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Potential genotoxixity impacts of a co-exposure of polypropylene microplastic and antibiotics to fresh water pearl mussel *Hyriopsis cumingii* (LEA, 1852)

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Abstract. Microplastics (MPs) are becoming ubiquitous, and their environmental fate is becoming an issue concern. MPs can adsorb antibiotics to coexist and accumulate in the aquatic environment in the form of complexes, resulting in unforeseeable adverse consequences. Herein, we investigated the genotoxic effects of coexist of polypropylene (PP) microplastic and two antibiotics of sulfamethoxazole (SMX) and oxytetracycline (OTC) at environmental concentrations on gill and gastrointestinal tract (GIT) cells of freshwater pearl mussel *Hyriopsis cumingii*. Results showed that coexist of PP and antibiotics SMX and OTC at environmental concentrations can cause the DNA damage (5.0 - 8.7 % in gill and 4.5 - 7.9 % in GIT) and micronucleus (10.8 - 20.9 ‰ in gill and 11.9 - 18.6 ‰ in GIT) for *H. cumingii*. By principal component analysis, we found that there was a strong relationship between microplastic accumulation in the GIT and biomarkers, including DNA damage and micronucleus, for *H. cumingii* co-exposed to PP microplastics in combination with only OTC antibiotic. Meanwhile, only a strong correlation was detected between microplastic accumulation in gill or GIT and DNA damage in those tissues, when *H. cumingii* was exposed to mixing antibiotics OTC and SMX along with PP microplastic. Our study contributed to improve the understanding of the adverse genetic impacts of co-existence of PP microplastics and antibiotics (SMX and OTC) in the environment as well as to provide essential information for ecological risk assessment of MPs and antibiotics pollution.

Key words: polypropylene, sulfamethoxazole, oxytetracycline, genotoxicity, *Hyriopsis cumingii*.

Classification numbers: 3.2.3, 3.3.1, 3.6.1, 3.6.2

1. INTRODUCTION

Microplastic occurrence and toxicological effects in some aquatic organisms like crustacean, fish and bivalve species could be documented in many studies [1 - 4]. Microplastic could also behave as chemical absorbents because of their large specific surface areas and

hydrophobicity, and then chemicals may reach even higher concentrations than measured in the water column [5 - 7]. Aquatic organisms can uptake microplastics with chemicals absorbed and the presence of microplastics could affect the biotoxicity of environmental pollutants [7 - 11]. Polypropylene (PP) microplastic is one of the common polymers observed in the aquatic ecosystems [12, 13]. PP microplastic pollution may be resulted from PP application for many fields such as composite biotechnology, catalysis, surface coating technology, flame retardants, food packaging and medical purpose [14].

Besides microplastics, antibiotics are also ubiquitous environmental pollutants with adverse effects on ecosystems and human health, sulfamethoxazole and oxytetracycline are two commonly used antibiotics that belong to the group of pharmaceuticals known as emerging contaminants. These antibiotics are widely used in both human and veterinary medicine for the treatment of bacterial infections and are excreted from the body in their unchanged form or as metabolites. Several global studies have reported the presence of sulfamethoxazole (SMX) in freshwater systems, ranging from few ng L^{-1} to more than 30 µg L^{-1} because it is an extensively-used antibiotic in humans and veterinary medicine [15 - 18]. Similarly, oxytetracycline (OTC) is a form of the antibiotic tetracycline with a broad spectrum of activity that is commonly used in aquaculture to treat bacterial infections in fish. Thus, OTC residues have been detected in freshwater systems with concentrations between a few ng L^{-1} to µg L^{-1} in aquatic ecosystems [19 - 22]. The presence of SMX and OTC antibiotics in aquatic systems was reported to cause adverse impacts on aquatic organisms such as fish and bivalve species [23 - 28]. Several endpoints and/or biomarkers were evaluated in previous studies about the joint toxicity of microplastics and single antibiotic SMX or OTC to fish species and algae, including bioaccumulation, histological and biochemical indices, gut microbial community, and gene expression [7, 11, 29, 30]. However, the effects of co-exposure of PP and antibiotics OTC and SMX on bivalve species at molecular levels are not thoroughly characterized. It is possible that PP microplastics could have additive or synergistic toxic effects on genetic materials such as DNA and nucleus of gill and gastrointestinal tract cells of freshwater mussel.

Hyriopsis cumingii plays an important role in aquatic ecosystems and fishery economy because of its potential value for water purification and edibleness. *H. cumingii* has also become a dominant species of cultivating pearl in freshwater because the pearls formed inside these mussels have a good quality including color, cleanness and shape. Thus, *H. cumingii* was selected in this study to assess the risk assessment of the environmental pollution by PP microplastics and antibiotics SMX and OTC. In this study, the comet and micronucleus assays were carried out for the first time on cells extracted from the gills and GIT tissues of freshwater pearl mussels *H. cumingii* in order to evaluate DNA damage and determine the frequency of micronucleated cells after exposing to PP microplastic and antibiotics SMX and OTC at environmental concentrations. Another attempt was also made in this study to find the relationship between MP bioaccumulation in GIT and gills of *H. cumingii* with the capable of inducing structural and/or numerical chromosomal damages.

2. MATERIAL AND METHODS

2.1. Chemicals and organisms

Polypropylene (PP: GF63935860-1EA, Aldrich) used for the experiment was purchased from Sigma-Aldrich Pte Ltd, with the nominal granule size of 4 mm and was then grounded using a VNS-800 grounder. PP powder was sieved to collect small-sizes < 200 µm. The purified

SMX ($C_{10}H_{11}C_3O_3S$) and OTC ($C_{22}H_{24}N_2O_9$) used in this study were purchased from the National Institute of Drug Quality Control. The stock solutions were prepared with 100 µg/L for both sulfamethoxazole and oxytetracycline. We analyzed the concentration of nominal sulfamethoxazole and achieved a 98.4 % recovery with relative standard deviation RSD < 0.9 %. In the meanwhile, the concentration of oxytetracycline was also analyzed and recorded with 96.5 % recovery achievement and RSD < 1.6 %. Analysis of the SMX and OTC was performed by ultra-performance liquid chromatography (ACQUITY UPLC, H-class, Waters, USA) in combination with a tandem mass spectrometer (Xevo TQD, Waters, USA). The detection limits of UPLC-MS/MS for sulfamethoxazole was 0.5 µg/L and 1.0 µg/L for oxytetracycline.

Freshwater pearl mussel (*Hyriopsis cumingii*) was chosen as a model organism due to their wide distribution, especially in coastal Viet Nam. Mussel *H. cumingii*, with size of 85.18 ± 5.37 mm in shell length, and 82.58 ± 6.12 g wet in weight, was collected from farming in Dong Hoa Lu ward, Ninh Binh province. Fifty-five mussels were adapted with the laboratory condition for five days in two glass tanks (20 L), which contained filtered freshwater before starting the exposure experiment. Mussels were eliminated fouling particles and debris that might have accumulated and they were acclimated at 24 ± 1°C, pH 7.5 ± 0.1 under the saturation oxygen condition with a 12 h light: 12 h dark cycle. During the assimilated period, mussels were fed with algae *Spirulina* sp., which was cultured in the laboratory of the Faculty of Aquaculture in Vietnam National University of Agriculture. Three initial mussels were preliminarily analyzed by MPs and counted MPs less than 2.31 items per individual.

2.2. Experiment design

Three treatments were set up including: (1) sulfamethoxazole (30 µg L^{-1}) + PP (10 mg L^{-1}) (SMX + PP), (2) oxytetracycline (20 µg L^{-1}) + PP (10 mg L^{-1}) (OTC + PP), (3) sulfamethoxazole (30 µg L^{-1}) + oxytetracycline (20 µg L^{-1}) + PP (10 mg L^{-1}) (SMX + OTC + PP). The tested concentrations of SMX and OTC were chosen according to the findings of the environmental concentrations of those antibiotics from the previous studies [18, 19, 31]. The control treatment without adding any PP microplastics or SMX/OTC was also prepared. Throughout the investigation, contact with laboratory plastics was eliminated except for the recirculation system which could not be avoided. The duration of experiment was 30 days and mussels were daily fed with algae *Spirulina* sp. at 10 o'clock, with the ratio of 1.0 % of mussel biomass for each tank. All treatments and control were conducted with triplicate and three mussels were cultured in each replicate during the experiment.

2.3. Microplastic extraction in organism

To extract the concentration of MPs inside freshwater mussels, we extracted MPs based on the method of Zhou *et al*. [28]. However, there were some modifications to optimize the digestion of our samples. About 30 - 50 mL solution of saturated KOH and NaClO with a ratio of 1:1 was added to samples depending on their weight and then placed into the incubator at 50 °C for 48 h to digest the organic matter. To make sure that organic matter was totally digested, about 1 - 2 mL H_2O_2 30 % was added to samples based on their weight for 24 h at 50 °C. This step can be repeated until the organic matter has been completely removed. After digestion, the sample was transferred to a sieve using 100 µm mesh size into a beaker. Saturated NaCl (density 1.18 g/cm^3) was used to float the microplastic from digestion samples. Microplastics were filtered using a cellulose nitrate membrane with pore size of 5 µm. Finally, the filtered membranes were observed under the Leica stereomicroscope (Model: DMIL LED, S/N 534699) to identify MPs that accumulated by mussels.

2.4. Biomarkers analysis

After 30-day exposure of freshwater mussels, two biomarkers of micronucleus and DNA damage were analyzed in the tissues of gills and GIT. In order to perform those biomarkers, cells were separated from freshwater mussel's gill and gastrointestinal tract (GIT) tissues according to the previous method of Mai *et al*. [32]. Dissociation solution of MEM (Minimum Essential Medium – Thermo Fisher Scientific) with Dispace II (Sigma Aldrich) 1.55 mg/L was prepared to dissociate the cells from gill and GIT tissues of mussel. We grinded the gill or GIT tissues with 1 mL of MEM solution only in an Eppendorf microtube 2 mL. Following, 1 mL dissociation solution was added and incubated at 37 °C for 45 minutes and shaking at 150 rpm. Eppendorf microtubes were centrifuged at 1000 rpm at room temperature for 10 mins to stop the dissociation process. After the dissociation process, 20 µL of cell suspension was check cell viability with 20 µL of Trypan blue (Sigma Aldrich) using Leica inverted microscope (Model: DM2500 LED, S/N 530676). Acceptable cell viability rate was 90 %.

The micronucleus test is to detect the micronucleus which breaks from the nucleus of a cellular. The method to determine the micronucleus was referred from the previous study [33], with modification of some steps to validate the right protocol in our laboratory. Briefly, we prepared a fixer solution (Carnoy mixture solution) using methanol and acetic acid with a ratio of 4:1 to fix the cells on the slide. 50 µL live-cell solution was mixed with 100 µL of Carnoy mixture solution and immediately transferred 50 µL of cell suspension onto the slide. All slides were left for drying under the fume hood for 24 hours. Micronucleus was observed using a Leica fluorescence microscopy (Model: DMIL LED Fluo, S/N 530875) after dying with 40 µL of acridine orange 0.003 %. The appearance frequency of micronuclei was calculated per 1,000 cells of each slide. Micronucleus was discovered as round, distinct from the main nucleus, its size was less than one-third of the main nucleus and has a green color similar to the main nucleus [33]. Cells that were stacked or stained orange-red were excluded and not counted.

DNA damage was analyzed through the neutral comet assay (CometAssay® HT – Trevigen®), which followed the protocol with Catalog number 4252-040-K, to determine the Tail DNA of cells after exposure to the toxicants. Low melting point agarose (LMPA) in a beaker of boiling water for 5 minutes at 37 °C water bath. Cells at $1x10^5$/mL were mixed with molten LMPA at a ratio of 1:10 and immediately transferred 30 µL mix solution onto 2 wells CometSlide™. Following the slides were placed at the cool temperature (2 – 8 °C) under the dark condition for 15 - 30 mins to adhere samples onto CometSlide™. The slides were then immersed in a pre-chilled Lysis solution at 4°C for 60 mins. After finishing Lysis step, CometSlide™ were gently immersed in 1X neutral electrophoresis buffer for 30 minutes at 4°C. For the CometAssay® ES tank, 950 ml prechilled 1X Neutral electrophoresis buffer was added into the tank and slides were placed in electrophoresis slide tray and covered with Slide Tray Overlay. The power supply for electrophoresis was set at 300 mA, 21V for 60 minutes at 4 °C, which allowed for better DNA migration and longer comet tail in DNA. Following, slides were immersed in DNA Precipitation Solution for 30 minutes at room temperature. The slides were then immersed in 70 % ethanol for 30 minutes at room temperature and then dried slides at < 45 °C for 15 minutes. 50 µL ethidium bromide 1X stock was used to color each well of slices for 30 minutes in a dark environment. All slices were observed under Leica fluorescence microscopy (Model: DMIL LED Fluo, S/N 530875) with x1000 magnification and an image analysis system (Comet Analysis Software: Cat# 4260-000-CS). DNA damage was expressed as a percentage of ''Tail DNA'', which was the percentage of total DNA that has migrated from the head [34]. A hundred randomly selected nucleoids were analyzed on one gel of each slide.

2.5. Data analysis

Statistical analyses were performed with SPSS 16.0. Data are indicated as mean ± standard error (SE). The normality of the data distribution was tested on data residues using the Shapiro-Wilk test ($p < 0.001$). Variance homogeneity was evaluated using the Levene test ($p < 0.05$). In cases of homogenous variance and normalized data, one-way ANOVA was performed, followed by the Tukey Post hoc test at $p < 0.05$. Furthermore, principal component analysis (PCA), based on KMO and Bartlett's Test, were conducted for each exposure condition to assess the relative involvement of the studied variables.

3. RESULTS AND DISCUSSION

3.1. Microplastic accumulation in mussels

In this study, thirty-six *H. cumingii* mussels accumulated PP microplastic in their gill and digestive system. The number of PP microplastics ingested or filtered by mussels varied amongst exposure conditions (Figure 1). Mussels exposed to microplastics and mixing antibiotics (SMX + OTC + PP) had highest number of PP microplastic in gill (15.4 item per individual) and GIT (6.7 items per individual), followed by co-exposure of OTC + PP (9.6 items per individual in gill and 4.1 items per individual in GIT), and co-exposure of SMX + PP (5.1 items per individual in gill and 1.0 items per individual in GIT). Interestingly, there was no significant difference in microplastic accumulation in gill and GIT between co-exposure SMX + PP and control treatment ($p > 0.05$). In contrast, microplastic accumulations in gill and GIT of mussels co-exposed to OTC + PP or SMX + OTC + PP were significantly higher than in gill and GIT of mussels co-exposed to SMX + PP ($p < 0.05$). This indicated that microplastic accumulation increased in gill and GIT of mussels *H. cumingii* exposed to SMX + PP with the addition of OTC antibiotic. The relatively highest PP microplastic abundance in gill and GIT of mussels exposed to mixing antibiotics and PP microplastics (SMX + OTC + PP) might be attributed to the co-existence of mixing pollutants in the environment, which might disturb the process related to detoxification and biotransformation of xenobiotics [30]. It is interesting to note that microplastic in the gill of *H. cumingii* is considerably higher than that recorded in the GIT of this mussel. Results from this study could provide further evidence of the filtration and ingestion of microplastics by a filter-feeding species like *H. cumingii* in the freshwater environment. We believe the interaction of mussel *H. cumingii* feeding behaviour and the joint toxicity of co-exposure to three contaminants (SMX + OTC + PP) in this study may have contributed to such a high incidence of filtration and ingestion of PP microplastics accidentally. Because *H. cumingii* are filter-feeding bivalve species and it has been suggested that *H. cumingii* may have filtered and ingested PP microplastics mixed with algae *Spirulina* sp. when foraging the food in the environment.

We performed the micronucleus assay to test the toxicity of PP microplastic in combination with antibiotics for the ability to form micronucleus, which is the result of a chromosome or a chromosome fragment not being incorporated into one of the daughter nuclei during cell division. Indeed, micronuclei usually is a sign of genotoxic events and chromosomal instability and is commonly seen in cancerous cells [35].

The results of this study showed that the presence of PP microplastics and antibiotics adversely affected gills and GIT of mussels through forming the micronucleus, which were clearly visible with a microscope. However, there was no significant difference in forming the

micronucleus in gill and GIT cells of *H. cumingii* mussels when they were exposed to PP microplastics in combination with single antibiotic SMX or OTC ($p > 0.05$). In the meanwhile, frequency of micro-nucleated cells was significantly higher in gill and GIT cells for mussels co-exposed to PP microplastics and mixing two antibiotics (SMX + OCT + PP) ($p < 0.05$) than for mussels exposed to PP in combination with single antibiotic only (SMX + PP or OTC + PP). This suggests that SMX + OTC + PP co-exposure induced the synergistic toxicity effects related to single/double DNA strand breaks and structural and/or numerical chromosomal damage (Figure 2).

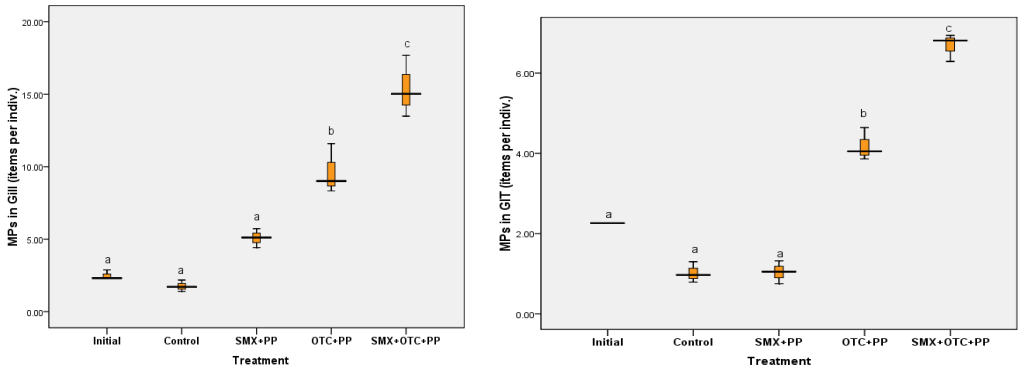

Figure 1. Polypropylene accumulation in gill and gastrointestinal tract of freshwater pearl mussels after 30-day co-exposed to antibiotics and polypropylene. Different letters denoted the significant differences between exposure conditions. The significant level is at $p < 0.05$ (ANOVA, Tukey'test).

3.2. Biomarker responses

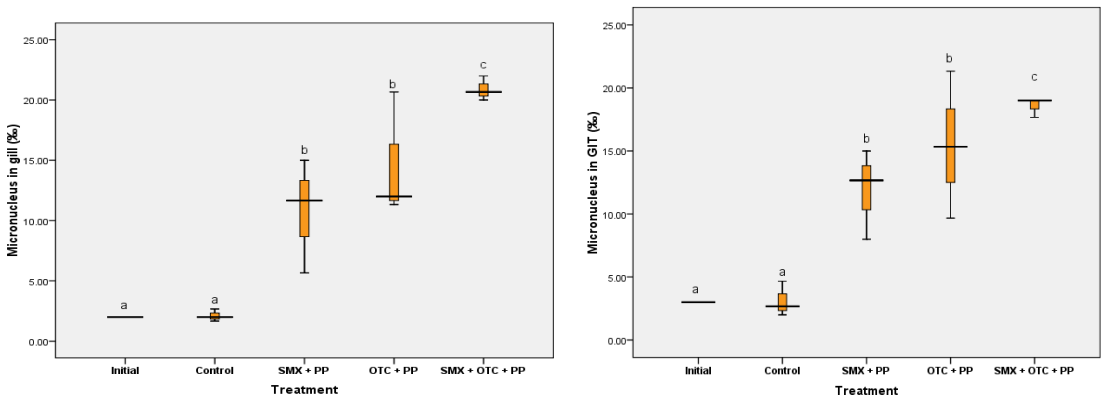

Figure 2. Micronucleus (‰) in gill and gastrointestinal tract of freshwater pearl mussels *H. cumingii* after 30-day co-exposed to antibiotics and polypropylene. Different letters denoted the significant differences between exposure conditions. The significant level is at $p < 0.05$ (ANOVA, Tukey'test).

The results of this study showed that the presence of PP microplastics and antibiotics adversely affected gills and GIT of mussels through forming the micronucleus, which were clearly visible with a microscope. However, there was no significant difference in forming the micronucleus in gill and GIT cells of *H. cumingii* mussels when they were exposed to PP microplastics in combination with single antibiotic SMX or OTC ($p > 0.05$). In the meanwhile, frequency of micro-nucleated cells was significantly higher in gill and GIT cells for mussels co-

exposed to PP microplastics and mixing two antibiotics (SMX + OCT + PP) ($p < 0.05$) than for mussels exposed to PP in combination with single antibiotic only (SMX + PP or OTC + PP). This suggests that SMX + OTC + PP co-exposure induced the synergistic toxicity effects related to single/double DNA strand breaks and structural and/or numerical chromosomal damage (Figure 2). It can be hypothesized that the process of cell division of the genetic material between the two daughter cells is disrupted because more chromosomes are broken or damaged by SMX + OTC + PP co-exposure. Therefore, more chromosomes may fail to be included in either of the two daughter nuclei, consequences of the genetic material that is not incorporated into a new nucleus may form its own "micronucleus". More study is necessary to better understand the mechanisms of forming the micronucleus in daughter cells after exposure to mixing pollutants in the environment.

Significant variations in DNA damage were observed for exposed mussels to PP microplastics in combination with single or mixing antibiotics (SMS and/or OTC) (Figure 3). After co-exposure to PP microplastics and antibiotics, a significant increase in DNA damage was detected in gill and GIT cells of freshwater pearl mussels ($p < 0.05$). This was suggested by high DNA breaks increased in the presence of single or mixing antibiotics combined with microplastics. Interestingly, we also observed that DNA was more damaged in cells of gill and GIT at co-exposure of PP with only OTC antibiotics and PP mixing two antibiotics SMX and OTC. DNA strand breaks reached 8.7 % in gill cells and 7.9 % in GIT cells compared to 2.1 % for gill and 1.5 % for GIT in the control ($p < 0.05$). It can be hypothesized that OTC antibiotics adsorbed onto the surface of PP microplastics were mainly responsible for the high toxicity to mussels, while little toxicity was detected to mussels after exposure to PP microplastic in combination with antibiotic SMX. The presence of OTC in combination with microplastics has been shown to be associated with alteration in the antioxidant enzyme activities such as increased superoxide dismutase (SOD) activity and decreased catalase (CAT) activity [7, 25]. These alterations represent a defense mechanism against oxidative stress and damage, which are known to cause DNA breaks [36]. Furthermore, antibiotic OTC was proved more toxic to aquatic species than antibiotic SMX [25, 37]. Therefore, this would explain why more adverse effects of PP microplastics with the addition of antibiotic OTC on DNA single/double strand were observed in gill and GIT cells of mussel *H. cumingii* than PP microplastics in combination with antibiotic SMX. To the best of our knowledge, there are no reports mentioning the effect of mixing antibiotics SMX and OTC in combination with PP microplastics.

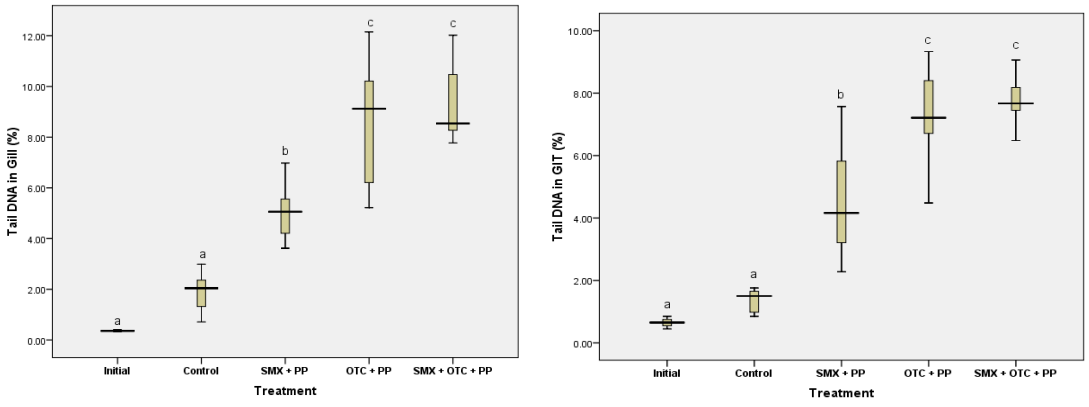

Figure 3. Tail DNA (%) in gill and gastrointestinal tract of freshwater pearl mussels *H. cumingii* after 30-day co-exposed to antibiotics and polypropylene. Different letters denoted the significant differences between exposure conditions. The significant level is at $p < 0.05$ (ANOVA, Tukey'test).

Recently, Li *et al*. [30] have documented the co-effects of single antibiotic SMX and nano-plastic polystyrene (PS) on the intestinal health and gene expression of medaka juvenile *Ozyzias melastigma*. These authors proved that PS might enhance the toxicity of SMX on the medaka intestine due to increased abundance of Proteobacteria that can cause damage to the intestinal epithelium. Another study of Yu *et al*. [29] indicated that binary-combined micro- and/or nano-plastic polystyrene (PS) and OTC increased the intestinal epithelial damage and diversity microbiome in the gut of zebrafish (*Danio rerio*). However, this study revealed that comet and micronucleus assays can provide significant insights into toxicity synergistic interaction between those contaminants for gill and GIT tissue of freshwater pearl mussels after exposure to PP microplastics in combination with antibiotics (SMX and OTC). Further data like biochemical and protein profiles are required to achieve a more comprehensive understanding on the molecular mechanism of microplastics and mixing antibiotics toxicity on freshwater pearl mussels.

3.3. PCA analysis

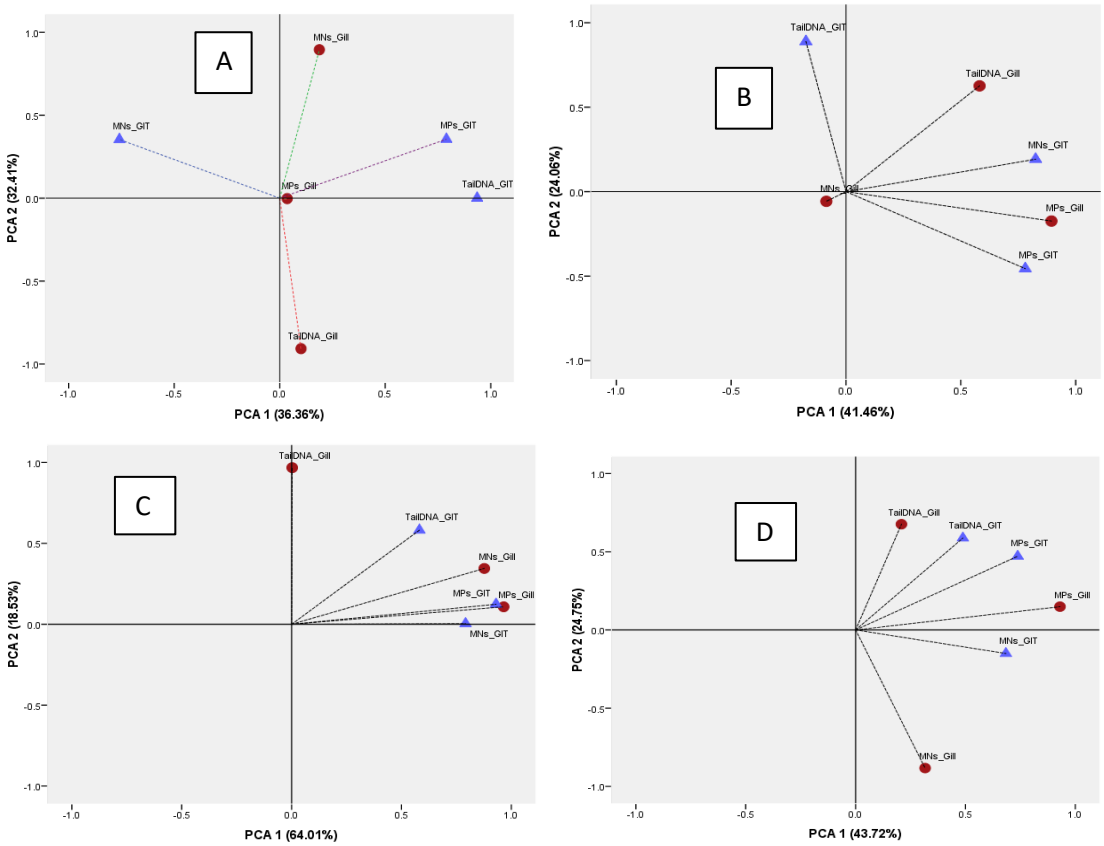

Figure 4. PCA represents the normalized coefficients on the three components for 6 variables: four biomarker variables (TailDNA_GIT = % DNA damage in gastrointestinal tract, TailDNA_Gill = % DNA damage in gill, MNs_Gill = micronucleus in gill, MNs_GIT = micronucleus in gastrointestinal tract, and two microplastic bioaccumulation variables (MPs_GIT = microplastic in gastrointestinal tract, MPs_Gill = microplastic in gill). (A) no exposure of neither antibiotics nor PP microplastics; (B) co-exposure of SMX + PP; (C) co-exposure of OTC + PP; (D) co-exposure of SMX + OTC + PP; blue triangle: variables represent for GIT tissues; red circle: variables represent for gill tissues.

More extensive and specific data are required to elucidate the potential ecotoxicological impacts to freshwater pearl mussels *H. cumingii* under the co-exposure of mixing antibiotics and PP microplastics. The correlation between the PP microplastic accumulation in gill and GIT with biomarker responses (Tail DNA and micronucleus) can be observed from the PCA analysis in Figure 4. The results showed that there was no correlation between microplastic accumulation in gill and GIT in the mussels exposed to neither environment without PP microplastics and any antibiotics (Figure 4A) nor environment to be added PP microplastics and antibiotic SMX (Figure 4B). According to the study of Qu *et al*. [28], the digestion glands of mussel *Mytilus galloprovincialis* showed higher tolerance to SMX. Li *et al*. [11] reported that SMX significantly decreased the toxicity of PP to marine algae *Skeletonema costatum* because of the effect from MPs adsorption. However, microplastic accumulation in GIT presented a correlation with DNA damage and micronucleus in the cells of GIT tissues for mussels *H. cumingii* exposed to PP microplastic in combination with antibiotic OTC, whereas microplastic accumulation in gill was positively correlated to micronucleus, but moderately correlated to DNA damage (Figure 4C). Microplastic accumulation, DNA damage and micronucleus exhibited significant contribution to the first component (PCA 1) of the PCA, which accounted for 64 % of the total variance within samples of gill and GIT cells of mussels *H. cumingii.* Interestingly, when mussels exposed to the mixed two antibiotics SMX and OCT with PP microplastics, PP accumulation in GIT tissue strongly correlated to DNA damage, and moderately correlated to DNA damage for gill cells was observed, whereas, there was no correlation between microplastic accumulation and forming micronucleus in gill and GIT cells of mussel *H. cumingii* (Figure 4D). Therefore, the relationships between microplastic accumulation and DNA damage were mainly observed in mussels exposed to PP microplastic with the addition of antibiotic OTC. Previous studies documented the size-dependent effects of single or co-exposure of PS nano- or micro-plastic and antibiotic OTC on histological damage, intestinal epithelial damage [7, 29]. This first observed correlation between microplastic accumulation in gill and GIT tissues of freshwater pearl mussels with biomarkers of DNA damage and/or micronucleus can highlight the necessary further work to obtain a deeper understanding on the relationships between the microplastic uptake/ingestion with damages of genetic materials for mussel *H. cumingii* when antibiotics present in aquatic environment.

4. CONCLUSION

In this study, co-exposure of PP microplastics and mixing antibiotics (SMX and OTC) significantly increased the accumulation of PP in the freshwater pearl mussels *H. cumingii*. The results showed that *H. cumingii* are likely extremely vulnerable to microplastic pollution in combination with mixing antibiotics and that OTC appears to be a major factor contributing to the toxicity impact in terms of DNA damage and micronucleus to the freshwater pearl mussels. Moreover, the results also indicated that the damages in DNA and forming micronucleus in the gill and GIT tissues of *H. cumingii* had a positive correlation with ingestion of microplastics in environments contaminated with antibiotics, making *H. cumingii* as a candidate species for monitoring microplastics and antibiotics in freshwater systems. Further research is needed to determine the mechanism responsible for the toxicity impact at molecular levels of co-exposure to multi-toxicants of *H. cumingii*.

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Credit authorship contribution statement. This manuscript was written through the contributions of all authors. Nguyen Ngoc Tuan: conceptualization, writing the manuscript, methodology, and statistical data analysis. Nguyen Danh Thien, Pham Gia Minh Tu: conducting the ecotoxicology experiment with freshwater clam and analysis of MP, comet assay and micronucleus test. Mai Huong: conceptualization, writing-review and editing of the manuscript contents.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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