Combinatorial immune and stress response, cytoskeleton and signal transduction effects of graphene and triphenyl phosphate (TPP) in mussel Mytilus galloprovincialis

Xiangjing Meng¹,², Fei Li²,³, Xiaoqing Wang²,³, Jialin Liu³, Chenglong Ji¹,², Huifeng Wu²,³,⁴

¹ CAS Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS), Shandong Key Laboratory of Coastal Environmental Processes, YICCAS, Yantai 264003, PR China
² Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, PR China
³ University of Chinese Academy of Sciences, Beijing 100049, PR China

GRAPHICAL ABSTRACT

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ABSTRACT

Owing to its unique surface properties, graphene can absorb environmental pollutants, thereby affecting their environmental behavior. Triphenyl phosphate (TPP) is a highly produced flame retardant. However, the toxicities of graphene and its combinations with contaminants remain largely unexplored. In this work, we investigated the toxicological effects of graphene and TPP to mussel Mytilus galloprovincialis. Results indicated that graphene could damage the digestive gland tissues, but no significant changes were found in the...

Abbreviations: AFM, atomic force microscopy; AHSA1, activator of 90 kDa heat shock protein ATPase homolog 1; AHSA2, activator of 90 kDa heat shock protein ATPase homolog 2; ANOVA, one-way analysis of variance; CAT, catalase; CDC37, hsp90 co-chaperone cdc37; CP450, cytochrome P450; DLC2, dynel light chain 2; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; DNAJC7, daz homolog subfamily C member 7; FKBP, f506-binding protein; FKBP4, f506 binding protein 4; FKBP5, f506 binding protein 5; FKBP6, f506 binding protein 6; FKBPL, f506-binding protein-like; GLMN, glomulin; GO, graphene oxide; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione s-transferase; HSD, hydroxysteroid (17-β) dehydrogenase; HSP90, heat shock protein 90; HSP90AA1, heat shock protein HSP90-alpha; Mgc, mgc79752 protein; MHC1, myosin heavy chain 1; MyD88a, myeloid diiferentiation factor 88a; NF-κB, nuclear factor-k-gene binding; NMs, nanomaterials; PMyo, para-myosin; PPI, protein-protein interaction; PTGES3, prostaglandin E synthase 3; qRT-PCR, reverse-transcription real-time PCR; S.D, standard deviation; SOD, superoxide dismutase; STIP1, stress-induced-phosphoprotein 1; STUB1, STIP1 homology and U box-containing protein 1; TMyo, tropomyosin; TPP, triphenyl phosphate; UNC45B, protein unc-45 homolog B; UNDCD2, nudc domain-containing protein 2; VTG, vitellogenin; WASL, neural wiskott-aldrich syndrome protein; β-actin, beta-actin

* Corresponding authors at: CAS Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS), Shandong Key Laboratory of Coastal Environmental Processes, YICCAS, Yantai 264003, PR China.

E-mail addresses: fl@yic.ac.cn (F. Li), hfwu@yic.ac.cn (H. Wu).

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1. Introduction

Coastal and estuarine ecosystems have been heavily influenced by anthropogenic activities through pollution and habitat loss throughout the world [1]. In recent years, the production of nanomaterials (NMs) has been increased and the global production of NMs will be up to 58,000 t by 2020 [2]. Graphene is an emergent engineered nanomaterial. Due to its special electrical, optical, mechanical and chemical properties, graphene and its derivatives have been widely used in many fields [3]. Due to the limitations of the detection method, there are few reports on the environmental concentration of graphene, but it is expected that large quantities of graphene-based wastes will end up in the environment, representing a risk for the marine environment [4,5]. Thus, it becomes extremely important to investigate the potential toxicity of graphene and its derivatives on marine organisms in order to provide relevant data for the ecological risk assessment of graphene.

Previous work revealed that graphene nanotoxicity mainly focused on influencing cell growth and inducing oxidative stress [6]. The toxic effects of graphene and its derivatives were related to the dose and species sensitivity [7], which had been studied in various organisms such as algae, invertebrates and vertebrates (Table 1). Alga can be used as appropriate models to examine the toxicity of graphene and graphene oxide (GO). GO at 0–10 mg/L intensively entrapped single-celled Chlorella vulgaris and reduced the cell permeability [8]. For the invertebrate, GO increased ROS production in exposed mussel M. galloprovincialis hemocytes at a wide range of concentrations (up to 25 mg/L) [9]. Studies showed that graphene could induce oxidative stress in brine shrimp (Artemia salina) at concentrations ranging from 0.5 to 1.0 mg/L [10]. The embryonic development of zebrafish (Danio rerio) is a common testing model for the developmental toxicity of chemicals. GO affected the development of heart and skeleton to cause deformity in zebrafish at concentrations ranging from 0.001 to 100 mg/L [11,12]. Researches on the model species, pseudomonas aeruginosa (Microcystis aeruginosa) also found that graphene could affect the enzyme activities associated with oxidative stress [13].

Once entering the environment, graphene can easily interact with other pollutants through hydrophobic, π–π stacking and hydrogen bonding interactions (such as heavy metals, organic pollutants, etc.), thereby affecting their environmental distributions and interactions with organisms [14]. Liu et al. showed that low levels of graphene (0.01 μg/mL) and GO (5 μg/mL) could increase the cell toxicity of arsenic [15]. In addition, Hu et al. found that GO and copper had significant antagonism against S. obliquus, and GO could reduce the ecotoxicity of copper even at environmentally relevant concentrations (1 mg/L) [5].

Triphenyl phosphate (TPP) was a high-production flame retardant that had been detected in many environmental media and biota [16,17]. Currently, there are no threshold limits for graphene and TPP issued by regulatory authority. The concentrations of TPP ranged from 7 to 209 pg/g dry weight (dw), with the geometric mean (GM) of 40 pg/g dw in the surface sediment from the Bohai Sea and Yellow Sea [18]. Studies showed that TPP could induce developmental toxicity, neurotoxicity and endocrine disruption at 0.01–100 mg/L [19]. Acute toxicity data for TPP were also available for algae (median effective concentration [EC50], 0.26–2.0 mg/L) [20]. Moreover, TPP contains three benzene rings, and easily lead to the adsorption by graphene. Graphene has high hydrophobicity and durability, which may increase the accumulation of TPP and transmit it to humans through the food chain. Our previous studies showed that combined graphene + TPP exposure could aggravate the damage of simulated bio-membrane induced by single graphene or TPP exposure [21]. Thus, it also becomes important to elucidate the joint effects of graphene + TPP exposure.

Mussel M. galloprovincialis is often used as a preferable bioindicator for the marine environmental pollutants and investigated as an experimental species in ecotoxicology [22,23]. As a filter-feeder, M. galloprovincialis has the ability to accumulate and tolerate huge amounts of pollutants. In this study, the effects of graphene, TPP and graphene + TPP on M. galloprovincialis were investigated by tissue sections, antioxidant status and reverse-transcription real-time PCR (qRT-PCR). The histopathological changes and antioxidant status of digestive gland tissues were used to evaluate the responses of M. galloprovincialis exposed to graphene and TPP. The expressions of certain functional genes including immune stress response, cytoskeleton, intracellular signal transduction and reproductive were determined after the graphene, TPP and their combined exposures. In addition, the network of protein interactions induced by the combined graphene + TPP exposure was clarified.

| NMs          | Species                              | Time | Concentration | Toxicity                                                                 | Ref       |
|--------------|--------------------------------------|------|---------------|--------------------------------------------------------------------------|-----------|
| GO           | Green alga (Raphidocelis subcapitata) | 24 h | 0.01–10 mg/L  | GO could lead to the increased ROS levels inside the algae cell, membrane damage, and lighting efficiency; graphene could induce oxidative stress | [8]       |
| GO           | Mytilus galloprovincialis             | 24 h | 0–100 mg/L    | GO caused invaginations and perforations of the plasma membrane and increased ROS production | [9]       |
| Graphene     | Brine shrimp (Artemia salina)        | 48 h | 0.5–1.0 mg/L  | graphene could induce oxidative stress                                   | [10]      |
| GO           | Zebrafish embryos                    | 96 h | 1–100 μg/L    | GO affected the development of heart and skeletal;                       | [11]      |
| GO           | pseudomonas aeruginosa (Microcystis aeruginosa) | 96 h | 0–5 mg/L     | GO caused zebrafish deformity, affected the heart rate;                  | [12]      |
| Cd^{2+} + GO | pseudomonas aeruginosa (Microcystis aeruginosa) | 96 h | 1–100 mg/L   | GO could increase ROS levels                                             | [13]      |

Abbreviations: GO, graphene oxide; NMs, nanomaterials.
2. Materials and methods

2.1. Test compounds and graphene characterization

Graphene (1.0 mg/mL) was purchased from Sigma-Aldrich (Shanghai, China), and TPP (purity > 98%) was obtained from Dr. Ehrenstorfer Co., Ltd. The shape and size of graphene were characterized by atomic force microscopy (AFM, Veeco Multimode 8 + bioscope catalyst, USA). The size and charge distribution were analyzed at 0 h, 6 h, 12 h and 24 h, using dynamic light scattering (DLS) and the zeta potentials analyzer (Malvern, Zetasizer Nano Sizer, Nano-2890, U.K.), respectively. Raman spectra were confirmed by a DMR Raman spectrophotometer (Thermal Fisher, USA) with a 633 nm laser source. Graphene was dispersed in the natural salt water (32‰ salinity), at room temperature (18 ± 1 °C) prior to the above characterization. To reduce the aggregation of graphene during the preparation, the graphene suspension was sonicated at 100 W (Shumei, KQ-5200DE, China) for 15 min [11].

2.2. Animals and treatments

Mussels (5.0 ± 1.0 cm in length) were obtained from the Zhifu Island in Yantai (Shandong, China). Experimental animals were acclimatized for 7 days under laboratory conditions with aerated seawater (500 mesh sieved), at 18 ± 1 °C and 32‰ salinity. Hu et al. reported that the environmentally relevant concentration of graphene oxide (GO) was 1 mg/L [5]. For the TPP, the EC50 values of the marine invertebrate were 0.26 ~ 2.0 mg/L [9]. Based on these, the effects of graphene and TPP at 0.5 mg/L to mussel Mytilus galloprovincialis were investigated in the present work.

For the experiments, twenty animals per replicate were assigned to 10 L glass-beakers and exposed to graphene, TPP and graphene + TPP for 7 days for each of the following treatments: Control (C), 0.5 mg/L graphene (G), 0.5 mg/L TPP (T), combined 0.5 mg/L graphene and 0.5 mg/L TPP exposure (G + T). Seawater was changed daily, and both graphene and TPP were re-dosed at a nominal concentration of 0.5 mg/L. During the acclimation and entire exposure periods, mussels were under a photo-period of 12 h light and 12 h dark, and fed with Chlorella vulgaris daily. No mortality of mussels was observed during the experiments. At the end of exposure period, the digestive gland was randomly dissected from 12 mussels for each treatment and used for the further study. The methods for histology and antioxidant status analysis of digestive gland tissues exposed to graphene and TPP could be found in the Supporting Information.

2.3. Concentrations of TPP in biota

Following the previous studies [18], the concentrations of TPP in biota were detected by gas chromatograph Agilent 7890A GC coupled with a triple quadrupole mass spectrometer Agilent 7010 MS (GC-MS/MS) equipped with a programmed temperature vaporizer (PTV) injector (Agilent Technologies, USA). The details were shown in the Supporting Information.

2.4. RNA extraction and qRT-PCR analysis

In order to investigate the possible mechanisms of toxicity caused by graphene and TPP, several gene expression levels were quantified by qRT-PCR technique. The target genes were involved in immune stress response (FKBP, HSP90, NF-κB and MyD88a), cytokoskeleton (Matrilin, DLC2, MHC1, Pmyo and TMyo), intracellular signal transduction (Cublin and Mgc) and reproduction (CP450, VTG and HSD). Gene-specific primers in this study were shown in Table 2. The methods for Total RNAs extraction and qRT-PCR analysis could be found in the Supporting Information. The interacting partners of proteins for all the selected functional genes were identified using STRING 11.0 database (https://string-db.org). The protein-protein interaction (PPI) networks were constructed and visualized using Cytoscape 3.7.1 (http://www.cytoscape.org/download.php).

Table 2

| Gene name | Full name | Correlation function | GenBank accession No. | Primer name F | Primer name R | Primer sequence (5'-3') |
|-----------|-----------|----------------------|----------------------|---------------|---------------|------------------------|
| β-actin | beta-actin | endogenous control | GT157817 | β-actin-F | β-actin-R | GCTATCCAGGGCTGTACCT |
| FKBP | Fk506-binding protein | immune stress response | AJ625569 | FKBP-F | FKBP-R | GCCGTGTTGTTGAAATGAG |
| HSP90 | Heat shock protein 90 | immune stress response | AJ625915 | HSP90-F | HSP90-R | AACGTGGCTGTCATATACCTG |
| NF-κB | NF-κB transcription factor Rel | immune stress response | HQ127223.2 | NF-κB-F | NF-κB-R | ATACCTTCTTCTGCCGTACATC |
| MyD88α | Myeloid differentiation factor 88α | immune stress response | JX112712.1 | MyD88α-F | MyD88α-R | TGGCGGTTGGAAGAATGTAG |
| Matrilin | Matrilin | extracellular matrix proteins | AJ625256 | Matrilin-F | Matrilin-R | ACAATATAGGCGGAAAGCCTCA |
| DLC2 | Dynein light chain 2 | motor protein light chains | AJ516886 | DLC2-F | DLC2-R | TCACACTCCGATGATTAGGCA |
| MHC1 | Myosin heavy chain 1 | myosin heavy chain | AJ249992.1 | MHC1-F | MHC1-R | GGGAGGACCATCAGGGATTAT |
| FMyo | Para-myosin | vice myosin | AB011670.1 | FMyo-F | FMyo-R | ATACCTTCTTCTGCCGTACATC |
| TMyo | Tropo-myosin | tropomyosin | AB000907.1 | TMyo-F | TMyo-R | TGTCACTTCGTGTCAGGTC |
| Cublin | cubulin | intracellular signal transduction | AJ626333 | Cublin-F | Cublin-R | CTTCTTAATCAATGGTTTAT |
| Mgc | mgc799752 protein | intracellular signal transduction | AJ624360 | Mgc-F | Mgc-R | CTTCTTCCGAGAATCGACAT |
| CP450 | cytochrome P450 | sex hormone synthesis | FL499705.1 | CP450-F | CP450-R | GATTAAGATGCCCTACGTAC |
| VTG | vitellogenin | vitellogenin | AJ625462.1 | VTG-F | VTG-R | CTTGGAATGCTTCTCCGAG |
| HSD | hydroxysteroid (17-β) dehydrogenase | hydroxyl steroid dehydrogenase | FL499705.1 | HSD-F | HSD-R | GATAAGGAGCCATGGTAC |

*Table 2: The primers for qRT-PCR in this study.*
2.5. Statistical analysis

All the data were reported as mean ± standard deviation (S.D.). Statistical differences in biological parameters between control and treatments were evaluated by one-way analysis of variance (ANOVA) using SPSS 22.0 software. Before ANOVA, the normality and homogeneity of variances were verified. Differences were considered statistically significant at a value of $P < 0.05$.

3. Results and discussion

3.1. Histological observation of digestive gland in mussels M. galloprovincialis exposed to graphene and TPP

As shown in Fig. 1, there was a minor degree of tissue loss in graphene-exposed mussels compared with control group. For the TPP-exposed group, no obvious damage was observed. The effect of graphene was related to its inherent surface characteristics [25]. As shown in the AFM pictures (Fig. S1A), the horizontal size of graphene layers was large and the edge was very sharp. After entering the digestive tract, graphene could cause damage to the digestive tract [26]. However, there was no obvious tissue damage in the mussels from graphene + TPP co-exposure group. In our study, the average TPP concentration in the co-exposed group (770.77 ± 425.69 μg/g fat weight) was higher than that in the TPP-exposed group (404.57 ± 258.22 μg/g fat weight) (about 1.9 times), presenting enhanced bioaccumulation of TPP. This might be explained that the adsorption of TPP reduced the surface sharpness of graphene. Hence, there was no significant change of the tissue damage in the graphene + TPP exposure group.

3.2. Antioxidant status of digestive gland tissue exposed to graphene and TPP

The antioxidant enzyme activities and the antioxidant contents can reflect the oxidation state of the organism [27]. As shown in Table 3, after the exposure of 0.5 mg/L graphene, the content of GSH and the activities of Gpx, SOD and CAT in the digestive gland tissues were increased significantly ($P < 0.05$). In the TPP-exposed group, the activities of CAT and GST were increased remarkably ($P < 0.05$). Mittal et al. [28] reported that the content of GSH and the level of ROS in human lung cells were increased obviously after exposure to graphene. Graphene also could enhance the oxidative stress in Artemia salina, and influenced the activities of antioxidant enzymes (GST, CAT, Gpx and SOD) and the content of MDA [29]. Moreover, Chen et al. found that TPP exposure aected the expressions of antioxidant enzymes and related genes (GPX, CAT and GST) [30]. Our findings were consistent with previous studies, indicating that both TPP and graphene could induce oxidative stress in mussels.

Interestingly, there was a significant decrease in the content of GSH and the activities of GST and CAT in the digestive gland tissues were increased significantly ($P < 0.05$). In the TPP-exposed group, the activities of CAT and GST were increased remarkably ($P < 0.05$). Mittal et al. [28] reported that the content of GSH and the level of ROS in human lung cells were increased obviously after exposure to graphene. Graphene also could enhance the oxidative stress in Artemia salina, and influenced the activities of antioxidant enzymes (GST, CAT, Gpx and SOD) and the content of MDA [29]. Moreover, Chen et al. found that TPP exposure affected the expressions of antioxidant enzymes and related genes (GPX, CAT and GST) [30]. Our findings were consistent with previous studies, indicating that both TPP and graphene could induce oxidative stress in mussels.

![Table 3](#)

| Groups          | Control | Graphene | TPP | Graphene + TPP |
|-----------------|---------|----------|-----|---------------|
| GSH             | 3.09 ± 0.27 | 4.15 ± 0.29<sup>a</sup> | 3.64 ± 0.33<sup>b</sup> | 2.26 ± 0.29<sup>c</sup> |
| GPx             | 65.48 ± 3.57<sup>b</sup> | 81.44 ± 3.45<sup>a</sup> | 45.56 ± 1.52<sup>c</sup> | 70.00 ± 1.41<sup>a</sup> |
| GST             | 40.88 ± 1.40 | 40.20 ± 2.99 | 49.92 ± 1.75<sup>a</sup> | 33.07 ± 1.08<sup>c</sup> |
| SOD             | 28.14 ± 0.71 | 31.30 ± 1.38<sup>a</sup> | 26.20 ± 1.19<sup>c</sup> | 31.26 ± 1.33<sup>c</sup> |
| CAT             | 35.53 ± 2.86 | 43.24 ± 5.79<sup>a</sup> | 39.98 ± 3.00<sup>c</sup> | 37.73 ± 1.75<sup>c</sup> |

* The data are presented as the mean ± S.D.
*<sup>a</sup> $P < 0.05$, compared with the control group.
*<sup>b</sup> $P < 0.05$, compared with the graphene-exposed group.
*<sup>c</sup> $P < 0.05$, compared with the TPP-exposed group.

GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione s-transferase; SOD, superoxide dismutase; CAT, catalase.

![Fig. 1](#)

Fig. 1. The histopathology images of the digestive gland tissues after exposures to graphene, TPP and graphene + TPP (× 400), scale bar is 20 μm.
inhibit the surface activity of graphene and thus reduce its toxicity.

Oxidative stress could cause tissue damage, cell membrane damage, mitochondrial dysfunction and other toxic effects [31]. To gain insights into the possible mechanisms of the combined effects of graphene + TPP in *M. galloprovincialis*, the expression levels of 14 genes involved in immune stress response, cytoskeleton, intracellular signal transduction and reproductive were determined as followed.

### 3.3. Effect of graphene and TPP on immune stress reaction gene expressions in the digestive gland tissue of mussels

The relative expressions of *HSP90* and *FKBP* genes were obviously up-regulated in the graphene-exposed group. In addition, *HSP90*, *NF-κB* and *MyD88a* genes were notably up-regulated in the TPP-exposed group (Fig. 2). Researches have demonstrated that GO could increase the oxidative stress parameters and the level of *HSP70* in *Acheta domesticus* [32]. According to Zhi et al., *C. elegans* could regulate gene expression as a response to oxidative stress due to the existence of graphene [33]. The oxidative stress and endocrine related genes in mice were influenced remarkably by TPP exposure [30]. Our findings were consistent with these previous results. These gene expression profiles indicated that both graphene and TPP could induce obvious immune stress in the digestive gland tissues of mussels.

The *MyD88a* gene encodes myeloid differentiation factor 88a, and significantly affects the muscle Toll receptor 9 (*TLR9*) signaling pathway. As the key components in immune signaling of invertebrates, Toll-like receptors are expressed in *M. galloprovincialis* hemocytes and digestive gland [34]. As shown in Fig. 2, a more considerable down-regulation in the level of *MyD88a* was observed in the graphene + TPP co-treatment group than that in graphene-exposed group. Liu et al. revealed that graphene could reduce the cell and genetic toxicity caused by PCB52, which was closely related to the induction of genuine autophagy [35]. Based on this finding, it was concluded that the combined graphene + TPP exposure could reduce the immune stress caused by graphene in tissues, which was consistent with the alteration of antioxidant enzyme activities.

### 3.4. Effect of graphene and TPP on intracellular signal transduction and reproduction gene expressions in the digestive gland tissue of mussels

Intracellular signal transduction plays an important role in the maintenance of cell normal biological function [36]. There was an increase in relative expressions of *Cubilin* and *Mgc* in mussel digestive gland tissues with exposure to 0.5 mg/L graphene and TPP, respectively (Fig. 3A). Cubilin is a receptor protein that on cell membrane encoded by *Cubilin* gene. As a receptor protein, Mgc 79752 protein plays an important role in signal transduction of cells [37]. It seemed that graphene and TPP could affect the intracellular signal transduction in the digestive gland tissues of mussels.

After graphene + TPP exposure, the relative expression of *Mgc* was significantly down-regulated in comparison with that in graphene-exposed group. However, the up-regulation of tubulin isoforms and Mgc protein were observed in mussels exposed to n-TiO₂ and the n-TiO₂/TCDD mixture, respectively [37]. It might be explained that the two receptor proteins, *Cubilin* and *Mgc* were involved in different signal transduction pathways, thus resulting in the different levels of gene expression induced by exogenous pollutants. Therefore, the specific mechanism needs further explorations.

The relative expressions of *CP450* and *VTG* genes in graphene-exposed group had no significant changes in comparison with the control group, while the expression of *HSD* was evidently up-regulated (Fig. 3B). For the TPP-exposed group, the relative expressions of *CP450*, *VTG* and *HSD* genes were significantly down-regulated. *CP450* gene encodes cytochrome P-450 protein participating in the foreign chemical hydrolysis, reduction and oxidation reaction, and also plays an important role in regulating the synthesis of steroids hormones in body (most sex hormones are steroids hormone) [38]. *VTG* gene encodes vitellogenin that is the precursor of yolk protein for almost oviparous animals [39]. Vitellogenin can provide amino acids, fat, carbohydrates, vitamins and other nutrients for the developing embryo [40]. 17β-HSDs (HSD) mainly express in digestive gland and gonads of mussels and can regulate the activities of sex hormones [41].

Liu et al. [19,42] showed that TPP could affect the balance of sex hormone in *Danio rerio*, and then affect the reproductive capacity. TPP could also inhibit the expression of cytochrome P-450 26a1 (*CYP26a1*), and induce the reproductive toxicity in *Danio rerio* [43]. Chen et al. [31] found that TPP could evidently inhibit the expression of cytochrome P450 cholesterol side-chain lyase (*CP450xccl*) and 17α-hydroxy steroid dehydrogenase (*17α-HSDa*) genes, showing obvious reproductive toxicity. These results showed that TPP could inhibit the expression of reproduction genes and thus influenced reproductive toxicity in mussels.

Graphene had a certain effect on reproductive related gene expression. Compared with the graphene-exposed group, significant decreases in the levels of *CP450* and *HSD* levels were observed in the graphene + TPP co-treatment group. After exposure with graphene + TPP, the expression levels of *CP450* and *HSD* were not consistent with other functional genes, and the mechanism should be further studied.

### 3.5. Effects of graphene and TPP on cytoskeleton gene expressions in the digestive gland tissue of mussels

The relative expressions of *Matrilin* and *MHC1* genes were significantly up-regulated in the mussel digestive gland tissue after exposure to 0.5 mg/L graphene and 0.5 mg/L TPP for 7 days, respectively (Fig. 4). It was reported that the bending stiffness of graphene was similar to the lipid bilayers of cells, which was beneficial to the close interactions between graphene and membrane proteins [44]. Smaller graphene with sharp edges could be engulfed by cells to interact and possibly to cut actin laments leading to destruction of the cytoskeleton [45]. Tian et al. found that graphene nanosheets could change the secondary structures of actin monomers, and affected cell microfilament structure, thus affected the material migration and transportation in human A549 lung cancer cell [46]. The expression profiles of these data are presented in Table 1.
genes indicated that both graphene and TPP affected the internal and external cellular structures of cells as well as the transport of substances in cells.

Compared with the graphene-exposed group, the relative expressions of DLC2, MHC1, and PMyo genes were evidently down-regulated in mussel samples after combined graphene + TPP exposure, which suggested that combined exposure of graphene and TPP could reduce the effect induced by graphene on the cell structure and material transport in mussel samples.

3.6. Construction of regulatory molecular pathways of graphene + TPP and M. galloprovincialis interaction

Systematic prediction of interaction network would help explore complex gene networking [47]. To elucidate the mechanism of action, online STRING 11.0 database was used to construct portable PPI networks based on the selected functional-related genes, and the inter-relationship was visualized by Cytoscape 3.5.1. As shown in Fig. 5, these genes associated with stress response (HSP90AA1, FKBP12, FKBP4, and FKBP5), cytoskeleton (UNC45B) and reproduction (FKBP6) interacted with each other.

The heat-shock proteins (HSP) are highly conserved family in all living organisms, and play an essential role in cell homeostasis [33]. In our study, the relative expression of HSP90 gene was obviously up-regulated in the graphene + TPP co-exposure group (Fig. 2). FKBP4, FKBP5 and FKBP12 proteins belong to immunophilins family and play roles in immune regulation. They are believed to be related with protein folding and transport, and participate in cellular stress responses [48]. The interactions between FKBP4/5L and HSP90 promoted the formation of steroid receptor heterocomplex, which might protect mussels from oxidative damage leading to the considerable changes of antioxidant levels in mussel digestive gland tissues (Table 3).
response (MHCII), cytokoskeleton (MHC1, PMyo and TMyo) and reproductive (CP450 and HSD) were significantly down-regulated in the combined graphene and TPP-treated group, comparing with those in the graphene-exposed group. Our research indicated that combined exposure of graphene and TPP could affect cytokoskeleton and stress response. The reproductive toxicity caused by graphene + TPP might be related to FKBP6, and the mechanism should be further studied. The study could provide valuable information for understanding possible toxic mechanisms of graphene and its combination with other contaminants to marine organisms.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.120778.

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