Supporting information

Chronic exposure to graphene oxide (GO) induced inflammation and differentially disturbed intestinal microbiota in zebrafish

Pan-Pan Jia[1,2], Tai Sun[1], Muhammad Junaid[1,3], Yang-Hui Xiong[1], Yang-Qing Wang[1], Li Liu[1,2], Shi-Ya Pu[1,2], De-Sheng Pei[1,2,4]*

[1]Key Laboratory of Reservoir Aquatic Environment, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing, 400714, China

[2]University of Chinese Academy of Sciences, Beijing 100049, China

[3]Key Laboratory for Heavy Metal Pollution Control and Reutilization, School of Environment and Energy, Peking University Shenzhen Graduate School, Shenzhen, 518055, China

[4]College of Life Science, Henan Normal University, Xinxiang 453007, China

*Corresponding authors.

E-mails: deshengpei@gmail.com and peids@cigit.ac.cn (D.S.P)

Summary: SI file contained the methods of Text S1-2, the results of Table S1, and Figure S1-8.

Text S1. The composition of electron microscope fix buffer was 2.5% glutaraldehyde solution, and the preparation was as following steps. Step 1 was to prepare the 0.2 M phosphate buffer solution, including the 40.5 mL A buffer of 0.2 M Na_2HPO_4 buffer solution(Na_2HPO_4•$7H_2O$ 26.83 g + DD H_2O 500 mL) and 9.5 mL B buffer of 0.2 M NaH_2PO_4 buffer solution (NaH_2PO_4•H_2O 13.8 g + DD H_2O 500 mL), and then regulated the pH of 0.2 M phosphate buffer solution to 7.4. Step 2 was to prepare the 2.5% glutaraldehyde solution, including 50 mL 0.2 M phosphate buffer solution (pH 7.4) + 10 mL 25% glutaraldehyde agent+DD H_2O to 100 mL.

Text S2. Total RNA was extracted from zebrafish gut tissues by using RNAiso Plus reagent (Takara, Japan) to detect genes expression, according to the manufacturer's instructions. The RNA quality was determined using the Agilent 2100 Bioanalyser system (Agilent, USA) and quantified using the NanoDrop ND-2000 (Thermo Scientific, USA). High-quality RNA samples (OD260/280=1.8~2.2, 28S:18S≥1.0) were prepared for the cDNA synthesis using Prime Script Reverse Transcription Reagent kit (Takara, Japan). The quantitative real-time PCR (qRT-PCR) used ABI 7500 system (Applied Biosystems, CA) and SYBR® Premix Ex Taq™ II (Takara, Japan) with a thermal running program initiated for 5 min at 95°C, 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s.

Table S1. The phenotype and properties of isolated bacterial strains from zebrafish in this study[a]

Orders	Colony descriptions	Closest bacterial strain * (16S ribosomal RNA gene)	Identity (%)	Length (bp)	Genus group	Accession No.
1	Milk white color, small size and round, half-transparent.	Pseudomonas pseudoalcaligenes strain CH 1-1-1 (KM871859.1)	>99.9	1409	*Pseudomonas*	MK178498
2	Light yellow color, middle size and round, non-transparent.	Aeromonas veronii strain MJ-4 (KC210767.1)	100	1411	*Aeromonas*	MK178499
3	Brown red color, middle size and round, non-transparent.	Shewanella sp. lam-5 (KR072679.1)	>99.5	1422	*Shewanella*	MK178500
4	Light yellow color, small size and round, half-transparent.	Vibrio cholerae strain DL3 (MG062859.1)	>99.6	1411	*Vibrio*	MK178501
5	Yellow color, middle size and round, non-transparent.	Microbacterium sp. 0702P1-2 (HM222654.1)	100	1404	*Microbacterium*	MK178502
6	Milk white color, middle size and round, non-transparent.	Rhodococcus erythropolis strain HX-2 (MG015900.1)	100	1393	*Rhodococcus*	MK178503
7	Red color, middle size and round, non-transparent.	Exiguobacterium sp. strain Firmi-40 (MH683129.1)	>99.9	1444	*Exiguobacterium*	MK178504
8	Milk white color, middle size and round, half-transparent.	Bacillus sp. (in: Bacteria) strain JSM 1685003 (MG893110.1)	>99.9	1430	*Bacillus*	MK178505

[a]All gut bacteria were isolated from a wild type adult zebrafish, which was maintained from embryos in our lab with standard culture condition. *Highest sequence identity was obtained from NCBI-BLAST with the best match of the total sequences from 16S ribosomal RNA sequences (Bacteria and Archaea) database.

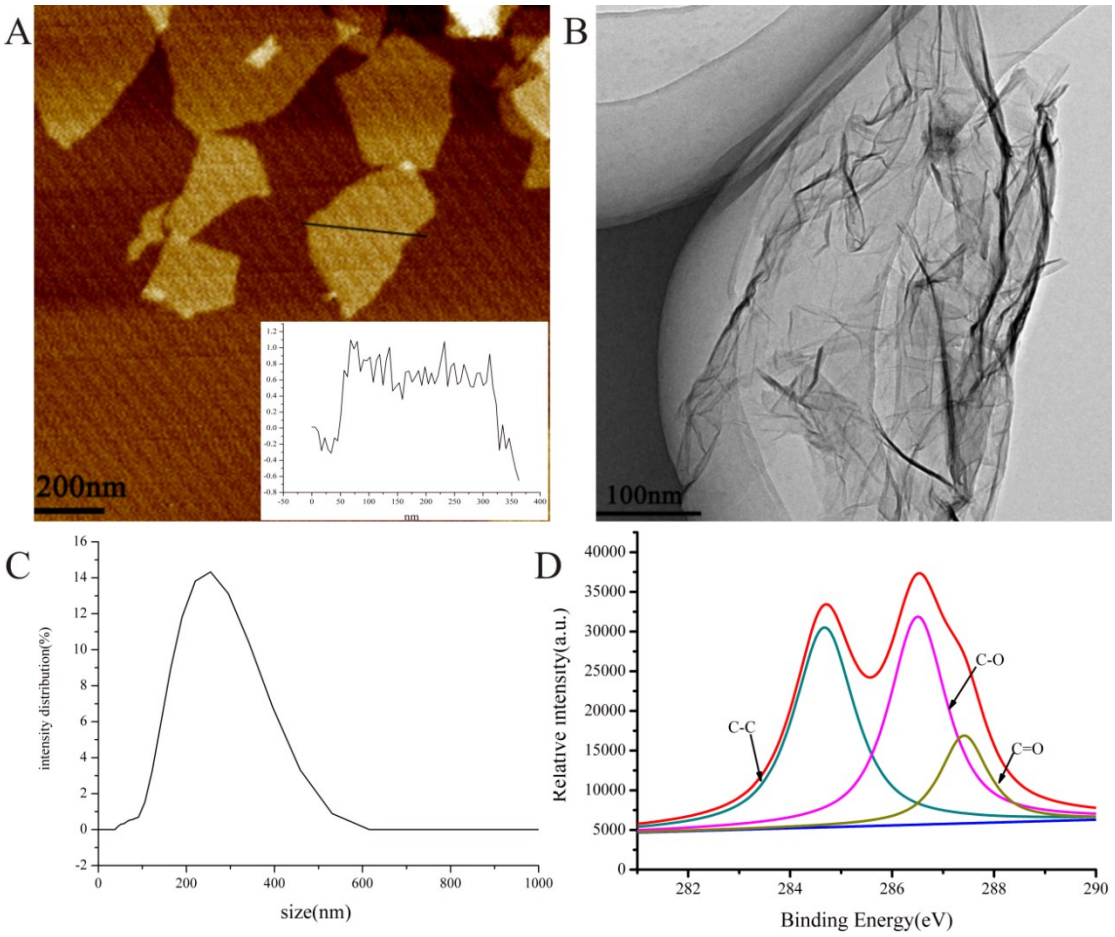

Figure S1. The characteristics of GO. (A) The superficial shapes, lateral dimensions, and thickness of GO were detected by AFM imaging with the scale bars of 200 nm. (B) Surface morphologies and sizes of GO were analyzed using TEM, with the scale bars of 100 nm. (C) The size distribution was determined using DLS on a Zetasizer Nano ZS (Malvern, UK). (D) C and O contents of GO material were quantified using XPS on a VG ESCALAB 250 spectrometer (UK) using an Al Ka X-ray source (1486 eV), X-ray radiation (15 kV and 10 mA), and hemispherical electron energy analyzer. The red line is the raw intensity, and the blue one is the background on the bottom. The green one stands for C-C peak, the pink and brown lines indicate the C-O and C=O peak, respectively.

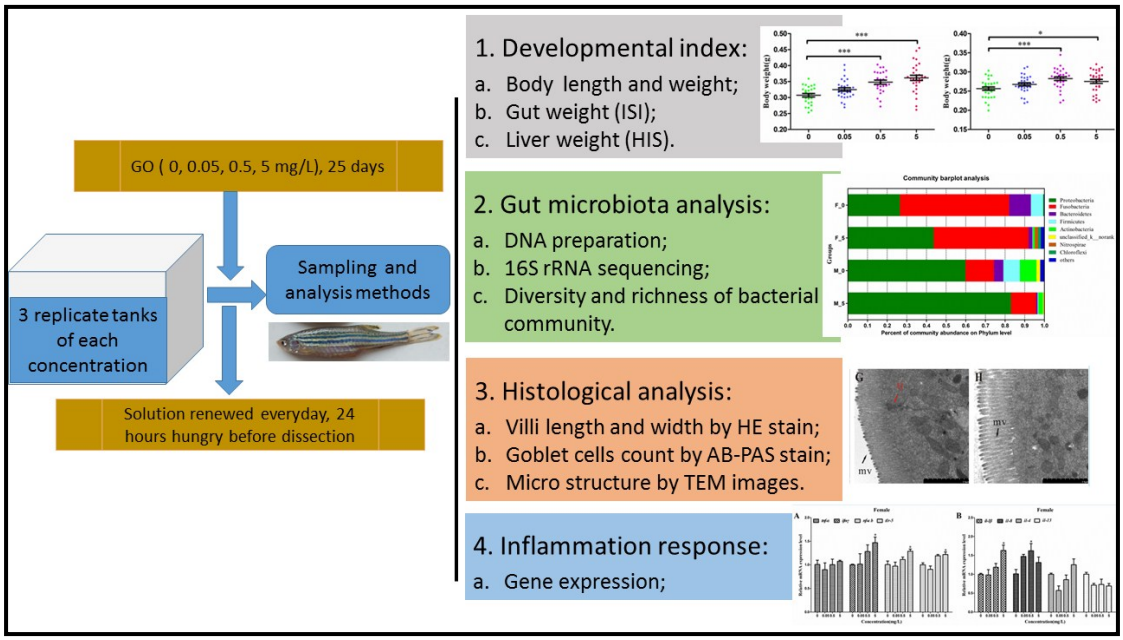

Figure S2. The scheme of exposure experiment in this study.

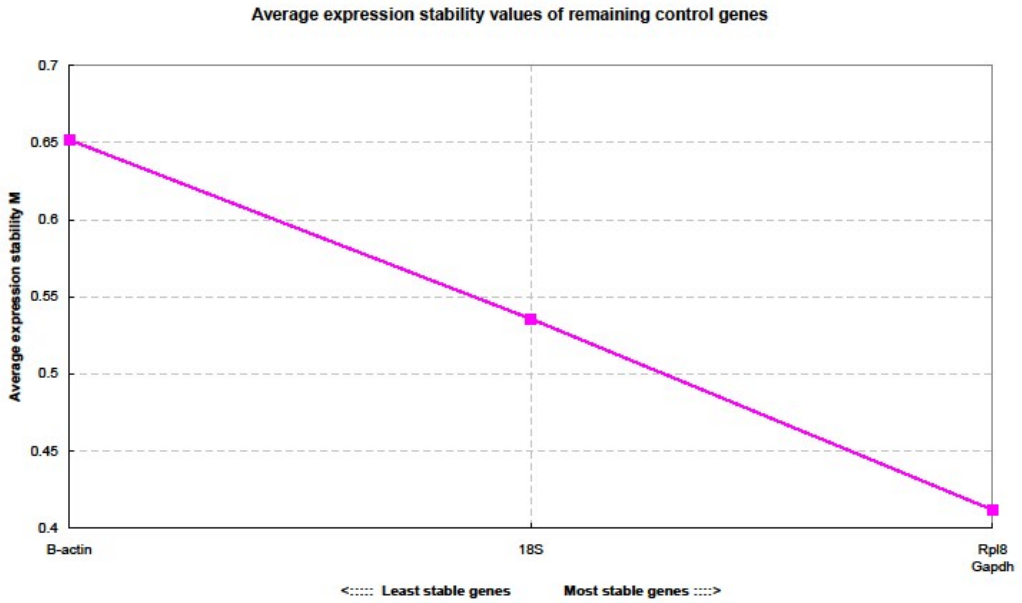

Figure S3. The screening housekeeping genes in zebrafish gut tissue after exposure to 5 mg/L GO for 25 days. Data were shown as the means ± SEM of three replicates in each group. The most stable genes upon GO treatment were *rpl8* and *gapdh*, while *rpl8* got a better score than other three housekeeping genes using geNorm v3.5 software. Thus, *rpl8* was selected as an internal control in this study.

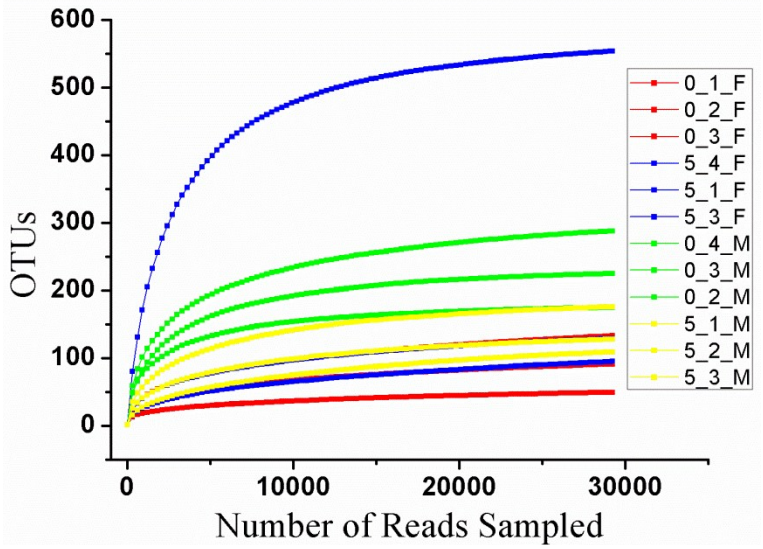

Figure S4. Rarefaction curve of OTUs at > 97% similarity level. Samples of control and exposed groups with three replicates were highlighted with different colors. For example, the 0_1_F indicated one randomly selected sample of female zebrafish from the control group, and the samples in other groups were named in the same manner.

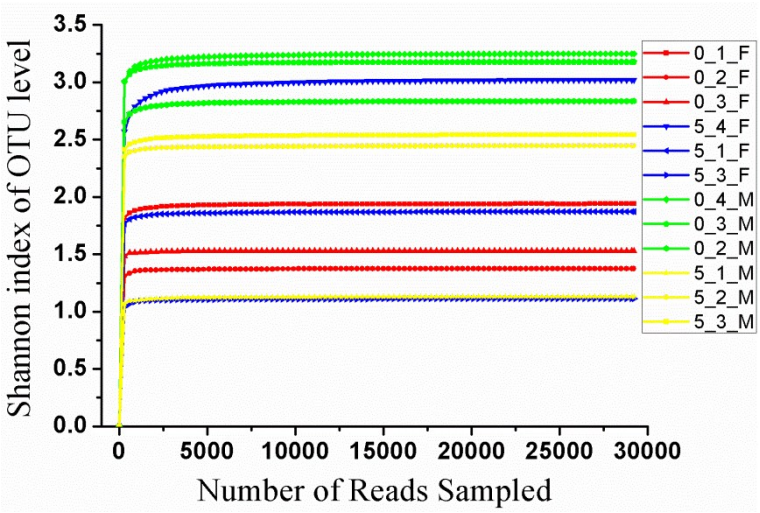

Figure S5. The Shannon rarefaction curve of OTUs (>97% similarity level). Samples of control and exposed groups with three replicates were highlighted with different colors. For example, the 0_1_F indicated one randomly selected sample of female zebrafish from the control group, and the samples in other groups were named in the same manner.

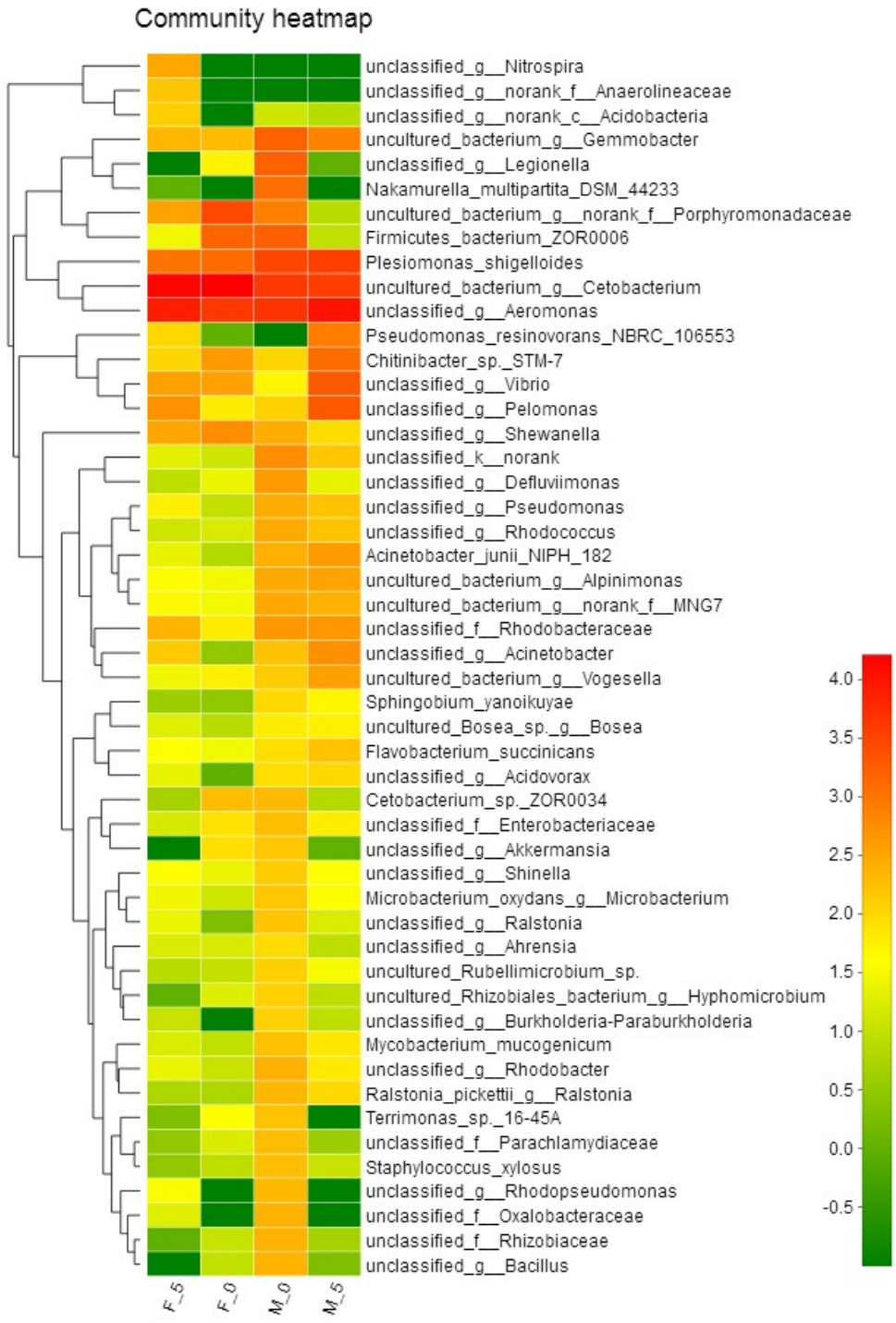

Figure S6. The heatmap presented the top 50 analyzed bacteria at the species level by the log transformation of bacterial abundance values in each group. The various species were clustered by the bacterial hierarchical genera with the average manner, and the green-yellow-red color bar presented the abundance changes of F-5 and M-5 groups. The species obviously influenced by GO chronic exposure in female

and male zebrafish were belonged to the same major genera with isolated bacterial strains, indicating the rich abundance and critical metabolic functions in the host.

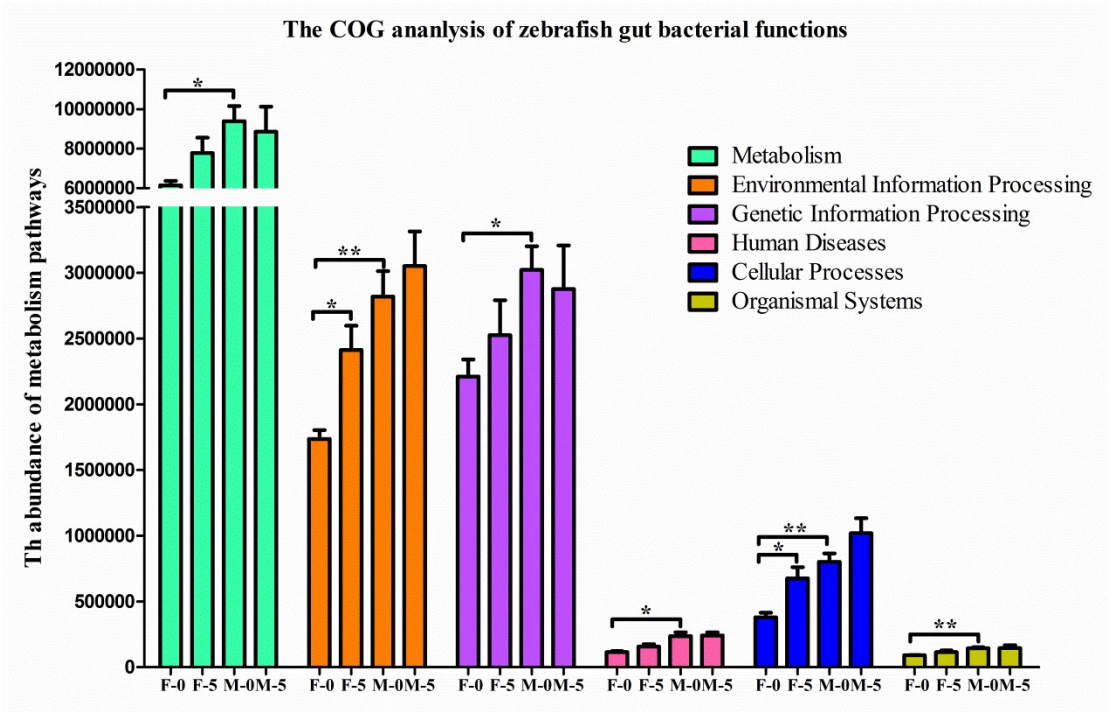

Figure S7. The relative abundance of different functions of intestinal microbiota in zebrafish by COG analysis. The different level of mechanism pathways calculated based on the MiSeq Illumina platform, and * denoted the significant differences between groups at *p*-value <0.05.

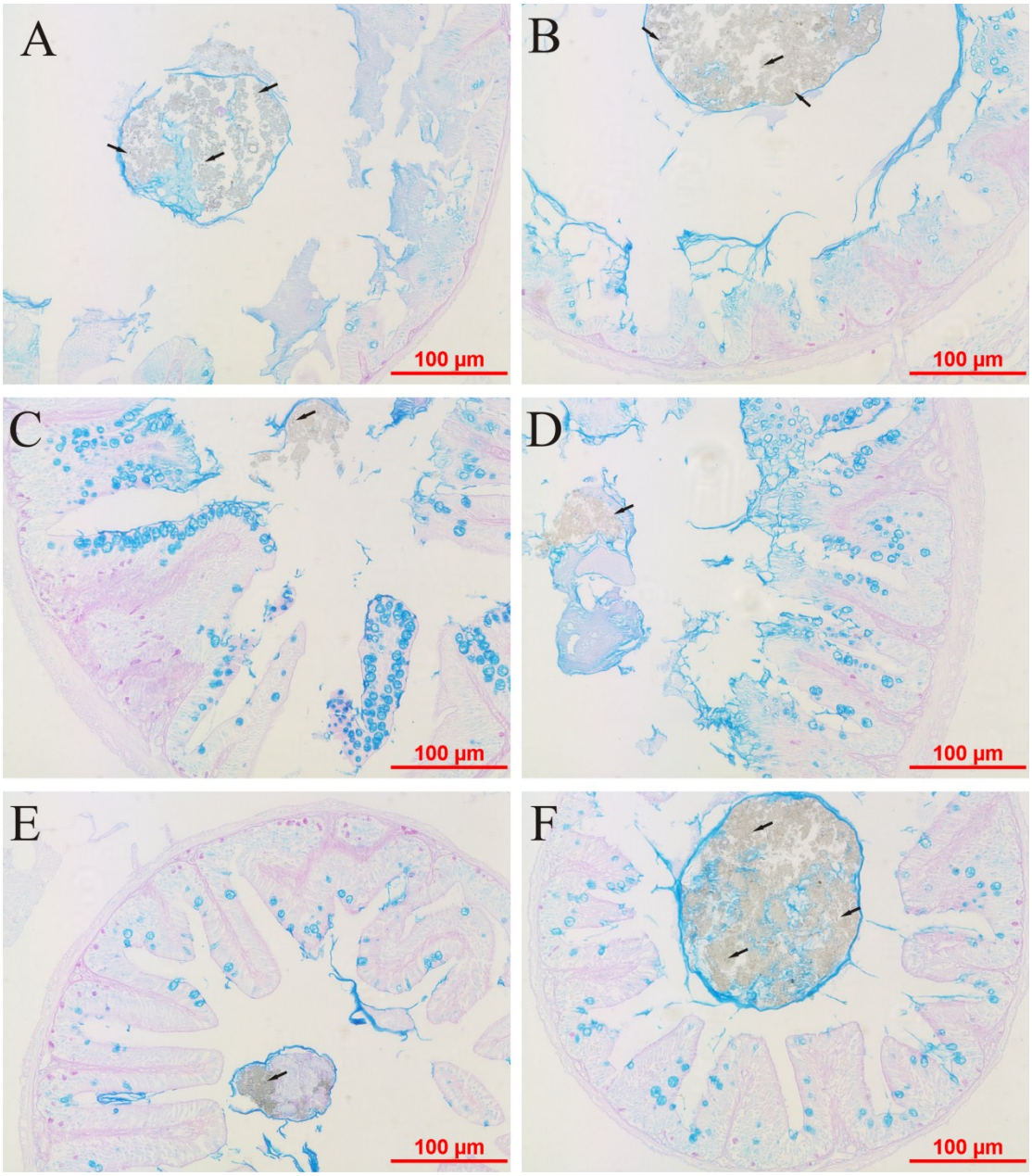

Figure S8. The histological analysis of anterior intestine female (A) and male (B) zebrafish at 5 mg/L GO treatment, the mid intestine in female (C) and male (D) zebrafish, and the post intestine in female (E) and male (F) zebrafish (intestinal contents: black arrow). The scale bar was 100 μm at 200 × images at all groups. After starvation for 24 hours, there were still intestinal contents left in tissues at 5 mg/L treatment group. Here, we speculated that GO treatment may affect the digestion or bowel movement speed in adult zebrafish.