Supporting Information

Biological Uptake, Distribution and Depuration of Radio-Labeled Graphene in Zebrafish: Effects of Graphene Size and Natural Organic Matter

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16 pages, 14 figures

Experimental Section

Graphene Characterization. X-ray Photoelectron Spectroscopy (XPS) measurements of graphene powder were performed on a PHI 5000 VersaProbe with a monochromatic Al Ka X-ray source. AFM images of graphene were recorded using a Multi Mode v8 scanning probe microscope (MM-SPM) (BRUKER, German). Samples for AFM were prepared by dropping the highly dispersed aqueous suspension of graphene on a freshly cleaved mica surface. After the samples were dried, the AFM images were measured using a ScanAsyst Mode. The obtained AFM images were analyzed using a NanoScope v6.13 Software (Veeco Instruments Inc, America). Raman Spectroscopy. FLG samples were resuspended in ethanol through sonication for approximately 1 min. Samples suspended in ethanol were prepared by drop-casting approximately 20 μL on a quartz microscope slide and drying. Raman spectra were collected on a Renishaw inVia Raman microscope using an excitation wavelength of 514 nm. Samples were scanned from 1,000 cm^{-1} to 1,800 cm^{-1} to visualize D and G band intensity changes from the degradation processes. All of the Raman spectra were collected with a 15 s exposure time and the results of five scans were averaged for each sample.

Preparation of natural organic matter solution. Suwannee River NOM (SRNOM) (RO isolation, International Humic Substances Society) was used as a model natural organic matter. The weight-average molecular weight of SRNOM was 23,300 g/mol. The SRNOM stock solution was prepared by dissolving 100 mg of the dry SRNOM powder into 50 mL of deionized water (DI water). This solution was then filtered through a 0.22 μm cellulose acetate membrane and was adjusted to pH 7.0 via addition of NaOH or HCl, after which point the stock solution was stored in

the dark at 4 °C. The total organic carbon (TOC) content of the SRNOM stock solution was determined to be (860 ± 1) mg/L (n=3) through high-temperature oxidation (TPC-L, Shimadzu). The background TOC content in the DI water was ≈ 0.3 mg/L.

Uptake Experiments. A 1.0 mg sample of ^{14}C-graphene was weighed (Mettler Toledo, XP56 Microbalance) and added to a 500-mL beaker containing 250 mL freshwater. The graphene stock suspension was prepared by probe sonication in ice-water bath. Probe sonication was performed using a 3 s "on"/ 2 s "off" pulse sequence with a probe tip that placed approximately 0.4 cm from the bottom of the beaker. The graphene stock suspension was diluted immediately with aerated freshwater to yield initial concentrations of approximately 50, 75 and 250 μg/L for uptake experiments. The exact graphene concentrations in each container were measured by mixing 1 mL water sample with 3 mL Gold Star scintillation cocktail (Meridian), followed by radioactivity measurements via liquid scintillation counting (LSC). One zebrafish was added to each container containing 250 mL of graphene suspension. There was no feeding during these experiments. Triplicate control containers (without zebrafish but with graphene) for each graphene concentration with 250 mL of exposure solution were used to monitor graphene settling during the exposure period. At each predetermined intervals (4, 12, 24, 48 and 72 h), five containers were sampled; organism mortality was not observed for any exposure condition. After removal from the container, zebrafish was washed using water, euthanized using 3-Aminobenzoic acid ethyl ester methanesulfonate (MS-222, 0.015% w/v), and the epidermis was peeled and fish fins was cut to minimize contributions from the graphene attached on the exterior of zebrafish to the total mass of graphene. Then, each freeze-dried (5 d using lyophilizer) fish from each container was weighed

(Mettler Toledo) and combusted in the biological oxidizer (BO) (OX-500; Zinsser Analytic, Germany) at 900 °C for 4 min under a stream of oxygen gas running at 360 mL/min. The $^{14}CO_2$ released during the combustion process was captured in 10 mL alkaline scintillation cocktail (Zinsser Analytic, Germany) and then the radioactivity was counted by LSC. The radioactivity from control samples (i.e. zebrafish unexposed to graphene) was subtracted from all of the uptake results. After fish removal, aqueous-phase radioactivity was also measured as described above to determine the concentration of graphene remaining in solution. The minimum detection limit of BO is detected as 0.2 ng ^{14}C graphene, corresponding to the signal from blank samples plus three times the standard deviation of the blank samples.

Experiments were also carried out using the same exposure setup and procedure described above to examine how the presence of NOM may influence the uptake of graphene by zebrafish. A 10 mg (TOC)/L NOM was added in each of the exposure suspensions of graphene (50, 75 and 250 μg/L). Triplicate control containers (without zebrafish but with graphene and NOM) for each graphene concentration with 250 mL of exposure solution were used to monitor graphene settling during the exposure period. At predetermined intervals (4, 12, 24, 48 and 72 h), aqueous-phase radioactivity was measured as described above to determine the concentration of graphene remaining in solution after fish removal.

Toxic Response of FLG in Liver and Gut. After exposure to an FLG suspension of 250 μg/L for 72 h, the livers and intestines of zebrafish were collected quickly and fixed in formalin (10%, w/v) for 24 h. Standard procedures of histopathological assessments were performed.[1] Briefly, the tissues were decalcificated in formic acid solution, dehydrated in gradient, hyalinized in xylene, and embeded in paraffin wax in order. Then the paraffin blocks were sectioned at 5 μm thickness,

stained with hematoxylin and eosin (HE), and finally were observed and photographed under an optical microscope equipped with a digital camera. Moreover, the time-related oxidative stress was evaluated by the quantification of malondialdehyde (MDA) and glutathione (GSH) and analysis of the antioxidant activity of superoxide dismutase (SOD) and catalase (CAT) in the liver. At predetermined exposure time (24 h, 48 h and 72h), zebrafish were quickly euthanized in melting ice and the liver tissue was removed quickly. The liver tissues were homogenized for 5 s using a manual homogenizer (5 mL, Jiangyin Elite Instrument Equipment Co., Ltd., China) on ice with ice-cold physiological saline (0.9% NaCl, w/w) to keep the temperature < 4 ºC, then centrifuged at 2000 *g* for 10 min at 4 ºC. The supernatant was collected and subjected to assay for lipid peroxidation (measured as MDA) and GSH content as well as for SOD and CAT activities using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MDA concentration was expressed as nanomoles per mg protein. The GSH content was expressed as micromole per g protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit the oxdation reaction by 50 % and was expressed as U/mg protein. One unit of CAT activity was defined as the amount of enzyme required to consume 1 μmol H_2O_2 in 1 s and was expressed as U/mg protein. The concentration of protein was determined by the coomassie brilliant blue method, using bovine serum albumin as a standard.

Impact of FLG on Intestinal Flora. Total genomic DNA was extracted from each of the three groups using FastDNA Soil Kit (MP Biomedicals, USA) following the manufacturer protocol. The concentration and quality of the extracted DNA were determined using spectrophotometry (NanoDrop, USA) to ensure that the DNA concentration in the samples was greater than 200 ng/μg

for the following experiments. The samples with >200 ng/μg DNA were selected and amplified by Polymerase Chain Reaction. The amplified DNA samples were purified using DNA fragment purification kit ver 4.0 (Takara, Japan) according to the recommended protocol.

Generated 16S rRNA gene sequences were processed using Mothur software. DNA was amplified by using the 515F-806R primer set, which targets the V4 region of bacterial 16S rDNA, with the reverse primer containing a 6-bp error-correcting barcode unique to each sample. All PCR reactions were carried out with high-fidelity PCR Master Mix (New England Biolabs). PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

Selected Bacterial Culture Method. *Escherichia coli* (*E. coli*) was grown in Luria Broth media (Tryptone 10 g L^{-1}, NaCl 10 g L^{-1} and Yeast extract 5 g L^{-1}) at 37 ºC and 150 rpm (15.7 rad/s) for 17 h with subsequently washing using sterile PBS for 3 times. *Aeromonas caviae (A. caviae)* was grown at 30 °C in a nutrient agar medium (Beef extract 3.0 g L^{-1} and Peptone 5.0 g L^{-1}). *Streptococcus mutans (S. mutans)* was grown at 30 °C in a solid Soybean Casein Digest Agar Medium (TSB) with 5% sheep blood. *Methylobacterium extorquens (M. extorquens)* was grown in a ATCC defined medium (K_2HPO_4 0.7 g, KH_2PO_4 0.54 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 4.0 mg, NH_4Cl 0.5 g, $ZnSO_4 \cdot 7H_2O$ 100.0 μg, $MnCl_2 \cdot 4H_2O$ 30.0 μg, H_3BO_3 300.0 μg,

$CoCl_2 \cdot 6H_2O$ 200.0 μg, $CuCl_2 \cdot 2H_2O$ 10.0 μg, $NiCl_2 \cdot 6H_2O$ 20.0 μg, $Na_2MoO_4 \cdot 2H_2O$ 60.0 μg, 1.0 L distill water) at 30 ℃ and 150 rpm (15.7 rad/s) for 72 h with subsequently washing using sterile PBS for 3 times.

MTT assay. 90 μL bacterial suspension was added in a 96-well flat bottom plate followed by the adding of 10 μL S-FLG or L-FLG to the bacterial culture yielding a final nominal graphene concentration of 250, 5000 and 10000 μg/L with 4 replicates for each concentration. The plates were incubated at 30 °C, 5% CO_2 in a humidified incubator for 1 day. Then 0.5 mg mL^{-1} of MTT solution was added to each well (20 μL per well) and the plates were continuously incubated for another 4 hours. 80 μL of 20% Sodium dodecyl sulfate (SDS) was added and incubated in the dark overnight. Absorbance was read at a wavelength of 595 nm. In addition, the S-FLG and L-FLG was treated similarly with the same incubation procedures (addition of MTT reagent, but without bacterial cells and measuring the absorbance) serving as control samples.

Bioinformatics and Statistical Analysis. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean according to the QIIME (V1.7.0) quality controlled process. The tags were compared with the reference database (Gold database) using UCHIME algorithm to detect chimera sequences, and then the chimera sequence were removed. Then the effective tags finally obtained. Sequences analysis were performed by Uparse software (Uparse v7.0.1001) and sequences with > 97% similarity were assigned to the same Operational Taxonomic Unit (OTUs). OTUs abundance information were normalized using a standard of sequence number corresponding to the same with the least sequences. Alpha diversity, including Observed-species, Simpson, ACE and Good-coverage, was applied to analyze the

complexity of species diversity for a sample. Beta diversity was calculated by QUUME software (Version 1.7.0) based on weighted Unifrac distances and non-metric multidimensional scaling (NMDS) was used to quantify and visualize compositional differences of species complexity. Mann-Whitney test was used for significance test of alpha diversity and beta diversity between groups. Linear discriminant analysis coupled with effect size (LEfSe) was performed to identify the bacterial taxa differentially represented between groups at genus or higher taxonomy levels.

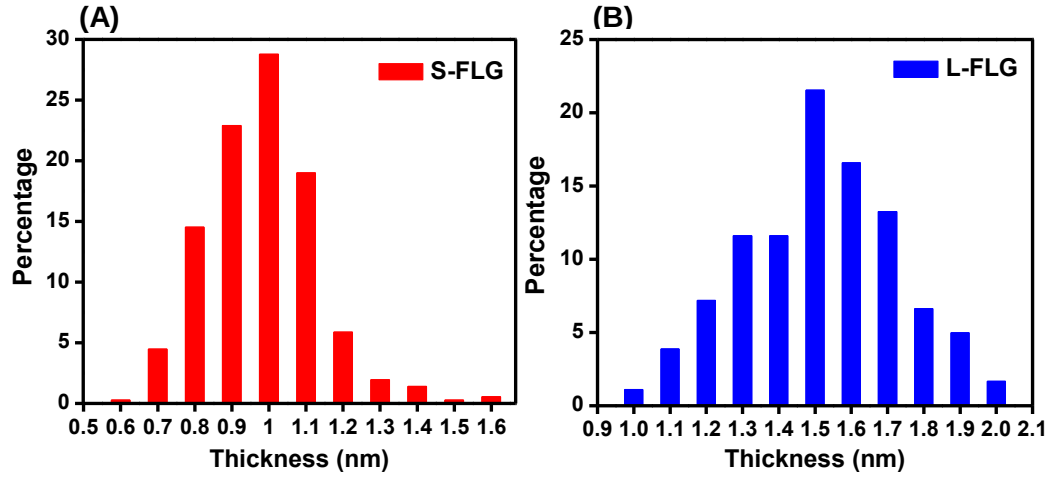


Figure S1. Analysis of the thickness of S-FLG (A) and L-FLG (B) through AFM (n = 230).

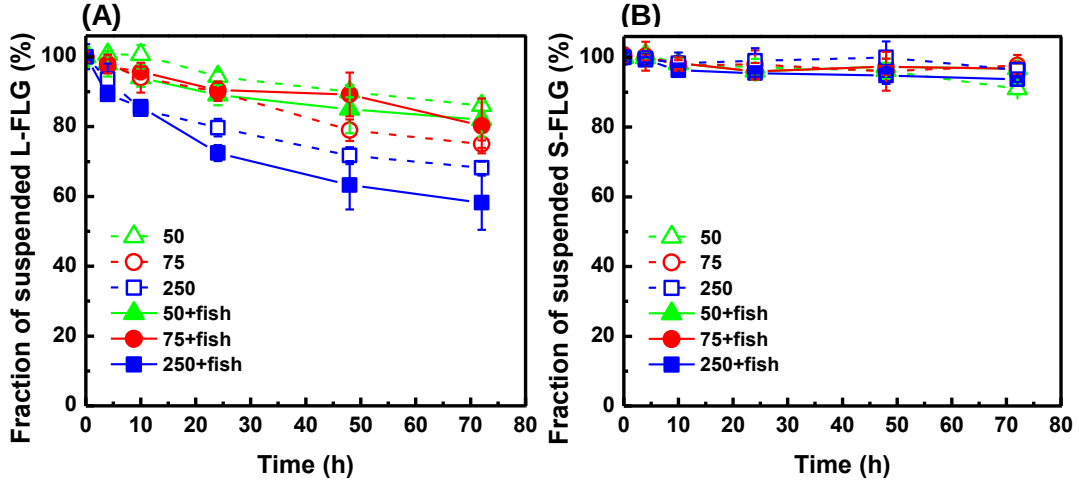


Figure S2. The fraction of the L-FLG (A) and S-FLG (B) concentration remaining in uptake experiment solution with the absence and presence zebrafish. Mean and standard deviation values were calculated from triplicate samples.

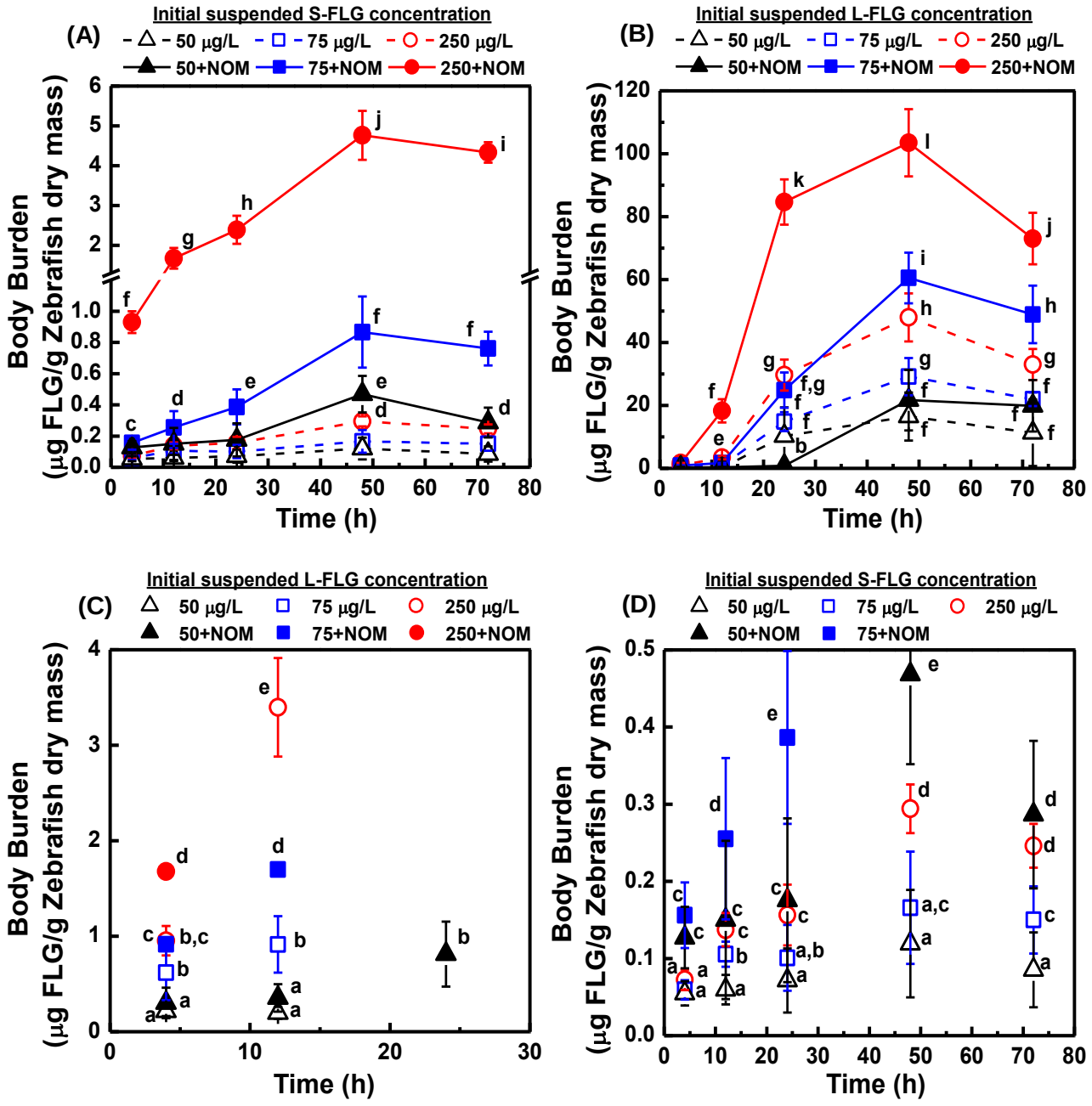


Figure S3. Effect of NOM on the uptake of L-FLG (A) and S-FLG (B) by zebrafish. Zebrafish were exposed to graphene in freshwater for 72 h with an initial suspended graphene concentration of 50, 75 or 250 μg/L and with the presence or absence of 10 mg (TOC)/L NOM. The y-axis (>4 μg/g) of Figure 3C and the y-axis (> 0.5 μg/g) of Figure 3D were cut off to clarify the statistical difference. Mean and standard deviation values were calculated from five duplicate samples.

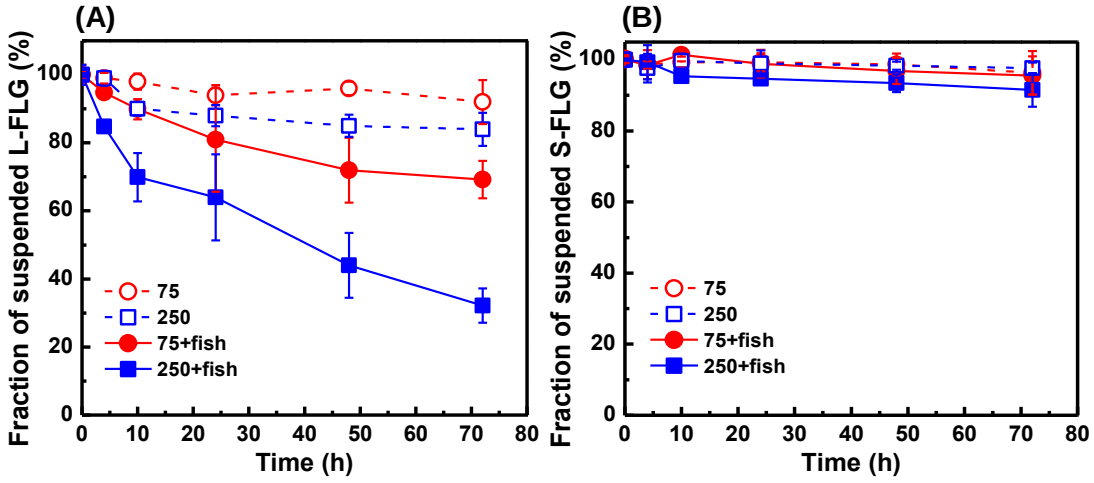


Figure S4. The fraction of the L-FLG (A) and S-FLG (B) concentration remaining in uptake experiment solution with presence of NOM. Mean and standard deviation values were calculated from triplicate samples.

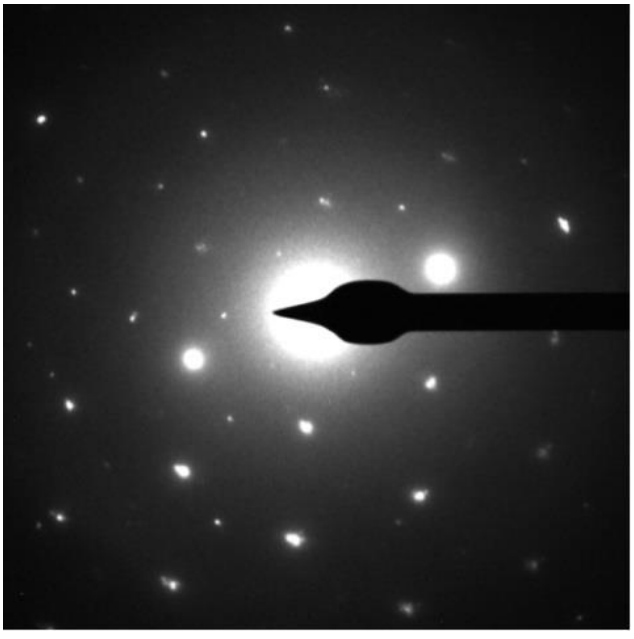


Figure S5. Selective area electron diffraction pattern (SADP) image of pristine graphene.

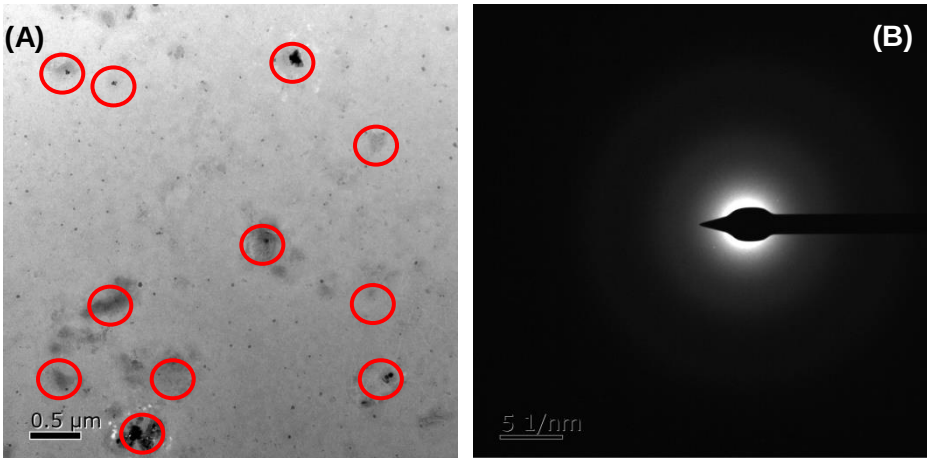


Figure S6. (A) TEM image of the sectioned hepatocytes isolated from the liver of the zebrafish of the control untreated group (not exposed to FLG). (B) SADPs taken from the red circles.

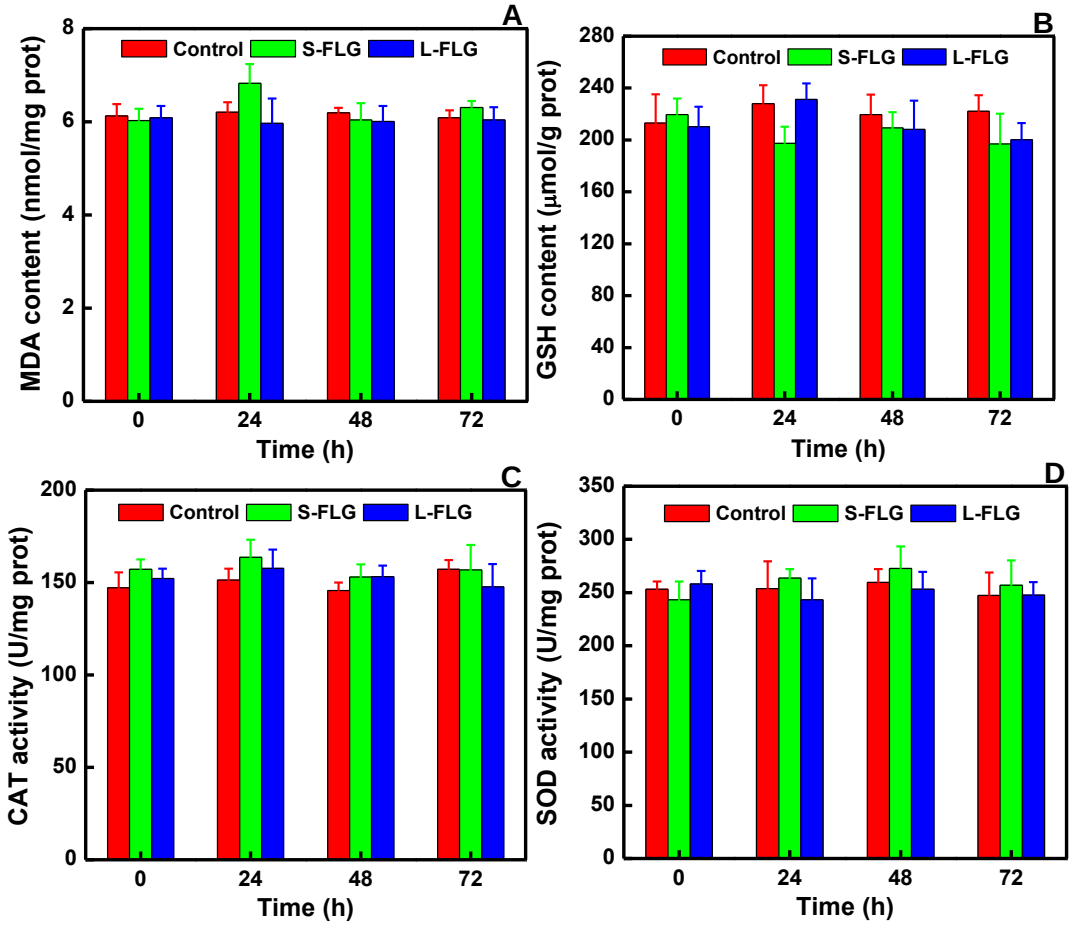


Figure S7. Time dependent alteration of (A) MDA, (B) GSH, (C) CAT, and (D) SOD in livers after exposure to a 250 μg/L FLG suspension. Values are presented as mean ± SD (n=3).

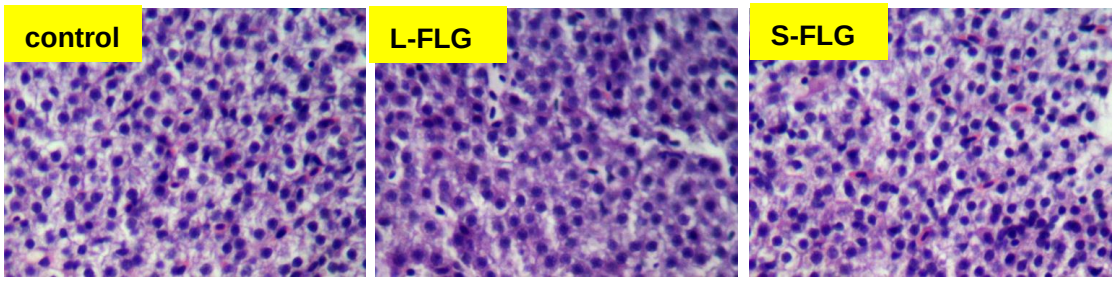


Figure S8. Representative HE stained images of liver collected from the control untreated zebrafish and FLG treated zebrafish at 72 h.

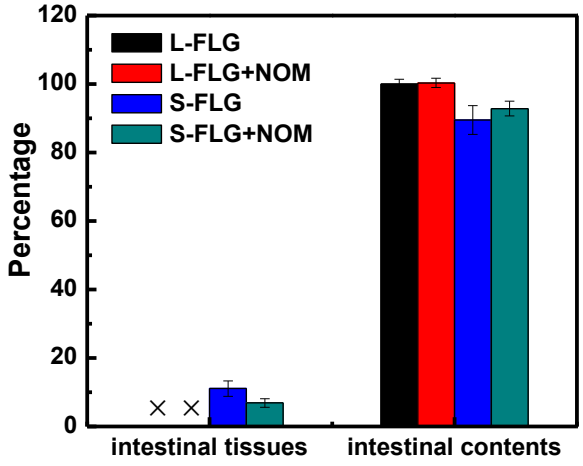


Figure S9. The relative distribution of the FLG (L-FLG and S-FLG) in gut and gut content of zebrafish, which was depurated for 120 h after being exposed for 48 h to a FLG suspension of 250 μg/L with or without the presence of NOM. Mean and standard deviation values were calculated from five duplicate samples. Symbols of "X" indicate "Not Detectable" concentration.

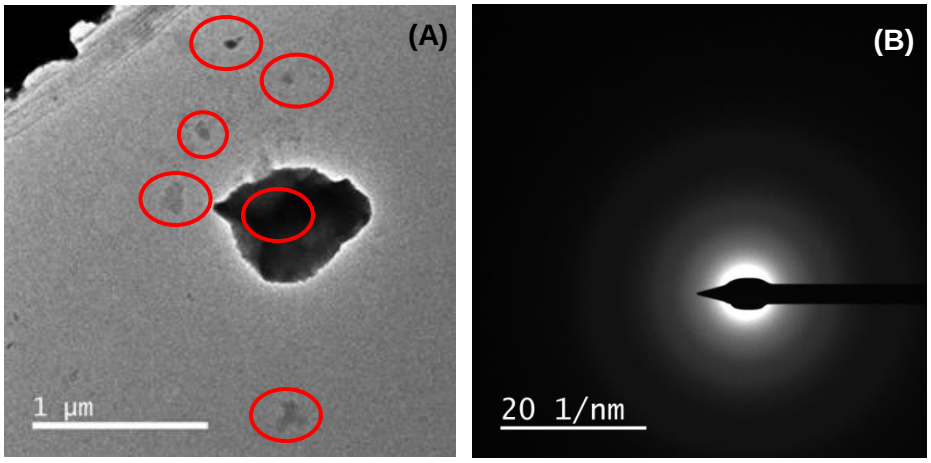


Figure S10. (A) TEM image of the sectioned intestinal epithelial cells isolated from the gut of the zebrafish of the control untreated group (not exposed to FLG). (B) SADPs taken from the red circles.

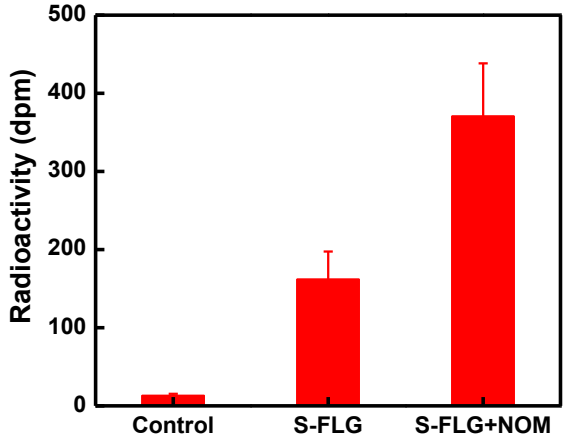
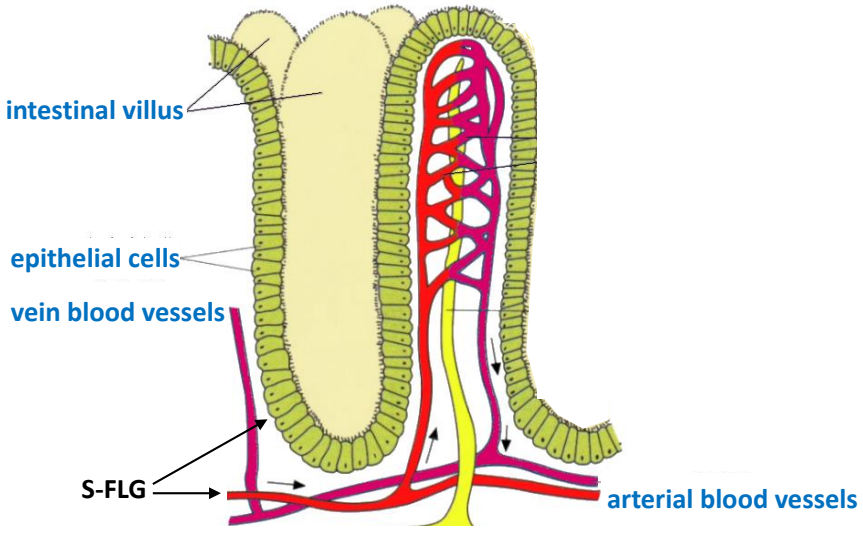


Figure S11. FLG content in blood collected from the fish of the control experiment or exposed to S-FLG without or with NOM (S-FLG+NOM) at an S-FLG concentration of 250 μg/L for 48 h, background radioactivity was not subtracted in this figure. Mean and standard deviation values were calculated from triplicate samples.

Scheme 1. Cellular distribution and translocation of S-FLG in gut.



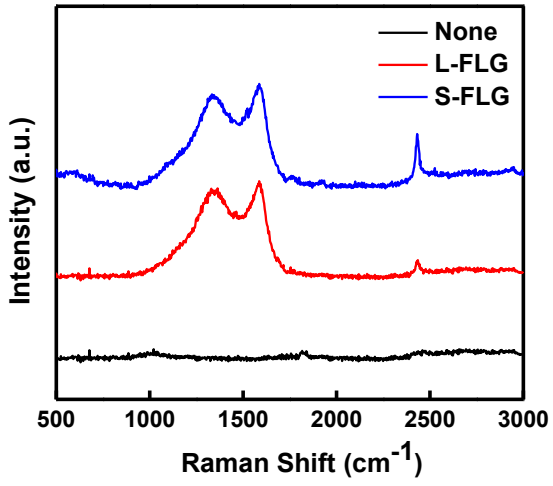


Figure S12. Raman spectra of the intestinal contents obtained from the Zebrafish treated by L-FLG (L-FLG), S-FLG (S-FLG) and control (None). Zebrafish were exposed to 250 μg/L FLG suspension for 48 h.

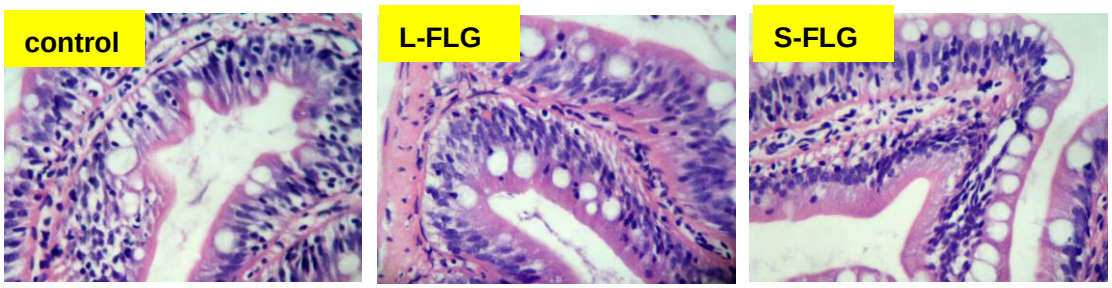


Figure S13. Representative HE stained images of intestine collected from the control untreated zebrafish and FLG (250 μg/L) treated zebrafish at 48 h.

1. Du, Z. K.; Wang, G. W.; Gao, S. X.; Wang, Z. Y. Aryl Organophosphate Flame Retardants Induced Cardiotoxicity During Zebrafish Embryogenesis: By Disturbing Expression of the Transcriptional Regulators. *Aquat. Toxicol.* **2015**, *161*, 25-32.