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# New view of graphene oxide biosafety on water environment using an eatable fish as a model

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**A comprehensive evaluation on the biosafety of graphene oxide (GO) was developed by combining 16S rRNA sequencing, gene expression detection, histology and scanning electron microscope assay on fish. GO does not affect the diversity and composition of gut microbiota, but down-regulates gene expression in fish liver, suggesting that it poses a potential risk to aquatic ecosystems.**

A food chain in a food web starts from "producer" species and ends at apex predator species, showing what the relationship between each species is. Each level of the chain represents a different trophic level. Any change in diversity at one trophic level can produce cascading changes in the ecosystem.[1] Fish, a significant link from aquatic to terrestrial organisms in evolution,[2] are an irreplaceable trophic level in the transportation of energy and nutrients in the ecosystem.[3] In modern society, fish have become an important source of energy and protein for human beings.[4] The water environment, where fish live, is a vulnerable ecological balance and is easily interrupted naturally or artificially.[5] In particular, increasing human activity can destabilise the aquatic environment and threaten human food safety, through factors like oil spills and unhandled pollutant emissions. Fortunately, these environmental hazards can be observed conspicuously in a short time through their damaging results such as the death of resident organisms or bad smells from polluted water, and urgent intervention could be carried out actively. On the other hand, some compounds related to materials and chemical reagents pose higher potential risk to biology and the environment, and yet are always ignored in our daily life due to their influence being unobservable by eye in a short period.

In recent years, numerous nanomaterials have been explored rapidly in order to develop advanced functional materials. It should not be denied that their fascinating advantages in unique structure, component and property would promote biotechnological development.[6] As a typical representative of nanomaterials, graphene has attracted substantial attention in various research areas owing to its extraordinary physicochemical properties.[7-9] Nevertheless, excellent dispersion of graphene is very difficult owing to relatively strong interactions among the nanosheets. In order to produce the mass-scale graphene-based nanomaterials to meet practical applications, chemical functionalization is still one of the most effective approaches. For example, Graphene oxide (GO) could be obtained by the sonic exfoliation of graphite oxide,[10] which is often considered as a significant precursor to achieve modified nanomaterials.[6] Compared with carbon nanotubes, GO can provide a larger surface area and better dispersibility in water due to the existence of polar oxygen functional groups on the GO surface, which could form stronger hydrogen bonds with water molecules, benefitting the creation of a stable GO colloidal suspension.[11] Until now, functionalized GO-based nanomaterials have been widely researched for exploring potential applications.[12, 13] Hence it is inevitable that GO would easily be introduced into water ecosystems, and the situation would become more and more serious. Therefore, the increased environmental exposure to GO would create a potential risk for organisms living in water.

So far, lots of studies have focused on the toxicity of GO.[14] For bacterial toxicity, the main results of these studies suggest that GO could be recognized as an antimicrobial nanomaterial as the nanosheets's sharp edges can induce membrane stress through direct contact, serving as "cutters" to physically damage bacterial cell membranes, and eventually lead to bacterial death by releasing of intracellular contents.[15, 16] Another probable reason for bacterial cytotoxicity being attributed to GO-based nanomaterials, is their capability to induce superoxide anion-independent oxidative stress on bacteria.[15] Despite the significant bacterial toxicity of GO and GO-based nanomaterials, Gram-negative bacteria with an

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outer membrane are more resistant than Gram-negative bacteria to such cell membrane damage.[14, 16] Some studies have even shown lack of GO toxicity to certain bacteria, such as *Shewanella* species.[17] For the results of GO toxicity to cell lines *in vitro*, different studies have resulted in divergent viewpoints.[14] For example, GO can cause oxidative stress and induce a slight loss of cell viability at high concentration.[18] In contrast to this study, GO in the form of film has been found to exhibit excellent biocompatibility and make mammalian cells attached more efficiently.[19] As for *in vivo* study, GO under high dosage exhibits visible toxicity to mice.[14] Though GO-coupled drugs might be excreted from a zebrafish's body rapidly, suggesting the advance of nanotherapeutics for biomedical applications,[20] the influence or biosafety of GO during its intake into the body of an aquatic living thing is still being ignored, especially on aquatic organisms *in vivo* in a complex water ecosystem. Better understanding of this issue is of significant benefit to the ecosystem. Herein we constructed a model of a water environment, using tilapia, one of the most important food fishes in the world, and developed a comprehensive and scientific evaluation method using 16S rRNA sequencing of the intestinal microbiota, gene expression detection, histology and scanning electron microscope (SEM) to assess GO's biosafety when it has been dispersed into the water environment and taken in by aquatic organisms.

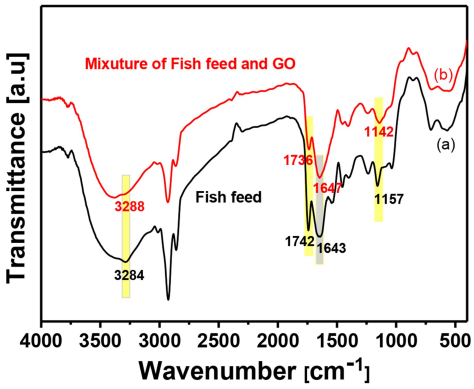


Fig. 1 Detection of the interaction between GO and fish feed by FT-IR spectra.

GO prepared by modified Hummers' method can be easily dispersed in water (Fig. S1). The detailed chemical structure, physicochemical characterization (Fig. S2), and dosage in experimental usage of GO can be found in the supporting information. In our model, fish feed contains many nutrients, composed of crude proteins (41%), crude fats (6%) and crude fibre (5.9%), etc. Generally, there is a large number of amide bonds in the chemical structure of proteins, so it is possible that the interactions between the fish feed and GO could be formed easily, which would increase the possibility of GO intake into fish. The FT-IR spectra confirmed the interaction between GO and fish feed by comparing the wavenumber shift. As shown in Fig. 1(a), the peaks at 3284, 1742, 1643 and 1157 $cm^{-1}$ can possibly be attributed mainly to carboxylic acid, carboxylate ester, amide and ether groups, respectively. The polar groups are prone to form hydrogen bonds and electrostatic forces, which is similar to formation of the double helix of DNA. Due to the polar groups existing, the adsorption bands significantly shifted by 4, 6, 4 and 15 $cm^{-1}$ after dry blending the fish feed and GO under a vacuum, strongly supporting the formation of interactions (Fig. 1(b)). The result confirms that the GO could be adsorbed onto the surface of feed in our model. Most importantly, it is a meaningful reminder that GO could be potentially adsorbed by the organic matter[21] which can be ingested by fish or other animals in a water environment.

The easiest way to estimate GO's impact on fish is to observe the difference in behaviour and growth between the fish fed with normal feed (control) and fish fed with GO-absorbed feed (experimental). In the case of the control fish, it swam leisurely, and could quickly return to the deeper water. The behaviour of the experimental appeared the same (Video S1). The average body weight of control fish (n = 6) was 34.2 g after 30 days. The average body weight of experimental fish (n = 6) increased to 35.3 g. This slight difference in growth is due to individual difference ($P$ = 0.668). Altogether, our data suggest that ingestion of a total of 4 mg of GO over a course of 30 days did not affect fish behaviour and growth.

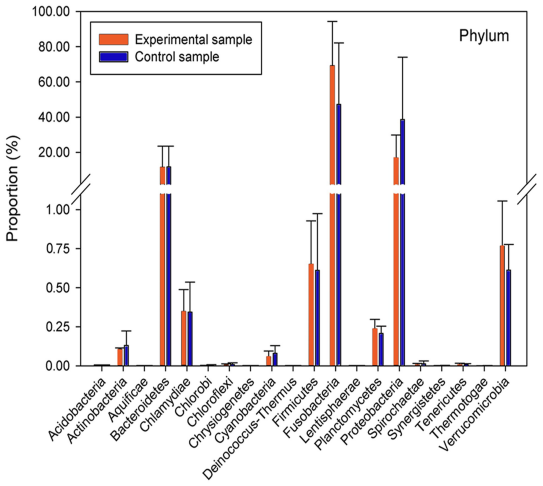


Fig. 2 Comparison of the top 20 taxonomic compositions in tilapia gut microbiota at phylum level in response to ingestion of GO-adsorbed feed. Data was expressed as the means + standard error.

The gut is an important organ with the functions of food digestion and nutrient absorption. The vertebrate intestinal tract is a complex ecosystem where diverse communities of microorganisms exist. Studies showed that the gut microbiota has become an integral component, and affects the health situation of its host.[22] As revealed in previous studies, 16S rRNA sequencing is a classical method to survey the composition of the gut microbiota.[23] To investigate and compare the gut microbiota in response to GO ingestion, we sequenced eight metagenomic DNA samples isolated from the gut of four control fish and four experimental fish. The results showed that the tilapia gut microorganism community could be classified into 27 known phyla, 55 known classes and 105 known families in total. In the control fish, Fusobacteria, Proteobacteria and Bacteroidetes were the dominant bacterial phyla (Fig. 2). The three most abundant bacterial phyla in the

experimental fish are same as the top three in the control. The experimental fish showed subtle differences in composition proportion from the control, but the data showed no significant differences ($P > 0.05$) at the analysed phylum, class and order level (Fig. 2, Fig. S3 and Tab. S1). Subsequently the gut microbiota composition profiles at order level are visually reflected by clustered heatmap (Fig. 3). As shown in Fig. 3, all the eight fish gut samples were divided into two clades, one is composed of A7 (control fish), A6 (experimental fish), A8 (experimental fish) and A9 (control fish); the other is B3 (control fish), B6 (experimental fish), B11 (control fish) and B10 (experimental fish). Obviously, the samples were not clustered according to whether there was intake of GO-absorbed feed. Similar results were also displayed at the phylum and class levels (Fig. S4). Overall, although the communities were sensitive to the diet of the fish,[23] no significant difference ($P > 0.05$) was shown in the composition and diversity of metagenome between the control and experimental fish.

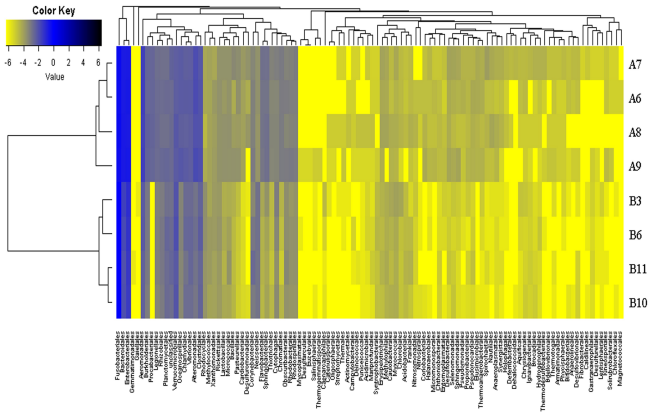


Fig. 3 Heatmap of bacterial distributions among the four experimental and the four control fish samples at order level. The relationship among the eight samples was determined by Bray distance.

A previous study showed that the viability of the bacterium *Escherichia coli* incubated with GO for 4 h can drop to 50%, as compared to 1 h incubation, and 91.6% of *E. coli* could be killed after incubation with GO at the concentration of 80 μg/mL, whereas only 10.5% could be killed at 5 μg/mL, indicating that the cytotoxicity of GO on bacteria can go up with increasing incubation time and concentration.[15] In this study, the daily GO intake is approximately 0.133 mg per fish, resulting in a maximum concentration of 80 μg/mL of GO in the gut tract (the inner-diameter and the length of the tilapia gut tract is assumed to be 2 mm and 50 cm). However it is almost in equal proportions between the experimental and control fish after 30 days. The distinct results in cytotoxicity of GO on bacteria between our and previous study could be mainly caused by the complexity of the fish digestive system. The previous study on cytotoxicity of GO on bacteria was conducted in a contiguous and limited space, where the damage could be induced directly on bacteria. For fish, however, GO has to undergo a long and complicated path to arrive in the gut, which in turn makes it difficult for direct or efficient interaction between the GO and host intestinal bacteria. This phenomenon suggests that the antibacterial activity of GO has limited influence in the aquatic ecosystems.

Gene expression is a fundamentally stochastic process, which is potentially involved in many aspects in organisms, including metabolism and responses to stress or disease.[24, 25] To further explore GO's impact on tilapia at the microscopic level, nine genes (Tab. S2) related to oxidative stress response and metabolism were selected for expression analysis from different tissues, including the liver, spleen, gill, intestine and muscle.

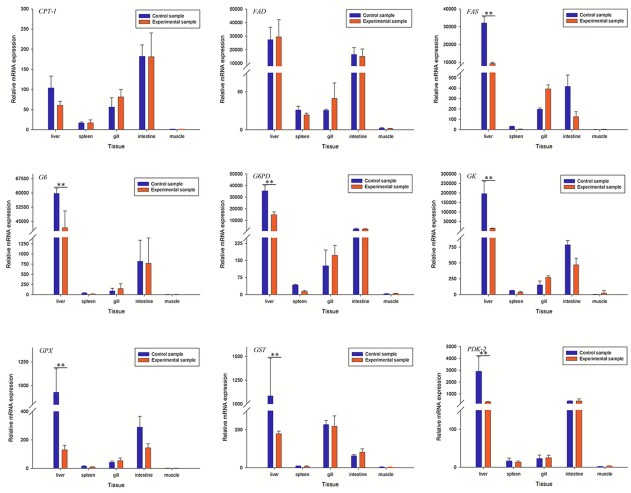


Fig. 4 Gene expression profiles in control and experimental fish tissues as revealed by quantitative RT-PCR. Asterisks indicate extremely significant differences ($P < 0.01$).

As shown in Fig. 4, after the intake of GO-adsorbed feed for 30 days, there was no significant difference in gene expressions of CPT-1 and FAD in the five tested tissues between control and experimental fish. Also, there was no significant difference ($P > 0.05$) in gene expression of the other seven genes in the spleen, gill, intestine and muscle. However, in the liver, the expressions of GST, GPX, FAS, G6, G6PD, GK and PDK-2 were significantly down-regulated by 83%, 86%, 71%, 30%, 59%, 93% and 88% on average, respectively, compared to control samples, even though no difference in behaviour and growth was observed between the control and experimental fish. Biomarkers, such as gene expression, have thus been proposed as indicators of the health status of organisms and used to assess nanomaterial toxicity.[26] For example, GST was considered as an indicator of the molecular response and toxicity in the bacterium *S. oneidensis* and the water flea *Daphnia magna* exposed to nanomaterials.[27] Our expression results should be paid attention to as decreased gene expression could be related to organism disease or stress.[28-30] Based on our results, we can infer that GO could be recognized as a foreign toxic chemical and may be able to induce genotoxicity in tilapia. Objectively, the risk could be enhanced if fish or other aquatic organisms containing "GO or GO-based pollutants" were predated. Hence the greatest impacts on aquatic environment will be increased in long-term exposures from nanomaterials.

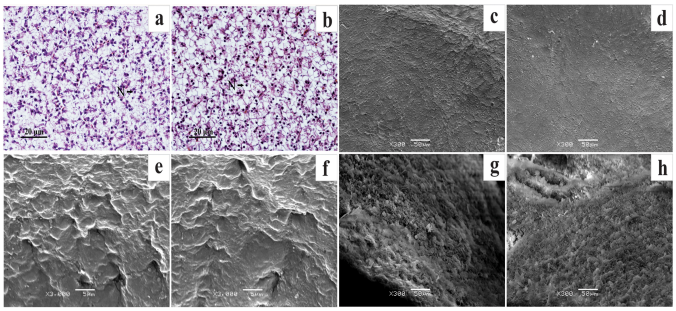


Fig. 5 Histology and SEM of the control and experimental tilapia liver samples after 30 days. a, c, e and g indicate the control fish liver samples. b, d, f and h indicate the experimental. a and b are stained by Hematoxylin and eosin. N: nucleus. c~h are SEM graphs: c and d show the surface of the liver with magnification of ×300. e and f, ×3000. g and h show the cutting surface with magnification of ×300.

In diagnostics, histology and SEM assay are effective ways to determine the pathological change. As differences of gene expressions were detected only in the liver, histological study and SEM assay were performed using control and experimental fish samples to further explore potential GO influence on the liver. As shown in the control samples of Fig. 5a, the tilapia liver consists of a large amount of closely arranged hepatocytes. The hepatocytes are polyhedral in shape and each contains a very basophilic central nucleus, which was stained in dark blue, and the border of each hepatocyte and the surrounding of the nucleus were stained red by eosin solution. Compared to the experimental samples in Fig. 5b, there were no differences in morphology. Next, the liver samples were subjected to SEM for detailed information on the surface and cutting surface. The control and experimental samples showed very similar results, such as mountain-like drape at the surface (Fig. 5c~5f) and cloud-like parenchymal cells at the cutting surface. All these results showed normal liver morphology, indicating that GO does not affect the appearance of the liver, or that it could be difficult to estimate pathology changes in a short time in our model.

In summary, although GO did not affect the composition and diversity of gut bacterial microbiota, the expression of most genes was significantly down-regulated in the liver. This should be paid attention to GO or GO-based nanomaterial utilization so as to maintain the sustainability of aquatic ecosystems.

All handling of fish was conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory, Singapore. The IACUC has specially approved this study within the project "Breeding of Tilapia" (approval number is TLL (F)-12-004). This research was supported by the internal fund of Temasek Life Sciences Laboratory, the China Scholarship Council (201303070320), Natural Science Foundation of China (51402151), and Zijin Intelligent Program by Nanjing University of Science and Technology. We are grateful to our colleagues Ms. May Lee for English editing.

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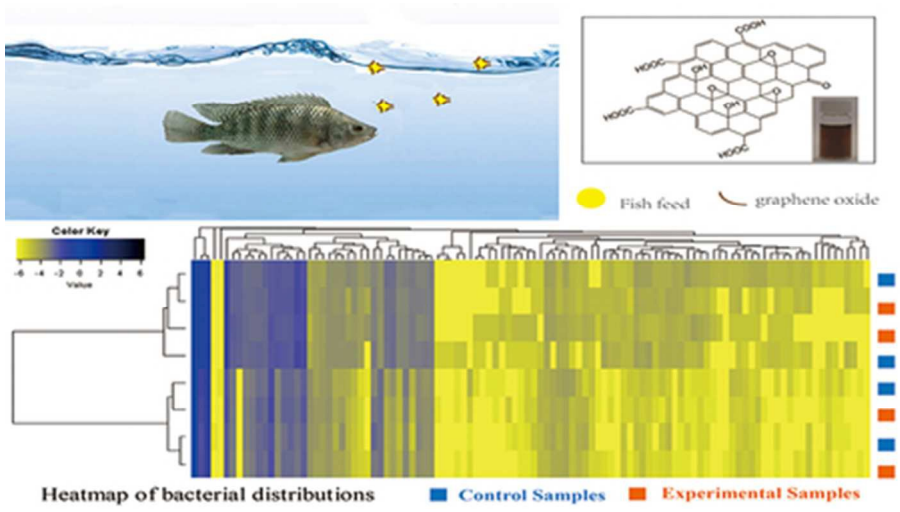


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