

2.1. Sonicate the stock solution of green fluorescently labeled polystyrene beads (10 mg/mL) with nominal diameter of 500 nm (excitation/emission: 460/500 nm) for 10 minutes.

2.2. Dilute the stock solution with 10% Hank's solution to produce the desired exposure solutions (0.1, 1, and 10 mg/L).

2.3. Always prepare the exposure solutions of microplastics before exposure.

NOTE: Caution should be taken when assessing the toxic effects of microplastics, because the presence of preservatives, such as sodium azide, in the commercial particle formulations, can be toxic to different organisms [16]. Therefore, these additives should be removed or accounted for in the controls before conducting toxicity experiment.

3. Microplastic exposure

3.1. Randomly select 6 newly fertilized embryos (4 hpf), and then transfer into each well of 6-well plate containing 5 mL of microplastic solutions with different concentrations. Include the control groups containing 10% Hank's solution.

3.1.1. Use triplicate wells (with a total of 18 embryos) are used for each treatment.

3.2. Incubate the embryos under the same light: dark cycle and temperature as adults (see 1.2) and observe every 12 hours. Remove the dead immediately.

3.3. Renew the microplastic solutions 90% every 24 h. During the exposure period, the fish are not fed.

NOTE: Generally, the hatching of embryo begins at 48 hpf and completes at about 72 hpf.

4. Assessment of microplastic distribution

4.1. At 24, 48, 72, 96, and 120 h post fertilization, randomly select the embryos/larvae (one from each of the three replicates) and rinse with 10% Hank's solution.

4.2. Transfer the larvae into a Petri dish and expose to 0.016% tricaine for anesthesia.

4.2.1. Prepare the stock solution of tricaine: 4 mg of tricaine powder is dissolved in 100 mL of double distilled water, and adjust the pH to 7.0 with Tris-HCl (pH 9.0). Store the stock solution in the freezer.

4.2.2. Prepare the working solution. Dilute the stock solution to the desired concentration (0.016%) with 10% Hank's solution at room temperature[14].

4.3. Arrange the embryos/larvae and prepare for observation.

4.4. Observe the fish with a fluorescence microscope and image with imaging software.

4.5. Quantify the fluorescence intensity in fish with ImageJ.

Representative Results

The distribution and accumulation of fluorescent microplastics are shown in **Figure 1** and **Table 1**. No visible fluorescence is observed in the unexposed group (control). However, an accumulation of fluorescence is found surrounding the chorion after exposure to different concentrations of microplastics (24 hpf). Green fluorescence is also detected in larvae, and the fluorescence levels appear to increase in a concentration- and time-dependent manner. The yolk sac, pericardium, and gastrointestinal tract are the main accumulated sites of microplastics (**Figure 2**).

Figure 1: Distribution of fluorescent polystyrene microplastics in embryos/larvae of zebrafish (40×). The fish are sampled from the control group, or the groups exposed to 500-nm microplastics at 0.1, 1 and 10 mg/L. Scale bar 100 μm

Figure 2: The sites of microplastic accumulation in zebrafish larvae (40×). This larva is sampled from the group exposed to 500-nm microplastics at 10 mg/L for 120 hours.

Table 1: The change of fluorescence level in zebrafish following exposure to fluorescent microplastics (n=3). Due to the influence of chorion on the absorption of fluorescent microplastics, the data are divided into two parts, that of the embryos (before hatching) and larvae (after hatching).

Discussion

According to the guideline on the protection of animals used for scientific purposes, such as EU Directive 2010/63/EU, animal ethics permission is not mandatory for an experiment with early life-stages of zebrafish until the stage of being capable of independent feeding (5 days post fertilization)[17]. However, best welfare practice is important for optimizing the use of zebrafish, and, for example, the humane methods of anesthesia and euthanasia should be of concern. Ethyl 3-aminobenzoate methanesulphate (MS-222, or tricaine), the routinely used agent in the most laboratories, is employed here for anesthesia and euthanasia.

Before observation under the microscope, the embryos and larvae should be rinsed since the microplastics adsorbed on the external surface might interfere with the results. In addition, the autofluorescence in the embryos/larvae, especially around the yolk sac, which has been reported occasionally, might be problematic. The presence of many biomacromolecules, such as flavins, nicotinamide-adenine dinucleotide (NAD), aromatic amino acids, lipofuscins, advanced glycation end products, and collage, will emit light when excited at the appropriate wavelength.

It is important to note that, as the particulate pollutant, the size of microplastic is considered as one of the determining factors of bioavailability, and toxicity[18]. The nominal diameter of microplastic used here is 500 nm, which is comparative to the pore size of the embryo chorion (within the range of 300 nm to 1 μm)[19]. Therefore, these microplastics are not expected to easily pass through the zebrafish chorion. Consistently, there is little fluorescence visible in the embryos before hatching (**Figure 1**). Since the chorion will act as an effective barrier against the particles with large size, the dechorionation process before exposure may be needed. Chorion can be removed easily using the forceps, but enzymatic dechorionation with pronase is preferred when the embryos are handled in bulk. However, although dechorionation will increase the bioavailability and facilitate the high-throughput screening for the toxicity of substances, the embryo with chorion intact is more recommended to assess the ecotoxicity of pollutants when considering the condition of exposure in the “real” world.

Although considerable efforts have been devoted to investigating the adverse effects of microplastics on fish, the current knowledge, including that of bioaccumulation, remain limited or even conflicting. These across-study inconsistencies are mainly attributed to the differences of properties of particles, including size, density, and surface characteristics (for example, surface charge). The behavior of microplastics in the solution is critical to the bioavailability as well. The physicochemical characteristics of microplastics should be tracked over the exposure duration, and the aggregation phenomenon that may occur should be recorded. In fact, for the exposures that require the microplastics to be suspended for an extended period, sonication or stirring with a magnetic bar is recommended.

Disclosures

The author declares no competing or financial interests.

Acknowledgements

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Figure1

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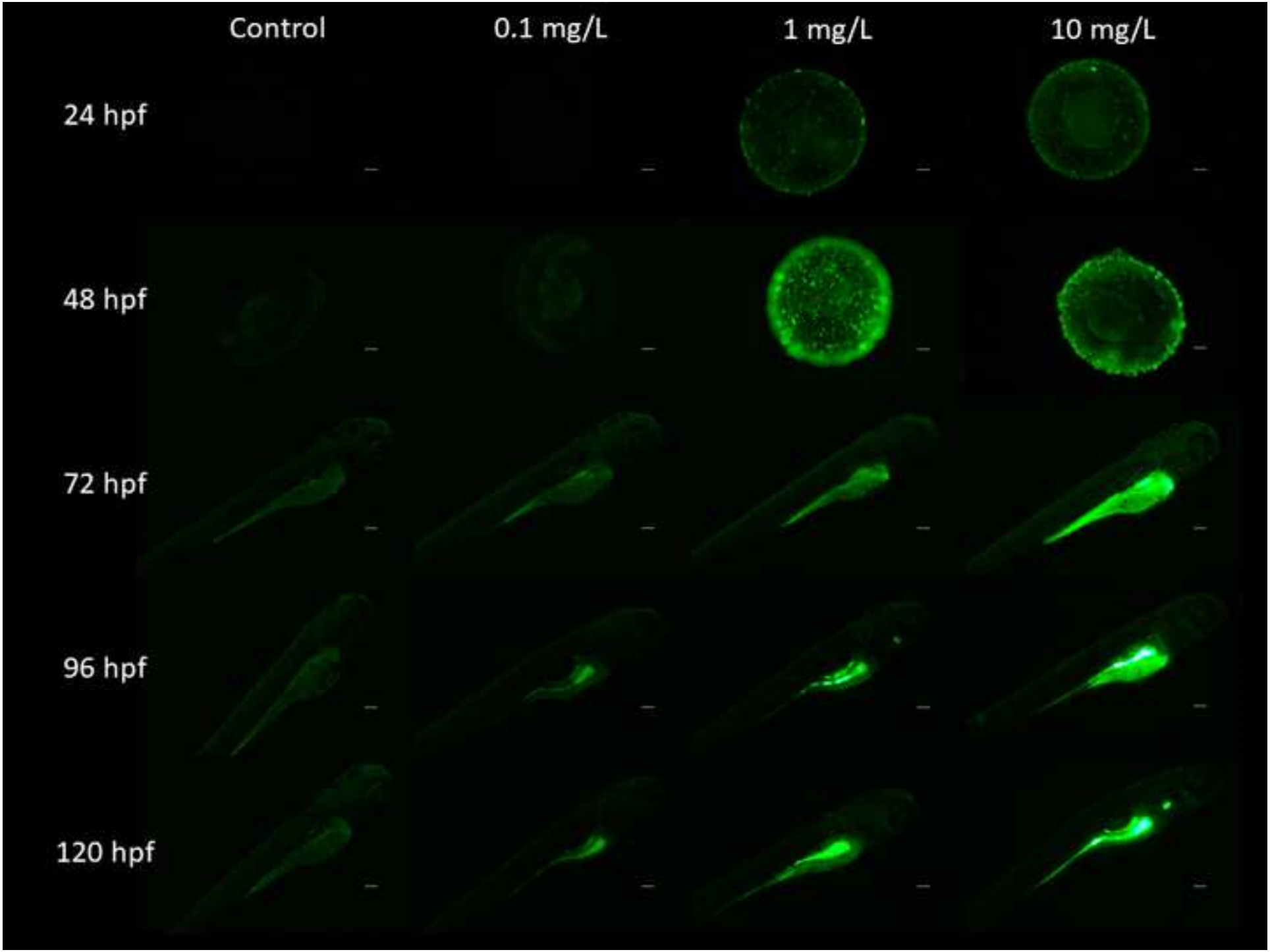

Figure2

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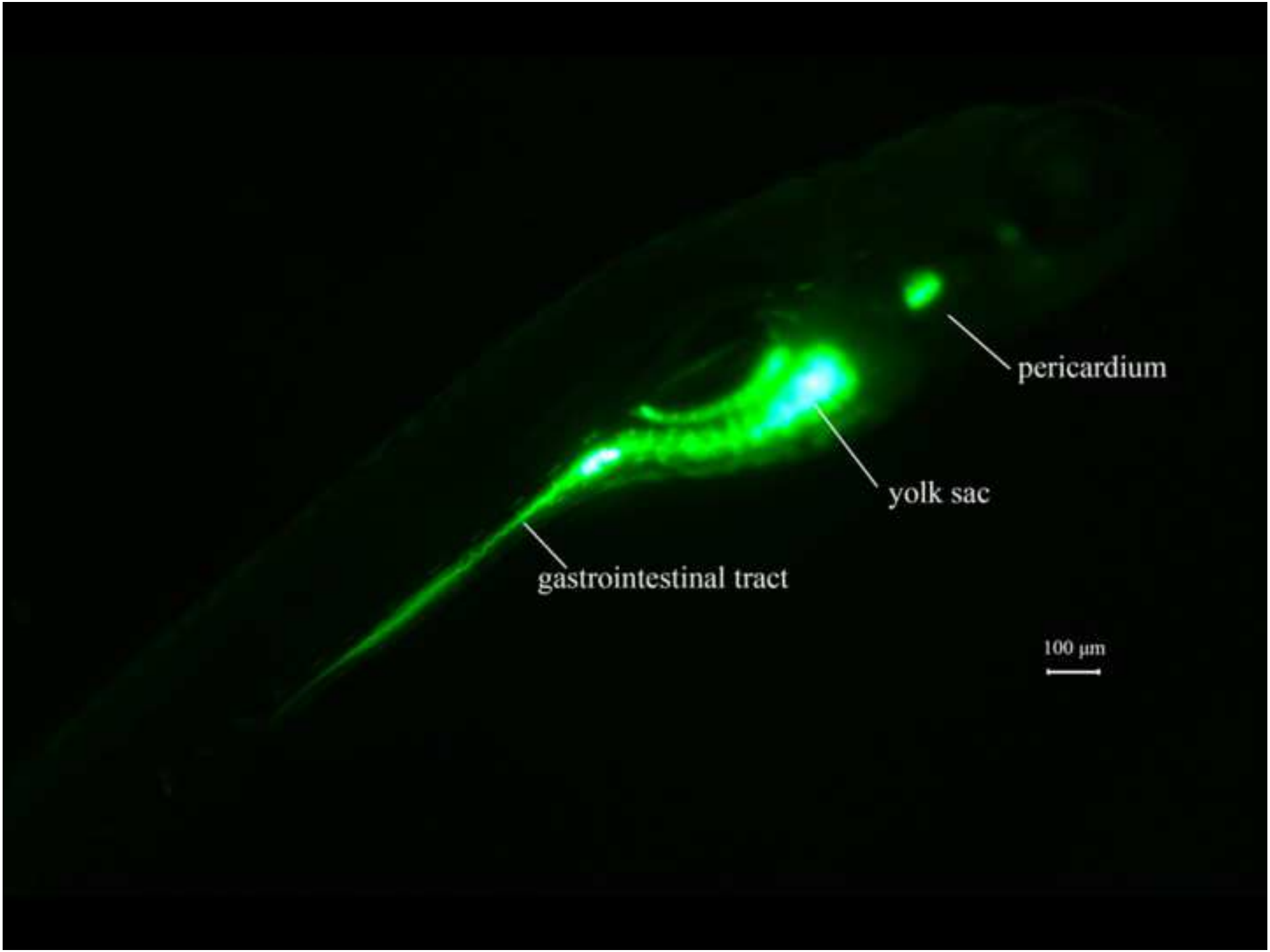

Concentration	Embryo		Larva			
(mg/L)	24 hpf	48 hpf a	48 hpf [b]	72 hpf	96 hpf	120 hpf
Cont.	1.2±0.1	2.6±0.3	2.2	3.0±0.2	2.6±0.7	3.3±0.3
1	1.2±0.2	5.0±0.1	5.3	7.5±0.5	8.7±0.5	10.0±1.9
0.1	7.0±0.9	26.1±2.9	8.9	18.4±0.7	16.3±2.8	25.7±2.7
10	9.1±1.1	82.3±5.3	30.4	32.7±3.2	41.6±0.4	44.1±0.9

[a]: only two embryos were assessed; [b]: only one larva was assessed.

Dear Dr. Nam Nguyen,

Please find the revised manuscript and video of **JoVE62117**.

According to your comments, the video has been re-edited, and we hope it could meet the standard of JoVE.

The responses to comments are given in the following pages.

Thank you for your consideration and we look forward to hearing from you.

Best regards,

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Changes to be made by the Author(s) regarding the video:

1. General Note on Video Transitions and Black Borders:

• All video footage & title cards should be scaled to fit the full video frame, no black bars or margins should be visible.

Re: The video has been re-edited.

• The motion effect video transitions that include scaling (zooming) and changing position of the video image should be removed all together. The video frame itself should be stationary and the video clips should not move around or zoom in any manner. Some effects were removed in the previous version, but many remain, and are listed below:

"• 02:08 Consider removing this motion effect on the overhead shot of the beaker. Simply start with the video frame stationary.

• 02:16 The edges of the frame ""grow"" from the center out, there should be no motion effects on the borders of the video, simply start with the video stationary. "

"• 03:10 The zoom-in of this bench with the 6-well plate, the Petri dish, and the beaker, should be stationary, and not zoom into place as it currently does.

• 03:49 04:02 04:15 04:32 These zoom-in effects should be removed.

• 04:39 Remove this ""fly-in"" effect, keep the simple and clear. Consider using a hard cut or a dissolve transition."

Re: All of the motion effect video transitions have been removed.

2. Audio Editing & Mixing:

• The audio volume levels should be reduced by 25%. Audio peaks should rise to no higher than -9 dB.

Re: The audio volume levels have been reduced.

• 01:58 There is a breath noise right after the word, "microscope." Consider seeing if you can trim the end of that clip closer to end of the word, "microscope" to

remove the puff.

Re: The audio has been re-edited, and the breath noise has been trimmed.

• 04:58 There is some lab noise here from the video clip of the microscope screen. This audio should be muted.

Re: The lab noise has been muted.

3. On-Screen Text:

• The main title card, ethics card, and chapter title cards should match the resolution (pixel size) of the video, which is 1920x1080. Right now, noticable blurring and pixelation can be seen on the title cards, especially where the edges of the letters meet the background. Please re-export the title cards (including the "Discussion" card at the end) at 1920x1080 so they won't appear blurry.

0:18: bioaccumulation is spelled wrong.

1:12, 2:23: degrees Celsius is spelled wrong.

Re: The title cards have been re-edited, and the typos have been corrected.