



Combined toxic effects of nanoplastics and norfloxacin on mussel: Leveraging biochemical parameters and gut microbiota

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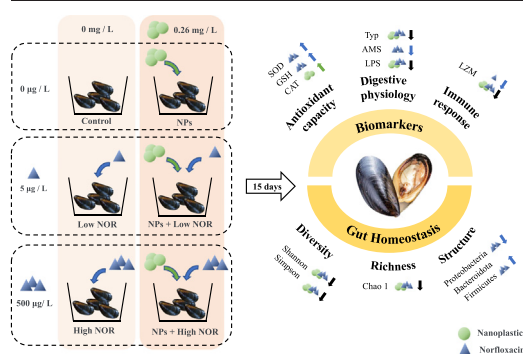
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HIGHLIGHTS

- Nanoplastics alone significantly inhibited SOD and AMS activity in mussels.
- Nanoplastics or norfloxacin increased CAT and GSH but co-exposure decreased Typ.
- Nanoplastics or norfloxacin reduced the richness and diversity of gut microbiota.
- SOD and AMS activities were correlated with COG functions of gut microbiota.
- The dual hazards of antibiotics and nanoplastics warrant more investigations.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics and nanoplastics (NPs) are among the two most concerned and studied marine emerging contaminants in recent years. Given the large number of different types of antibiotics and NPs, there is a need to apply efficient tools to evaluate their combined toxic effects. Using the thick-shelled mussel (*Mytilus coruscus*) as a marine ecotoxicological model, we applied a battery of fast enzymatic activity assays and 16S rRNA sequencing to investigate the biochemical and gut microbial response of mussels exposed to antibiotic norfloxacin (NOR) and NPs (80 nm polystyrene beads) alone and in combination at environmentally relevant concentrations. After 15 days of exposure, NPs alone significantly inhibited superoxide dismutase (SOD) and amylase (AMS) activities, while catalase (CAT) was affected by both NOR and NPs. The changes in lysozyme (LZM) and lipase (LPS) were increased over time during the treatments. Co-exposure to NPs and NOR significantly affected glutathione (GSH) and trypsin (Typ), which might be explained by the increased bioavailable NOR carried by NPs. The richness and diversity of the gut microbiota of mussels were both decreased by exposures to NOR and NPs, and the top functions of gut microbiota that were affected by the exposures were predicted. The data fast generated by enzymatic test and 16S sequencing allowed further variance and correlation analysis to understand the plausible driving factors and toxicity mechanisms. Despite the toxic effects of only

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one type of antibiotics and NPs being evaluated, the validated assays on mussels are readily applicable to other antibiotics, NPs, and their mixture.

1. Introduction

Plastic production is fast-increasing (Kurniawan et al., 2021) and large amounts of plastic waste end up in marine ecosystems (Andrady, 2011; Wayman and Niemann, 2021). In natural environments, plastics are broken down by mechanical (e.g., waves), chemical, UV, or biological factors into smaller pieces, named microplastics (MPs, <5 mm) (Thompson et al., 2004; Thompson et al., 2009). Microplastics have been found in various marine environments, e.g., Antarctica and the Southern Ocean (Cunningham et al., 2020), the Pacific Ocean (Ding et al., 2022), Southern Caspian Sea Coasts (Mataji et al., 2020), and Southern Mediterranean coasts (Missawi et al., 2020). Numerous studies have shown the uptake or negative effects of microplastics on marine organisms, e.g., invertebrates (Wang et al., 2020b), fish (Feng et al., 2019), whale (Besseling et al., 2015), and turtle (Nelms et al., 2016). Nanoplastics (NPs, <1000 nm), which are several orders of magnitude smaller than microplastics, have been shown more easily ingested and enriched in aquatic organisms (Mattsson et al., 2015; Alimi et al., 2018), causing generally higher toxicity than microplastics across different taxonomic groups (Matthews et al., 2021). For example, it was shown that exposure to 50 nm NPs resulted in significant membrane damage in *Proteobacterium Halomonas alkalicola*, while 1 μ m microplastics led to moderate damage (He et al., 2018). The acute toxicity of NPs to mussels (*Mytilus* spp.) was related to the enhanced immune response and oxidative stress (Cole et al., 2020).

In addition to the direct effects of MPs and NPs, previous studies also showed their carrying potential for other emerging contaminants (Alimi et al., 2018; Bhagat et al., 2021). Among many different types of emerging contaminants, antibiotics are one of the most concerned groups, due to their high biological activity even at low environmental concentrations (Tang et al., 2019; Iwu et al., 2020). Antibiotics are a commonly used class of drugs that can be used as effective agents to kill infectious bacteria, nevertheless, overuse of antibiotics will lead to bacterial resistance in the host (Silbergeld et al., 2008; Makary et al., 2018). Residual antibiotics in the natural environment may also affect the normal functions of environmental organisms (Ojemaye and Petrik, 2019). For example, norfloxacin (NOR), a fluoroquinolone antibiotic, can act on pathogenic DNA gyrase and hinders bacterial DNA replication (Mathur et al., 2021). NOR is widely used in agriculture, medicine, and other fields, and has been detected in natural water and wastewater (Giger et al., 2003; Costanzo et al., 2005; Tong et al., 2011) and marine species like oysters (He et al., 2019). In a laboratory experiment, short-term exposure to NOR resulted in the homeostasis imbalance of intestinal flora of juvenile large yellow croaker *Pseudosciaena crocea*, which affected immunity, inhibited metabolism and growth, and even led to death (Wang et al., 2020a). Environmental residues of NOR were immunotoxic to the mussel *Elliptio complanata*, inducing 10 % mortality, and loss of blood cell viability after 14 days of exposure (Gagne et al., 2012). The occurrence of both antibiotics and plastic particles has been detected in various marine environmental samples (Zheng et al., 2021; Goncalves and Bebianno, 2021). Because of the special characteristics of plastics surface, both MPs and NPs are considered carriers of emerging contaminants antibiotics, modifying their bioavailability and toxicity (Hermabessiere et al., 2017; Amelia et al., 2021). The adsorption ability of NOR on the surface of NPs has been reported, which may be attributed to the hydrophobic interaction (Zhang et al., 2020).

Given the co-occurrence of different types of emerging contaminants and anticipated differences in their toxicity and environmental impacts, there is a need to establish and apply an efficient toxicological system to evaluate the potential adverse effects of emerging contaminants alone and in combinations. The marine mussels (*Mytilus* spp.) are filter-feeding bivalves that are known to readily accumulate micropollutants from the

environment, such as trace metals (Stankovic and Jovic, 2012), antibiotics (Le Bris and Pouliquen, 2004), plastic particles (Hanna et al., 2014), etc. Compared with other species, mussels are highly adaptable to environmental conditions with a relatively high tolerance to environmental contaminants (Lacroix et al., 2015). There is a growing use of mussels as indicators of marine environmental pollution and toxicological model organisms (Li et al., 2016; Pastorino et al., 2021). Moreover, it has been found that the gut microbiota of mussels is closely related to the immune system, metabolic functions, and overall health status of mussels (D'Aversa et al., 2013; Yoo et al., 2020), complementary to the conventional direct toxicological endpoints (e.g., survival rate, reproduction, and biochemical parameters) in mussels.

Due to its advantage of high throughput, cost-effectiveness, and high precision, 16S rRNA sequencing has been widely applied in basic research (Di Bella et al., 2013; Langille et al., 2013; Douglas et al., 2018). The continued development and refinement in sequencing techniques and computational ability make 16S rRNA sequencing a promising and advanced tool for exploring and characterizing complex microbial communities (Johnson et al., 2019; Wensel et al., 2022). The objective of this study was to combine commercially available enzymatic activity assays and gut microbiota 16S sequencing to evaluate the interactive effects of NPs and NOR on *Mytilus coruscus*. Changes and associations in antioxidant enzymes, digestive enzymes, and gut microbiota were quantified and compared between *M. coruscus* treated by NPs, NOR, and their combination. Our study improves the understanding of the combined toxicity of polystyrene NPs and NOR in mussels and offers a set of validated toxicological assays for future evaluation of other types of NPs and antibiotics.

2. Materials and methods

2.1. Mussels

The thick-shelled mussels were collected from Gouqi Island, Shengsi County, Zhoushan City, Zhejiang, China (N30°43'1.64", E122°46'3.25") in December 2020. Once the mussels were transported to the lab, they were washed to remove the mud and scraped off any attached organisms on the surface. Five hundred mussels were cultured for 7-day acclimatization and checked twice a day to remove dead mussels. Before experiments, mussels of similar size (length: 77.1 ± 2.4 mm) were transferred to 21 clean tanks (20 L). Each tank contained 15 L of fully aerated artificial seawater and 20 mussels. The tank conditions were set as below: salinity (25 psu), temperature (20 °C), pH 8.1, dissolved oxygen >6 mg·L⁻¹, 12 h/12 h dark/light cycle. Mussels were fed daily with microalgae *Chlorella vulgaris* (5×10^4 cells/mL) twice. Aerated artificial seawater was daily renewed 2 h after feeding.

2.2. Chemicals and nanoplastics

Norfloxacin (NOR, purity >98 %, CAS number: 70458-96-7) and dimethyl sulfoxide (DMSO, AR, CAS number: 67-68-5) were purchased from Shanghai McLean Biochemical Technology Co., LTD. Nanoplastics (polystyrene nanobeads, size: 80 nm, maximum excitation wavelength: 470 nm, maximum emission wavelength: 526 nm) were purchased from Tianjin Bessler Co., LTD. Scanning electron microscope (SEM) and micro-Fourier transform infrared spectroscopy (m-FTIR) were used to characterize the size and polymer composition of the NPs (Fig. S1). Ultrasonic oscillation was used to suspend and prevent the agglomeration of NPs. DMSO was used as the solvent of NOR (final 0.01 % v/v in 15 L exposure solution), which followed the OECD guideline (OECD, 2002). NOR exposure solution was prepared at two concentrations (5 μ g·L⁻¹ and 500 μ g·L⁻¹). 5 μ g·L⁻¹

represented a high concentration detected in the coastal environment (Zou et al., 2011), and $500 \mu\text{g}\cdot\text{L}^{-1}$ was chosen as a biological effective concentration to understand toxicity mechanisms on mussels.

2.3. Exposure design

The 15-day exposure consisted of 7 groups, and each group included 3 replicated tanks with 20 mussels in each tank. The 7 groups were: (1) DMSO solvent control group (0.01% v/v); (2) Seawater control group; (3) Low concentration NOR group ($5 \mu\text{g}\cdot\text{L}^{-1}$); (4) High concentration NOR group ($500 \mu\text{g}\cdot\text{L}^{-1}$); (5) NPs group ($0.26 \text{ mg}\cdot\text{L}^{-1}$, based on two previous studies, which was used as an exposure concentration to simulate realistic levels of environmental pollution) (Barboza et al., 2018; Zhou et al., 2020); (6) Low concentration combined group: NOR ($5 \mu\text{g}\cdot\text{L}^{-1}$) + NPs ($0.26 \text{ mg}\cdot\text{L}^{-1}$); (7) High concentration combined group: NOR ($500 \mu\text{g}\cdot\text{L}^{-1}$) + NPs ($0.26 \text{ mg}\cdot\text{L}^{-1}$).

2.4. Enzyme activity assay

On days 5, 10, and 15, 2 mussels were taken from each tank (i.e., 6 mussels in each treatment or control group). The digestive glands of each mussel were collected to detect the activity of digestive enzymes and gut microbiota, and the gills were dissected to detect the activity of antioxidant and immune enzymes. The digestive glands and gills were removed into micro tubes and immediately frozen in liquid nitrogen. 0.1 g of tissue was weighted, and normal saline was added in a ratio of mass: volume (mL) = $1:9$; the mixture was bathed on ice, and then centrifuged at 4°C , 2500 g for 10 mins ; and the tissue homogenate was stored at -80°C .

The activities of 7 enzymes including lipase (LPS), trypsin (Typ), amylase (AMS), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and lysozyme (LZM) were measured by commercial kits (Nanjing Jian Cheng Bioengineering Research Institute, Nanjing, China). The optical density values were determined with a microplate reader (FlexStation 3, Molecular Devices, USA). The total protein content of the tissue sample

was determined by the Coomassie brilliant blue staining method (Bradford, 1976).

2.5. Gut microbiota analysis

The sequencing and data analysis of the whole intestine samples on day 15 was completed at Shanghai Ouyi Biomedical Technology Co., LTD. Briefly, DNA was extracted using a DNA extraction kit, and the concentration of DNA was detected using agarose gel electrophoresis and NanoDrop2000. The corresponding regions for bacterial diversity identification were the 16S V3-V4 region (Primer 343F: $5'\text{-TACGGTAGGCAG CAG-3'}$ and Primer 798R: $5'\text{-AGGTATCTAATCCT-3'}$). Tks Gflex DNA Polymerase (Takara LTD) performs PCR to ensure efficiency and accuracy. PCR products were detected by electrophoresis and then purified by magnetic beads. After repeated purification, the samples were sent for sequencing.

Vsearch (Version 2.4.2) software was used for OTU classification of valid tags obtained from quality control with a similarity of 97% (Rognes et al., 2016). The sequence with the largest abundance in each OTU was selected as the representative sequence. Then, RDP Classifier Naive Bayesian classification test was used to compare and annotate the representative sequence with Silva(V138) database (Wang et al., 2007). A flower plot provided the cluster results of unique and common OTUs. Based on the OTUs data, the community richness of Chao1 and the diversity of Shannon and Simpson were calculated. Kruskal-Wallis test was applied to test the differences between diversity indices.

The PICRUST predicted metagenomes based on 16S rRNA gene sequencing data and the cluster of orthologous groups of proteins (COG) database was used to predict the functional profiling of gut microbiota. COG clusters were generated by using the COGNITOR program and the abundance of COG functions was calculated according to the OTU abundance of the groups. According to the Kruskal-Wallis test, the predicted COG results were considered statistically different at $p < 0.05$.

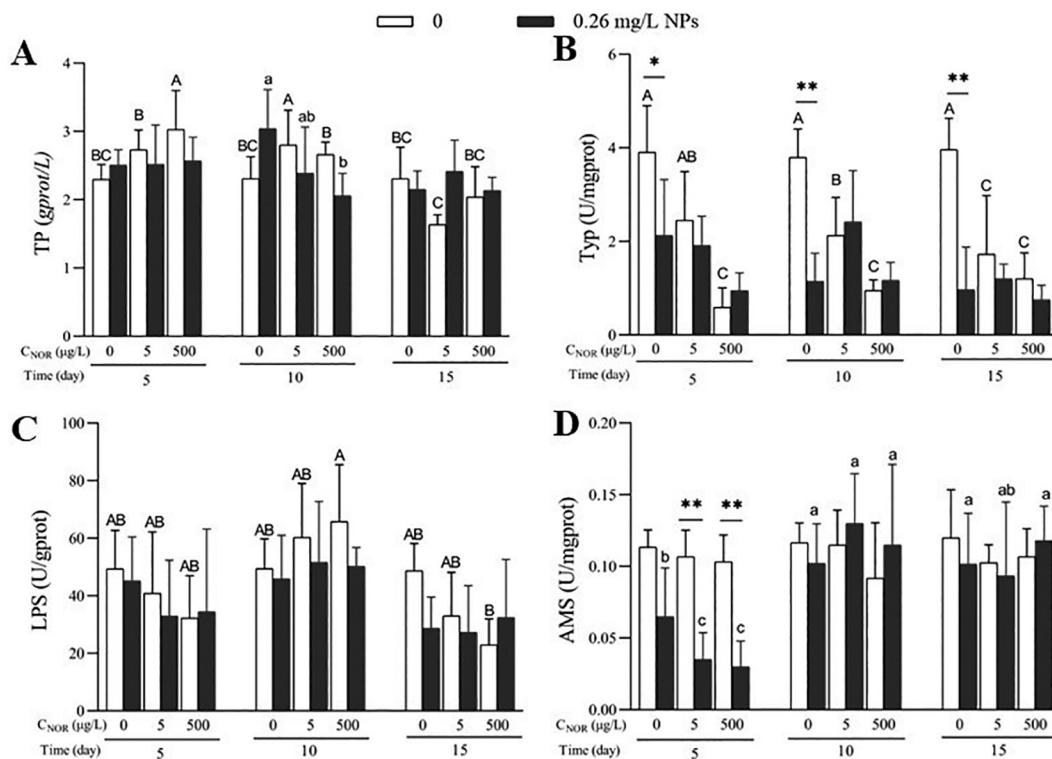


Fig. 1. The changes of total protein (A), Trypsin (B), Lipase (C), and Amylase (D) activity in the digestive glands of *M. coruscus* during 15-day exposure to NOR and NPs. Different capital letters indicate the significant difference of NOR group at each time point in the absence of NPs. Different lowercase letters indicate significant differences among time points within each NOR concentration in the presence of NPs. “*” indicates the significant difference between the presence and absence of NPs at the same NOR concentration and time point ($p < 0.05$), while “**” indicates a highly significant difference ($p < 0.01$).

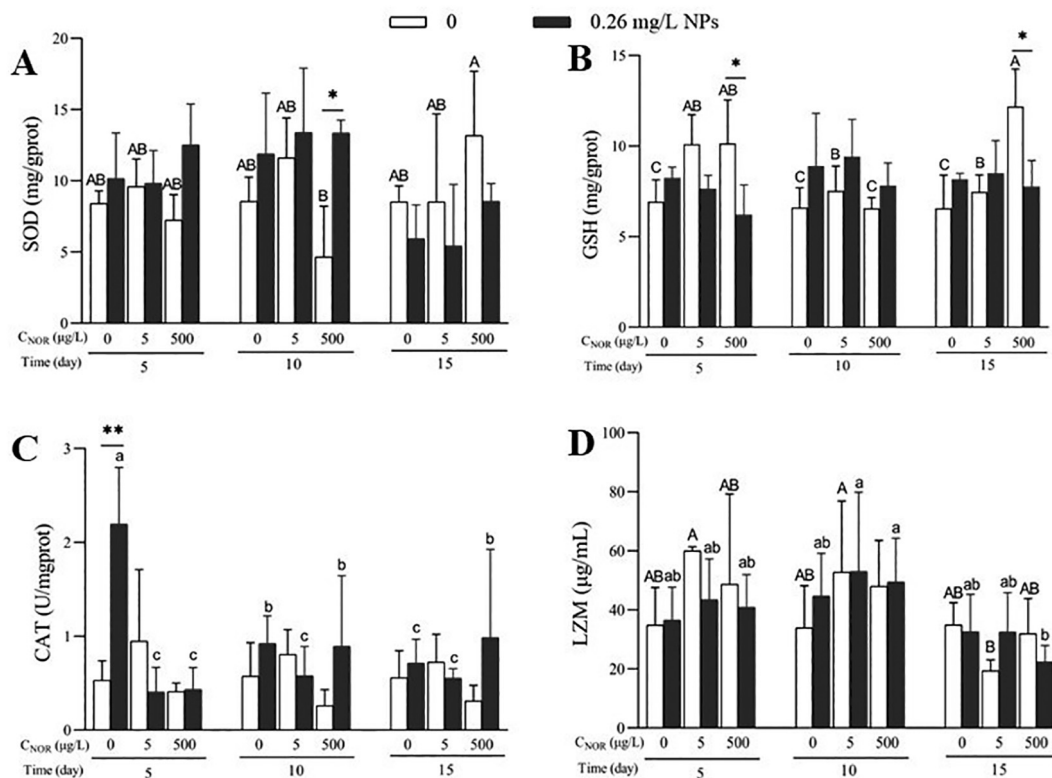


Fig. 2. The changes of Superoxide dismutase(A), Glutathione (B), Catalase (C), and Lysozyme (D) activity in the gills of *M. coruscus* during 15-day exposure to NOR and NPs. Different capital letters indicate the significant difference of NOR group at each time point in the absence of NPs. Different lowercase letters indicate significant differences among time points within each NOR concentration in the presence of NPs. “*” indicates the significant difference between the presence and absence of NPs at the same NOR concentration and time point ($p < 0.05$), while “**” indicates a highly significant difference ($p < 0.01$).

2.6. Statistical analysis

IBM SPSS Statistics 23 was used for statistical analysis. Normal distribution and the homogeneity of variance were checked, followed by two-way ANOVA. A three-factor ANOVA was used to evaluate the effects of NOR, NPs, time, and their interactions. Principal component analysis (PCA) was performed by integrating the 7 enzymatic activities (Typ, LPS, AMS, LZM, CAT, SOD, and GSH) and the 3 time points (day 5, 10, 15) in Origin Pro 2018C. GraphPad Prism was used to test the correlation between biomarkers and COGs. All data were presented in the form of mean $\pm$ standard deviation (means $\pm$ SD), with $p < 0.05$ (*) and $p < 0.01$ (**) indicating significant differences among groups.

3. Results

3.1. Responsive enzymatic activities

Fig. 1A shows the changes in total protein (TP). Compared with the control mussels, Typ was decreased by NOR or NPs exposure as early as on day 5 (Fig. 1B). The high concentration of NOR significantly reduced LPS activity on day 15 (Fig. 1C). Exposure to NPs and NOR resulted in a significant decrease in AMS activity with increasing NOR concentration on day 5. AMS activity was not significantly affected by NOR in the last five days (Fig. 1D).

On day 15, SOD and GSH activities in the NOR group increased significantly. The addition of NPs significantly increased the SOD activity in the high concentration NOR group on day 10 but significantly decreased the GSH activity on day 15 (Fig. 2A, B). NPs exposure alone resulted in a significant increase in CAT on day 5 and then a gradual decrease (Fig. 2C). LZM had a significant decrease in the low concentration NOR group and the high concentration combined group (Fig. 2D).

There were no significant differences between the DMSO solvent control group and the seawater control group (Table S1).

3.2. Gut microbiota changes

The seven experimental groups, i.e., the seawater control, solvent control, NPs, low concentration NOR, high concentration NOR, low concentration

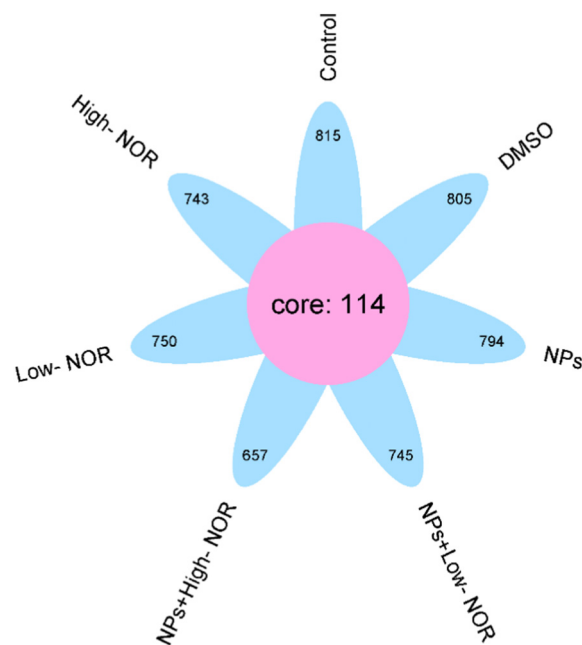


Fig. 3. Flower plot of OTUs. The number in the Core of the figure represents the OTUs common to all samples (Core OTUs), and the number on the petals represents the total OTUs of each sample minus the number of OTUs common to all samples.

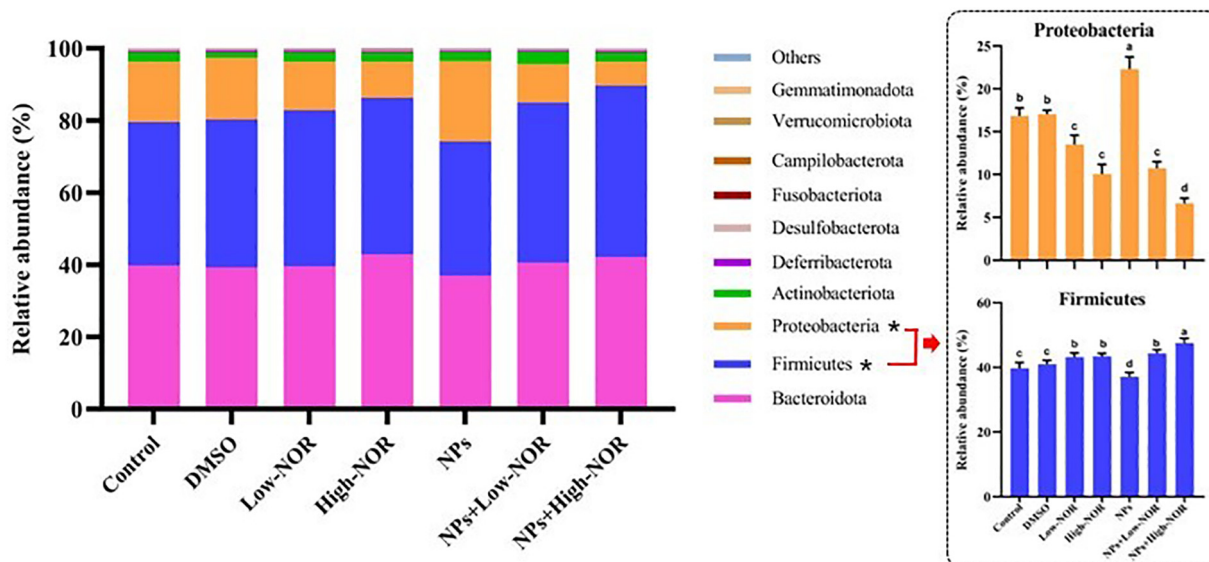


Fig. 4. The top 10 relative abundance of the gut bacteria in *M. coruscus* from different treatments at the phylum level.

combined, and high concentration combined group, contained 815, 805, 794, 750, 743, 745, and 657 OTUs, respectively, resulting in a total of 114 core OTUs (Fig. 3).

Fig. 4 shows the top 10 relative abundance of the gut microbial phyla. Proteobacteria, Bacteroidota, and Firmicutes were the three dominant species in the intestinal tract of mussels. Compared with the control groups, the relative abundance of Proteobacteria decreased with the increase in NOR concentration. On the contrary, in the NPs group, the relative abundance of Proteobacteria increased; when combined with NOR, it showed varying degrees of decrease.

The alpha diversity of the gut community was not significantly different between the seawater and the solvent control group. The community richness decreased with NOR, NPs, and combined exposure. Shannon and Simpson index also decreased in all treatment groups. The coverage indexes were >99 % in all groups (Table 1).

Furthermore, biological functions of the gut community affected by different treatments were predicted based on 16S sequencing data and Clusters of Orthologous Groups (COG). The three main affected processes were cellular processes and signaling, metabolism, and information storage and processing (Table S2). Functional groups COG 1132, 1309, 4753, 1653, and 0395 were predicted to be significantly enhanced by NPs, and such enhancement was more significant by high concentration NOR and combined exposure groups (Fig. 5).

3.3. Variance, correlation, and principal component analysis

Three-factor analysis of variance revealed that SOD and AMS activities were significantly affected by NPs exposure, while a significant interaction effect was detected for TYP. There was also an interaction effect under combined stress for GSH activity (Table S3).

The PCA combined 7 biomarkers and 3 sampling time points. PC1 explained 27.5 % of total variances, separating the NOR groups from groups

without NOR; PC2 explained 23.47 % of total variances, which separated the presence or absence of NPs groups (Fig. 6).

There was a significant positive correlation between SOD and a COG function, i.e., defense mechanisms ($r = 0.828$, $p = 0.004$, Fig. 7A), and AMS was significantly and negatively correlated with energy production and conversion ($r = 0.704$, $p = 0.018$, Fig. 7B).

4. Discussion

Superoxide dismutase (SOD) acts as a first-stage detoxification enzyme in cells to combat reactive oxygen species (Esposito et al., 1999). In the present study, SOD in the high concentration NOR group increased significantly on day 15. Continuous exposure to NPs may aggravate oxidative damage of cells, destroy the ability of the body to resist free radicals, and decrease the activity of SOD (Letendre et al., 2008). In another short-term study, exposure to 50 nm polystyrene NPs resulted in increased SOD activity in mussels after 24 h but decreased after 7 days (Cole et al., 2020). A chronic study of *Mytilus galloprovincialis* showed that exposure to 40 nm polystyrene NPs for 42 weeks reduced 65 % SOD activity than controls (Hamm and Lenz, 2021). Our results showed a significant interaction between NPs and the high NOR group on day 10, and NPs significantly stimulated SOD activity in the high NOR concentration group, suggesting SOD as a potential biomarker for NPs exposure in mussels.

Glutathione (GSH) plays important roles in maintaining intracellular homeostasis and scavenging free radicals, and a decrease in GSH activity is often accompanied by an increase in cell membrane peroxidation (Jozefczak et al., 2012). Our results showed that GSH was not significantly affected by the changes in NPs or NOR concentrations, but there was an interaction effect under combined exposure. In the high-concentration NOR + NPs group, the significant inhibition on GSH might be due to the increased bio-distribution of NOR in mussels carried by NPs, which leads to stronger cellular peroxidation, resulting in cell damage and inhibition.

Table 1

Summary of intestinal microbiota alpha diversity (Chao1, Shannon, Simpson) in *M. coruscus* under different treatment conditions. NPs, nanoplastics; NOR, norfloxacin.

Groups	OTUs	Chao1	Shannon	Simpson	Coverage
Control	929 ± 28.32 ^a	1397.99 ± 88.60 ^a	6.78 ± 0.0038	0.9479 ± 0.2221	99.57 ± 0.06 %
DMSO	919 ± 54.48 ^a	1359.22 ± 88.73 ^a	6.67 ± 0.1331	0.9487 ± 0.0063	99.55 ± 0.09 %
Low-NOR	864 ± 20.43 ^b	1284.08 ± 112.08 ^{ab}	6.55 ± 0.0233	0.9430 ± 0.0031	99.58 ± 0.01 %
High-NOR	857 ± 36.59 ^b	1260.63 ± 27.46 ^b	6.66 ± 0.2756	0.9437 ± 0.0103	99.54 ± 0.05 %
NPs	908 ± 54.24 ^{ab}	1275.77 ± 43.51 ^b	6.52 ± 0.1217	0.9423 ± 0.0046	99.55 ± 0.03 %
NPs + Low-NOR	859 ± 26.10 ^b	1229.34 ± 36.92 ^{bc}	6.46 ± 0.1240	0.9394 ± 0.0126	99.56 ± 0.04 %
NPs + High-NOR	771 ± 7.94 ^c	1065.43 ± 28.34 ^c	6.43 ± 0.0654	0.9393 ± 0.0025	99.61 ± 0.00 %

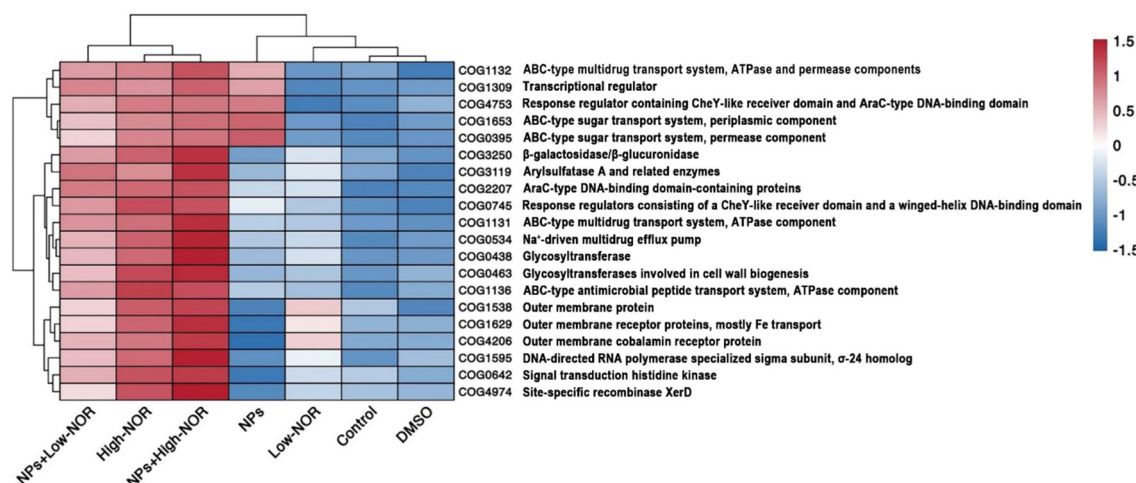


Fig. 5. The heatmap of 16S COG functions predicted from different treatments. Red indicates increased abundance and blue indicates decreased abundance.

Similarly, Guo et al. (2021) evaluated the effects of combined stress of 80 nm polystyrene NPs and ciprofloxacin (CIP) on *Corbicula fluminea* and found that the toxic effects of CIP were exacerbated by the presence of NPs. Also, co-exposure of three antibiotics, oxytetracycline (OTC), florfenicol (FLO), and sulfamethoxazole (SMX) with 500 nm polystyrene NPs inhibited GSH and down-regulated detoxification-related genes in thick-shelled mussels (Han et al., 2021). Catalase (CAT) is a common antioxidant enzyme present in most aerobic tissues, but CAT can damage cells at high concentrations (Ercal et al., 2001). We observed that the short-term NPs exposure led to a significant activation of CAT in mussels, showing a strong oxidative toxic effect, while the effects appeared to be alleviated over exposure time. Thus, it is speculated that chronic exposure to low concentrations of NPs may cause reversible oxidative damage as a sign of slow adaptation, which needs further investigations. Exposures to 50 nm or 70 nm NPs have also been reported to cause significant increases in CAT in other mussel studies (Wang et al., 2021; Capolupo et al., 2021). Taken together the results of CAT, SOD, and GSH, we proposed in general that under NOR and NPs exposures, oxygen free radicals and hydrogen

ions form hydrogen peroxide under the action of SOD, and the increase in SOD results in excessive hydrogen peroxide, requiring more CAT enzymes. Only the high NOR exposure group showed a significant increase in GSH, suggesting that more GSH was needed to remove the accumulated oxygen free radicals under higher NOR stress.

Trypsin (Typ) as a proteolytic enzyme, plays an important role in purification and anti-inflammation (Walsh et al., 1964). In the present study, Typ was found responsive to NPs or NOR exposure in mussels. Lipase (LPS) can hydrolyze triglycerides into glycerol and fatty acids that are converted, absorbed, and utilized by organisms (Layer and Keller, 2003). LPS was significantly reduced after 15-day exposure to the high concentration of NOR but no significant changes were found for NPs exposure. A recent study showed that different types, sizes, and concentrations of MPs did not affect LPS activity in *Mytilus galloprovincialis* (Trestrail et al., 2021). Amylase (AMS) is a general term for enzymes that hydrolyze starch and glycogen (van der Maarel et al., 2002). Our results showed that the AMS activity of mussels was significantly reduced within 5 days and gradually recovered, suggesting that the toxicity of NPs or NOR did not exceed the recovery capacity of mussels. Hypothetically, upon initial exposure to NOR or NPs, the stress response and insufficient energy supply of mussels decreased AMS activity, which gradually returned to normal levels over time. Also, the inhibited feeding of mussels (e.g., insufficient nutrients) under environmental stress conditions might be related to decreased digestive function and lower digestive enzyme activity.

Lysozyme (LZM) is an alkaline enzyme produced by animals that can hydrolyze mucopolysaccharides in pathogenic bacteria, and its antibacterial, anti-inflammatory, and antiviral effects form part of the innate immune system of the animal (Ho and Ellermeier, 2022). In the present study, when mussels were exposed to NOR, immune damage occurred and LZM activity increased. Continuous exposure to NOR could lead to a breakdown of the immune defense system of mussels. Similar immunotoxicity of NOR was also found in zebrafish (Liang et al., 2020). Another study reported that MPs caused immunotoxicity, accompanied by altered microbial profiles in juvenile *Eriocheir sinensis* (Liu et al., 2019).

Gut microbiota is the largest and most dynamic micro-ecosystem of living organisms (Sekiroy et al., 2010). Gut microbiota plays an important role in digestive physiology, metabolism, and immunity (Tremaroli and Backhed, 2012). The flower plot in Fig. 3 shows that mussels contain shared OTUs, known as the core gut microbiota (Shade and Handelsman, 2012) under different treatment conditions. The core gut microbiota involves in identifying pathogens and improving survival, which has been used to assess the health risks of mussels (Southwick and Loftus, 2003). In the present study, it was observed that both NOR and NPs exposures induced significant inhibitory effects on the richness and diversity of gut microbiota, and such effects were more significant under combined exposure. Specifically,

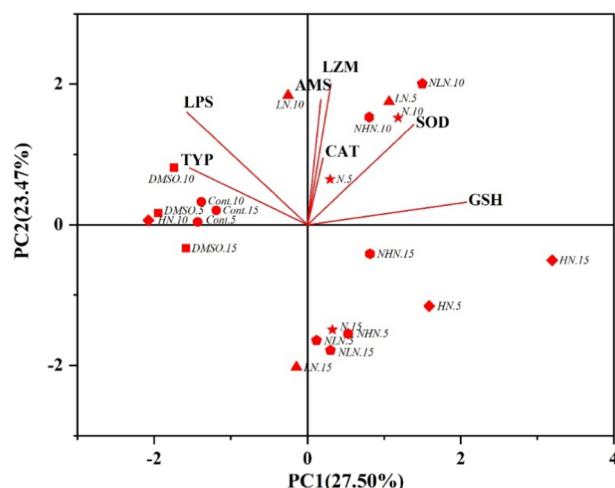


Fig. 6. Principal component analysis (PCA) on 7 enzyme activities (TYP, LPS, AMS, LZM, CAT, SOD, and GSH) and 3 time points under 7 treatment conditions (■DMSO.5, 10, 15: DMSO 0.1 % (v/v) on day 5, 10, 15; ●Cont.5, 10, 15: NPs 0 mgL⁻¹ × NOR 0 μgL⁻¹ on day 5, 10, 15; ▲LN.5, 10, 15: NPs 0 mgL⁻¹ × NOR 5 μgL⁻¹ on day 5, 10, 15; ◆HN.5, 10, 15: NPs 0 mgL⁻¹ × NOR 500 μgL⁻¹ on day 5, 10, 15; ★N.5, 10, 15: NPs 0.26 mgL⁻¹ × NOR 0 μgL⁻¹ on day 5, 10, 15; ●NLN.5, 10, 15: NPs 0.26 mgL⁻¹ × NOR 5 μgL⁻¹ on day 5, 10, 15; ●NHN.5, 10, 15: NPs 0.26 mgL⁻¹ × NOR 500 μgL⁻¹ on day 5, 10, 15).

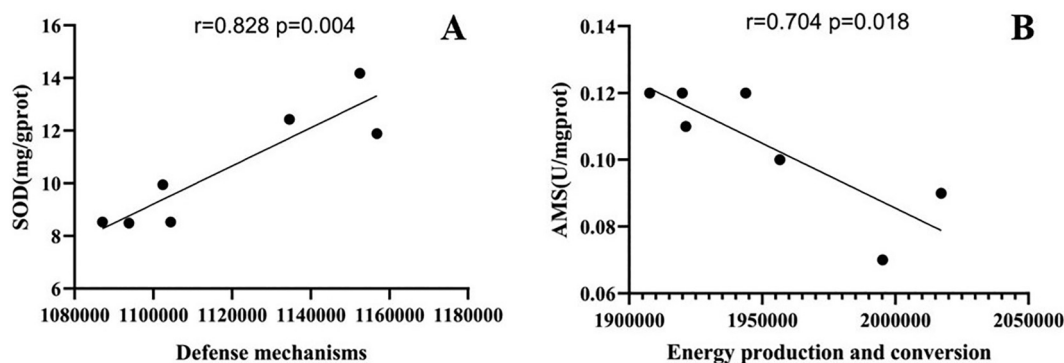


Fig. 7. functions. (A) SOD and defense mechanisms; (B) AMS and energy production and conversion.

the relative abundance of Proteobacteria in mussels' gut was decreased after NOR exposure. NOR is a known fluoroquinolone antibacterial agent against a variety of pathogenic bacteria belonging to Proteobacteria (Meireles et al., 2019). In contrast, the relative abundance of Firmicutes was increased after NOR exposure, which may be related to the relative decrease of Proteobacteria. Firmicutes can accelerate the formation of fat droplets in the intestinal epithelium and liver, facilitating fat absorption (Semova et al., 2012).

The impact of NOR and plastic particles on gut microbiota has also been reported in other organisms. For example, after 14 days of NOR exposure, the diversity of gut microbiota of large yellow croaker *Pseudosciaena crocea* was significantly reduced (Wang et al., 2020a). Juvenile sea cucumber *Apostichopus japonicus* was fed NOR for 45 days and its growth was affected, which was related to the changes in intestinal microbiota structure (Zhao et al., 2019). Li et al. (2020a) showed a decrease in gut microbiota diversity after 6 weeks of MPs exposure, and the abundance of pathogenic bacteria remained high after the recovery period. In *Larimichthys crocea*, after exposure to 100 nm NPs for 14 days, gut microbiota structure was significantly changed, further manifested as increased mortality and decreased growth rate (Gu et al., 2020). Different from the above studies that focused on either NPs or antibiotics, our combined exposure showed an additive effect of NOR + NPs on intestinal homeostasis, which was indicated by OTUs, Chao1, Shannon, and Simpson.

According to PCA (Fig. 6), two principal components accounted for 50.97 % of the total composition. PC1 separated the presence of NOR from the absence, accounting for 27.5 % of the total variances, while PC2 separated the presence or absence of NPs, accounting for 23.47 % of the total variances. The PCA results indicated that antioxidant, immune, and digestive enzymes co-responded to the combined stress of NOR and NPs, which was consistent with the results of Brandts et al. (2018) and Li et al. (2020b). Significant correlations of biochemical parameters and COG functions were also found (Fig. 7). The reduced activity in the antioxidant enzyme SOD and the digestive enzyme AMS might be related to the reduced abundance of bacteria that are involved in oxidative defense and energy storage.

While enzymatic assays efficiently assessed the responsive of selected biomarkers to NPs and NOR exposures, the results of gut microbiota 16S rRNA-seq enabled the prediction of COG functions and biological processes. Although further confirmatory data is needed, the three primary processes - cellular processes and signaling, metabolism, and information storage and processing - are predicted to be impacted by NPs and NOR exposures. The correlation between enzyme activities and COG functions indicates the potential to integrate both approaches as a standard and efficient method for environmental hazard assessment.

5. Conclusion

Antibiotics and NPs, as emerging contaminants or pollutants in the marine environment, have been gradually acknowledged. In the present study, integrating enzymatic assays and gut microbiota 16S rRNA-seq, we

observed combined toxic effects of NOR and NPs on mussels, including disrupted enzymatic activities, gut homeostasis, and physiological damage. The standardized enzymatic assays and gut microbiota 16S rRNA-seq can also be applied to assess the combined or individual effects of other micropollutants. Given the anticipated increasing environmental exposure of both antibiotics and NPs, the direct dual hazards of contamination by antibiotics and NPs warrant more investigations in other environmental organisms, meanwhile, the health risks associated with indirect hazards, such as antibiotic-associated resistance genes, also need to be explored in the future.

CRediT authorship contribution statement

Ran Xu: Formal analysis, Visualization, Methodology, Writing - original draft. **Li'ang Li:** Formal analysis, Investigation, Software, Writing - review & editing. **Jiahui Zheng:** Investigation, Data curation, Writing - review & editing. **Chenglong Ji, Huifeng Wu:** Project administration, Writing, Validation. **Xiang Chen, Yuchuan Chen:** Investigation, Data curation. **Menghong Hu:** Supervision, Validation. **Elvis Genbo Xu, Youji Wang:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors state that they have no known competing financial interests or personal relationships that could affect the work reported in this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.163304>.

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