Combined Toxicity and Toxicity Persistence of Antidepressants Citalopram and Mirtazapine to Zooplankton Daphnia Magna

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Research Article

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Abstract

Citalopram (CTP) and mirtazapine (MTP) are two typical psychoactive drugs used for the treatment of depression. As emerging pollutants, CTP and MTP are of widely concern because they are active substances for organisms. However, the studies about the toxicity potential of CTP/MTP pollution to aquatic organisms were limited. In the present study, the effects of CTP and MTP on the feeding behavior, heartbeat, nutritional enzymes and related gene expressions of *Daphnia magna* were investigated under single and mixed environmental stress. Meanwhile, the recovery of exposed *D. magna* was studied to analyze the toxic persistence of those pollutants. After 24 h of exposure, the ingestion rate decreased significantly under 1.45 mg/L of CTP. In the mixed treatment groups, no significant synergistic effect of CTM and MTP on daphnia' feeding inhibition was found. After 24 h of recovery, the feeding behavior of *D. magna* was stimulated by compensatory stimulation. At exposure period, the heartbeat rate of exposed *D. magna* increased significantly, and was recovered during the recovery period. The activity of α-amylase (AMS) and trypsin, and their relative gene expression were significantly changed in most of the exposed daphnia, both in the exposure period and recovery period. But there were different responses between gene transcription with enzyme activity. In general, psychoactive drugs have an obvious toxic threat to aquatic organisms, and after acute exposure, the physiological function of *D. magna* could be recovered to a certain extent. The results were helpful to evaluate the ecological risk of psychotropic drugs in aquatic environments.

1. Introduction

The use of antidepressants has increased considerably since 2000, and they were inevitably released into the environments and caused serious environmental pollution (Sehonova et al., 2018). As emerging contaminants, their removal efficiency in wastewater treatment plants was low (Kuzmanovic et al., 2015; Petrie et al., 2015). CTP and MTP are typical antidepressant drugs, which are widely used for the treatment of depression (Cipriani et al., 2018). Previous studies have found that more than 10% of psychoactive drugs were excreted into the aquatic environments in their active form (Balakrishna et al., 2017), and about 12% of CTP was excreted into the aquatic environments (Bergheim et al., 2012).

Recently, the use of CTP and MTP continues to increase, both drugs can be found in most of the environment compartments, such as sediments, surface water, groundwater, etc (Silva et al., 2015; Faggio et al., 2016). Golovko et al. (2020) reported that the average concentrations of CTP and MTP in surface water of Lake Ekoln in Sweden were 0.59 and 1.1 ng/L, respectively. The detection frequency of MTP at a university hospital in Ioannina located in northwestern Greece was > 73% and the average concentration was 8.3 ng/L (Kosma et al., 2019). CTP was detected in untreated sewage in Denmark, with its concentration range of 0.19–10.3 μg/L (Kosma et al., 2019). Antidepressants have potentially highly dangerous for aquatic ecosystems (Minguez et al., 2016). The risks of disturbances in behavior, endocrine system may be expected in fish exposed to a psychotropic drugs-contaminated environment (Giang et al., 2018). However, the potential impacts of CTP/MTP pollution on aquatic organisms were limited.

The aquatic environmental risks of CTP and MTP have been concerned. For example, Bachour et al. (2020) observed a significant decrease in swimming activity of zebrafish exposed to CTP with a concentration of 373 μg/L. The inhibition rate of ACHE activity was73% of *D. magna* exposed to CTP with a concentration of 1 g/L (Yang et al., 2017). Assessment of individual chemical is a common tool for ecological risk assessment of pollutants. However, in the aquatic environments, many pollutants usually exist at the same time. When aquatic organisms were exposed to a mixture of pollutants at the same time, the toxicity of those pollutants to organisms may be superimposed or reduced. (Lari et al., 2017; Liu et al., 2018; Bachour et al., 2020; Hossain et al., 2021). It was revealed that the 1:1 binary mixture of CTP and tramadol caused significant decrease swimming activity of zebrafish during dark conditions in comparison with individuals CTP and tramadol (Backhaus, 2016). As a compound drug, the combined toxicity of CTP and MTP was worthy of attention.

Because of the characteristics of easy cultivation, short life cycle and high sensitivity to pollutants, *D. magna* is a good model organism for the aquatic environment pollution evaluation (Chai et al., 2021; Tkaczyk et al., 2021). *D. magna* have been used to assess the acute toxic effects of MTP or CTP (Yang et al., 2017), but studies about the damage of CTP and MTP on daphnia's behavior were limited. Toxicology studies have suggested that the feeding behavior and heartbeat of *D. magna* were used to assess the sub-lethal effects of pollutants (Bownik, 2017, 2020). The concentration of pollutants in the environment is constantly changing. But in what concerns CTP/MTP, few studies analyzed post-exposure effects in *D. magna*. In previous studies, Yan et al. (2018) revealed that after 7days of exposed-recovery to sulfamethazine (SMZ), the activities of SOD and MDA of zebrafish were reversed. However, after 1-week of exposed-recovery to rifampicin, bacterial communities of *Gambusia affinis* were not able to recover in terms of diversity or composition (Carlson et al., 2017). It is worth studying whether the aquatic organisms could fully recover when the pollutants were removed. Thus, the studies of the abnormal behavior of *D. magna* caused by CTP and MTP were performed to understand the aquatic ecological risks of these two substances.

Thus, to improve the understanding of the potential toxicity of CTP and MTP, the individual and combined toxicity of them on the feeding behavior, heart rate, nutritional enzymes, and related gene expressions of *D. magna* was thoroughly studied in this study during exposure and recovery periods. There were three aims in this study: 1) the effects of CTP and MTP on the feeding behavior and heartbeat of *D. magna* were studied under single and mixed environmental stress; 2) the recovery of *D. magna* after exposure was study to evaluate the toxicity persistence

of CTP and MTP; 3) the potential toxic mechanism of CTP and MTP was studied by monitoring the digestive enzymes and related genes of *D. magna.* The findings were helpful to evaluate the potential risk of CTP and MTP in aquatic ecosystems.

2. Materials And Methods

2.1 Chemicals

Citalopram (CTP; CAS: 59729-33-8) was purchased from Sichuan Kelun Pharmaceutical Co., Ltd (China), and Mirtazapine (MTP; CAS: 85650-52-8) was from N.V. Organon (Netherlands). The physiochemical properties of the target compounds are listed in Table S1 (*Supporting Information*). The assay kits for measuring the digestive enzyme activity of α-Amylase (AMS) and trypsin were purchased from Nanjing Jiancheng Bioengineering Institute (China). Trizol reagent was purchased from New Cell &Molecular Biotech Co., Ltd (Suzhou, China). Reverse transcriptase was purchased from Vazyme Biotech Co., Ltd (Nanjing, China). The concentrations of CTP and MTP in each group were detected by HPLC (S0D-M 20A, Shimadzu, Japan), equipped with DAD detector and Baseline C-18 column (4.6 mm × 150 mm, 5 μm). The mobile phase was methanol: water (50:50, V/V). The detection wavelength was 237 nm for CTP and 240 nm for MTP, the flow rate was 1.0 mL/min, the column temperature was 35 ℃, and the injection volume was 20 μL. The retention time of CTP and MTP was 5.2 and 2.7 min, respectively.

2.2 Culture program of *Chlorella pyrenoidosa* and *D. magna*

Chlorella pyrenoidosa (*C. pyrenoidosa*) was obtained from Institute of aquatic biology, Chinese Academy of Sciences (Wuhan, China), cultured in the BG-11, and maintained at the temperature of 25.0 ± 1.0°C with a light-dark cycle of 16:8 h. Before the experiment began, *C. pyrenoidosa* were harvested by centrifuging at 4000 rpm for 10 min. *D. magna* was cultured in medium hardness water and maintained at the temperature of 20.0 ± 1.0°C with a light-dark cycle of 16:8 h. *C. pyrenoidosa* was used as the food source for *D. magna*.

2.3 Feeding behavior experiment

Feeding tests were conducted with 7-day-old *D. magna* in exposure and recovery for 24 h. According to our pre-experiment, the 24-h EC_{50} values for CTP and MTP were 28.93 and 20.59 mg/L, respectively. The 1/80 EC_{50} and 1/20 EC_{50} of CTP (0.36 and 1.45 mg/L) and MTP (0.25 and 1.03 mg/L) were used for the further experiment. The summary of the experimental process is listed in Table 1. Five replicates were set for each group, and five *D. magna* were employed in each replicate. The tests of feeding inhibition were conducted in 100 mL beakers containing 60 mL of exposure solution. *D. magna* was fed with *C. pyrenoidosa* which density was 1×10^6 cells/mL. To avoid the growth of microalgae, all groups were conducted in the dark condition. The solution before and after 24 h of exposure in each beaker was shaken fiercely to resuspended the *C. pyrenoidosa* cells, and the algal density was detected by ultraviolet spectrophotometry at 680 nm for calculating the feeding rate (Li et al., 2020).One *D. magna* was taken from each beaker, and the heartbeat of *D. magna* was recorded by video under Nikon SMZ1000 stereomicroscope for 2 minutes. The others *D. magna* were transferred into 60 mL of uncontaminated medium containing 1×10^6 cells/mL of *C. pyrenoidosa*. The solution before and after 24 h of recovery in each beaker was shaken fiercely to resuspended the *C. pyrenoidosa* cells, and algal density was detected. Then, one *D. magna* from each beaker was used to measure the heartbeat from each beaker.

Table 1
Summary of the treatment process applied for single and mixture compound(s) feeding tests during exposure and restores period.

Group		Nominal concentration (g/L)		Actual concentration (mg/L)		Starvation treatment(h)	Exposure time (h)	Starvation treatment (h)	Restores time(h)
		Citalopram	Mirtazapine	Citalopram	Mirtazapine				
Control		0	0	0	0	24	24	24	24
Citalopram	C-L	0.36	0	0.34	0	24	24	24	24
	C-H	1.45	0	1.29	0	24	24	24	24
Mirtazapine	M-L	0	0.25	0	0.17	24	24	24	24
	M-H	0	1.03	0	1.02	24	24	24	24
Mix (Citalopram and Mirtazapine)	Mix-L	0.18	0.125	0.18	0.08	24	24	24	24
	Mix-H	0.72	0.52	0.54	0.44	24	24	24	24

2.4 Measurements of enzyme response assay

Under the same conditions as the feeding assays, 200 *D. magna* were cultured at the same time for the enzyme response experiment for each group. After 24 h of exposure, *D. magna* was collected and mixed with normal saline in the ratio of 1:9 and then homogenize thoroughly on ice.

Then the homogenates were centrifuged at 8000 rpm at 4 ^{o}C for 10 min, the supernatant was taken out to measure the total protein (TP) content, and the activities of AMS and trypsin, according to the instructions of respective assay kits. All the operations were carried out on the ice. Steps of the measurement are provided in detail in the S1-3 (*Supporting Information*).

2.5 Extraction of total RNA and reverse transcription

D. magna were performed as experimental materials to detect gene expression in different stress conditions, three replicates were set for each group. 15 *D. magna* were contained in each replicate for detecting the gene transcription of AMS and trypsin. After 24 h exposure and 24 h recovery, *D. magna* were removed from the culture medium and washed twice with double distilled water, and then transferred to a homogenizer containing Trizol reagent to extract total RNA (4°C). Nanodrop 2000 was performed to detect the concentration of total RNA (Thermo Fisher Scientific, USA). The concentration of total RNA was determined by Nanodrop 2000 (Thermo Fisher Scientific, USA). To obtain cDNA, 2 μg of total RNA was used for reverse transcription with HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) following the manufacturer's instructions.

The gene expression of *D. magna* exposed to CTP and MTP was analyzed by real-time quantitative polymerase chain reaction (qPCR). β-actin gene was used as the internal standard gene and the specific primers were designed for three target genes (trypsin and AMS). The primers for qPCR were designed in National Center for Biotechnology Information according to the known sequences. The primer specificity information is listed in Table S2 (*Supporting Information*). The expressions of these genes were conducted by SYBR Green PCR Master Mix (Vazyme, Nanjing, China) according to the instruction. Cycling parameters were set as follows: one cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Then, the formation of specific products was determined by melting curve analysis. The target gene expression values were calculated by the 2 - △ CT method (Livak and Schmittgen, 2001). Three independent biological repeats were performed in these experiments.

2.6 Data analysis

Statistical analyses were performed by SPSS statistics 26.0 software. The difference between the experimental group and the control group was performed by T-test. The correlation between activity of digestive enzymes and their corresponding genes was analyzed by bivariate correlation analysis. The experimental data were present as mean ± standard deviation. *$p < 0.05$ and **$p < 0.01$ were set as the significance levels of all calculations. Data visualization was performed using Origin 8.0 software.

3. Results And Discussion

3.1 Feeding inhibition of *D. magna* caused by psychoactive drugs

The feeding inhibition experiments of *D. magna* was used to study the single or joint toxicity of CTP/MTP to aquatic organisms during exposure period and recovery period, and the results are shown in Fig. 1A and 1B, respectively. After 24h exposure, the ingestion rates of *D. magna* were $(2.37 \pm 0.02) \times 10^5$ cell/ind./h in control group. The feeding rates of *D. magna* significant decreased for high concentrations of CTP and mixed pollutants in Group C-H and Mix-H ($p < 0.01$ or 0.05), which were $(1.56 \pm 0.06) \times 10^5$ cell/ind./h and $(1.86 \pm 0.12) \times 10^5$ cell/ind./h, respectively. There was no significant decrease in feeding rates of *D. manga* when they exposed to MTP [low concentration group: $(2.24 \pm 0.13) \times 10^5$ cell/ind./h; and high concentration group: $(2.39 \pm 0.14) \times 10^5$ cell/ind./h)], low concentrations of CTP [$(2.51 \pm 0.09) \times 10^5$ cell/ind./h] and low concentrations of mixed pollutants [$(2.49 \pm 0.10) \times 10^5$ cell/ind./h] ($p > 0.05$). In general, compared with the control, there were no obvious differences in feeding behavior when exposed to MTP for 24 h, which indicated no inhibitory effect of MTP in feeding behavior when its exposure concentrations ranged from 0.25 to 1.03 mg/L. CTP had a more effective significant effect on the feeding behavior of *D. magna* than MTP after 24 h exposure, which showed that *D. magna* was more susceptible to CTP than MTP. Food consumption of *D. magna* decreased with increasing CTP concentration, which indicated a concentration-response relationship (Lari et al., 2017).

After 24 h of recovery, the ingestion rates of *D. magna* were $(3.50 \pm 0.07) \times 10^5$ cell/ind./h in control group. The feeding rates increased significantly for low concentrations of MTP and high concentrations of CTP in Group M-L and C-H ($p < 0.01$), which were $(5.42 \pm 0.009) \times 10^5$ cell/ind./h and $(5.74 \pm 0.04) \times 10^5$ cell/ind./h, respectively. Compared with the control, there were no obvious differences in daphnia feeding behavior when recovery for 24 h in Group Mix-L [$(3.15 \pm 0.05) \times 10^5$ cell/ind./h], Mix-H [$(5.74 \pm 0.04) \times 10^5$ cell/ind./h], M-H [$(3.18 \pm 0.09) \times 10^5$ cell/ind./h] and C-H [$(3.54 \pm 0.08) \times 10^5$ cell/ind./h] ($p > 0.05$). The results showed that the feeding behavior of *D. magna* was stimulated by the low concentration of MTP and high concentration of CTP after 24 h of recovery. This may be because of the mechanism of overcompensation (Lv et al., 2018; Liu et al., 2019). The feeding inhibition reversed into hormesis from the exposure period to the recovery period. The overcompensation is an exceeding compensation after the animals suffer damaging stress which helps to restore its nutritional status(Liu et al., 2018) .

3.2 Heart rate

CTP is a selective 5-HT re-uptake inhibitor that blocked the re-uptake of transporters, while MTP could enhance noradrenergic and serotonergic neurotransmission (Salomone et al., 2011). Heartbeat was significantly stimulated by psychoactive drugs (CTP/MTP), which can be used to reflect the toxicological effects on individuals (Liang et al., 2017). Figure 2A and 2B shows that effects of CTP and MTP on heartbeats of *D. magna* after 24 h of exposure and 24 h of recovery, respectively. Generally, In the 24 h of exposure period, except Group Mix-H, the heartbeats of *D. magna* significantly increased in the exposed groups ($p < 0.01$). For MTP, the heartbeats of *D. magna* increased with the increase of pollutant concentration, showing a concentration dependent manner. On the contrary, the heartbeats of *D. magna* decreased with the increasing concentration of CTP and mixed drugs. Those may be related to the higher sensitivity of *D. magna* to CTP which was consistent with the findings of Liang et al. (2017), in which heartbeat was significantly stimulated by low concentrations of perfluorooctane sulfonate (PFOS) and inhibited by high concentrations of PFOS (the change from a low-dose stimulation to a high-dose inhibition).

After recovery for 24 h, there were no obvious differences in heartbeat between control group and exposed groups. It was interesting to note that the heart rate decreased slightly with the increasing concentration of MTP, CTP and their mixed drugs after 24 h of recovery. It was possible that *D. magna* was damaged after exposure to high concentrations of pollutants, but whether the damage decreased with time needs to be further studied.

3.3 Enzyme activity

The effects of CTP and MTP on the digestive enzymes (AMS and trypsin) of *D. magna* were investigated to identify the potential mechanism for the feeding inhibition, and the results are shown in Fig. 3. AMS are involved in fibrin and starch digestion in *D. magna*, while trypsin in protein digestion (Perera et al., 2012; Huang et al., 2017), all of them are the typical digestive enzymes (Houde et al., 2013). Figure 3A and 3B shows that the activities of AMS after 24 h of exposure and 24 h of recovery, respectively. Compared with the control, the AMS activities of exposed daphnia increased significantly in Group M-L, M-H and Mix-H after 24 h of exposure ($p < 0.05$), the increase activities of AMS could be an adaptation to maximize utilization of the limited amount of food ingested (Seyoum et al., 2021). Those in Group C-L and C-H decreased significantly ($p < 0.05$), which associated with damage to the digestive system of *D. magna*. The previous study also found that the activity of AMS was decreased significantly after exposed to azithromycin, and the concentration-response relationship was present (Li et al., 2020). There was no significant difference in the AMS activities between Mix-L and control group ($p > 0.05$). Those indicated that the different effects on the activity of AMS may be related to different pollutants. After recovery for 24 h, the AMS activities of exposed daphnia increased significantly in Group M-L and M-H, while decreased significantly in Group C-H, Mix-L and Mix-H ($p < 0.05$), and there was no significant change in Group C-L ($p > 0.05$). Those indicated that the digestive system of *D. magna* was injured by contaminants, then the AMS activities were not well recovered.

Figure 3C and 3D shows that the activities of trypsin after 24 h exposure and 24 h recovery, respectively. After 24 h exposure, the trypsin activities of exposed daphnia increased significantly in Group M-L and Mix-L ($p < 0.05$), which suggested that tissue protein may undergo proteolysis. Protein was reported to serve as an alternate source of energy under extreme stress conditions (Suryavanshi et al., 2009). Stimulation the activity of trypsin may be an adaptive response of *D. magna* to extreme conditions (Dai et al., 2014). There was no significant difference in trypsin activities between Group M-H, CTP and Mix-H and control group ($p > 0.05$). The activities of trypsin were negatively correlated with the concentration in the MTP and mixed groups, and changed from stimulative effects to inhibition effects with the increase of concentration, which possibly related to the excitatory effect (Liu et al., 2012; Rhee et al., 2013). However, the activities of trypsin were positively correlated with the concentration in the CTP, which might be related to the assimilation processes (Lv et al., 2017). After 24 h recovery, the activity of trypsin was decreased significantly in Group C-L and increased significantly in Group Mix-H ($p < 0.05$), and there was no significant change in the rest exposed groups ($p > 0.05$)

3.4 Gene transcription of *D. magna*

There might have a relationship between up- or down-regulation of genes expression and digestive system disorder (Schwarzenberger and Fink, 2018). Figure 4A shows that the up-regulated significantly of expression of AMS genes in all the exposed groups ($p < 0.01$). This indicated that CTP, MTP and their mixture stimulated the expression of AMS genes during exposure period. This was consistent with the results of the Houde et al. (2013), in which the expression of AMS genes was stimulated in the groups added with hexachlorocyclopentadiene (HCCPD). However, this was contrary to the results of Zhao et al. (2019), in which the expression levels of AMS gene were inhibited in the groups with BDE-47, BDE-99 and their mixture. Those indicated that the different effects on the expression of AMS gene may also be related to different pollutants. In Fig. 3A, the activities of AMS increased significantly in MTP exposed daphnia after 24 h of exposure, and the gene expression of AMS was up-regulated significantly (Fig. 4A). However, there was no significant correlation between gene expression and enzyme activity of AMS ($R^2 = 0.1753$, $p = 0.266$, Fig. 5A). The activity of AMS of daphnia was decreased significantly in Group Mix-L and Mix-H after 24 h recovery, and the gene expression level of AMS in those groups was decreased significantly ($P > 0.05$) (Fig. 4B). Correlation analysis was conducted with enzyme activity and gene transcription, and the results showed no significant correlation between the two ($R^2 = 0.0002$, $P = 0.787$, Fig. 5B). Recovery period, the damage of the target chemicals on *D. magna* was alleviated, but whether this damage can be completely removed still need further study. Houde et al. (2013), showed that the activity of AMS was decreased significantly while *D. magna* exposed to HCCPD, and the gene expression levels of AMS were decreased significantly. Schwarzenberger and Fink (2018) found that there was significant correlation between

enzyme activity and gene expression. These results suggested that enzyme activity and gene expression may exhibit different responses to various environmental stress.

Figure 4C shows that the gene expression levels of trypsin significantly increased in Group M-L, C-L, C-H, Mix-L after 24 h exposure ($p < 0.01$), but the activity of trypsin shows no significant difference in Group C-L and C-H (Fig. 3C). After 24 h of recovery, the gene expression levels of trypsin increased significantly in the groups of M-H, C-H, but there was no significant difference in the trypsin activities. Correlation analysis was conducted with enzyme activity and gene expression of trypsin, and the results showed no significant correlation between them after 24 h of exposure ($R^2 = 0.4734$, P = 0.111, Fig. 5C) and 24 h of recovery ($R^2 = 0.0024$, P = 0.872, Fig. 5D). The digestive system was damaged on *D. magna* exposed to the target pollutants at the gene transcription level. However, the mechanism of these effects needed to be paid more attention to.

4. Conclusions

In this study, the toxicity of CTP, MTP and their mixtures on the feeding behavior and bodily functions of *D. magna* was investigated, and the aquatic toxicity of the two psychotropic drugs from two stages of exposure and recovery was evaluated. After exposure, the feeding behavior of *D. magna* was significant inhibited in the Group C-H and Mix-H, and the heart rate increased significantly in all treatment groups. The changes in enzyme activity and gene expression levels of AMS and trypsin indicated that CTP/MTP and their mixture had different effects on the physical function of *D. magna*. However, there were different responses between enzyme activities with gene transcription. In the recovery period, obvious overcompensation effect in feeding behavior was found in Group M-L and C-H. There were no obvious differences in heartbeat and the damage of the target chemicals on *D. magna* was alleviated at the genomic level. Those results demonstrated that there were obvious toxicity effects of psychoactive drugs on *D. magna* at the molecular level under single and mixed environmental stress. Further researches are needed to determine the toxic pattern of those compound contaminations and investigate the potential mechanism of their toxicity.

Declarations

5.1 Ethics approval and consent to participate

Not applicable

5.2 Consent for publication

Not applicable

5.3 Availability of data and materials

Not applicable

5.4 Competing interests

The authors declare that they have no competing interests

5.5 Authors' contributions

Yunfeng Ma: carried out experiments and analyzed experimental results; Chenyang Li and Shu Wei: developed the methodology; Ruixin Guo: analyzed experimental results; Yang Li: developed the methodology; Jianqiu Chen and Yanhua Liu: managed and coordinated responsibility for the research activity planning and execution.

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Figures

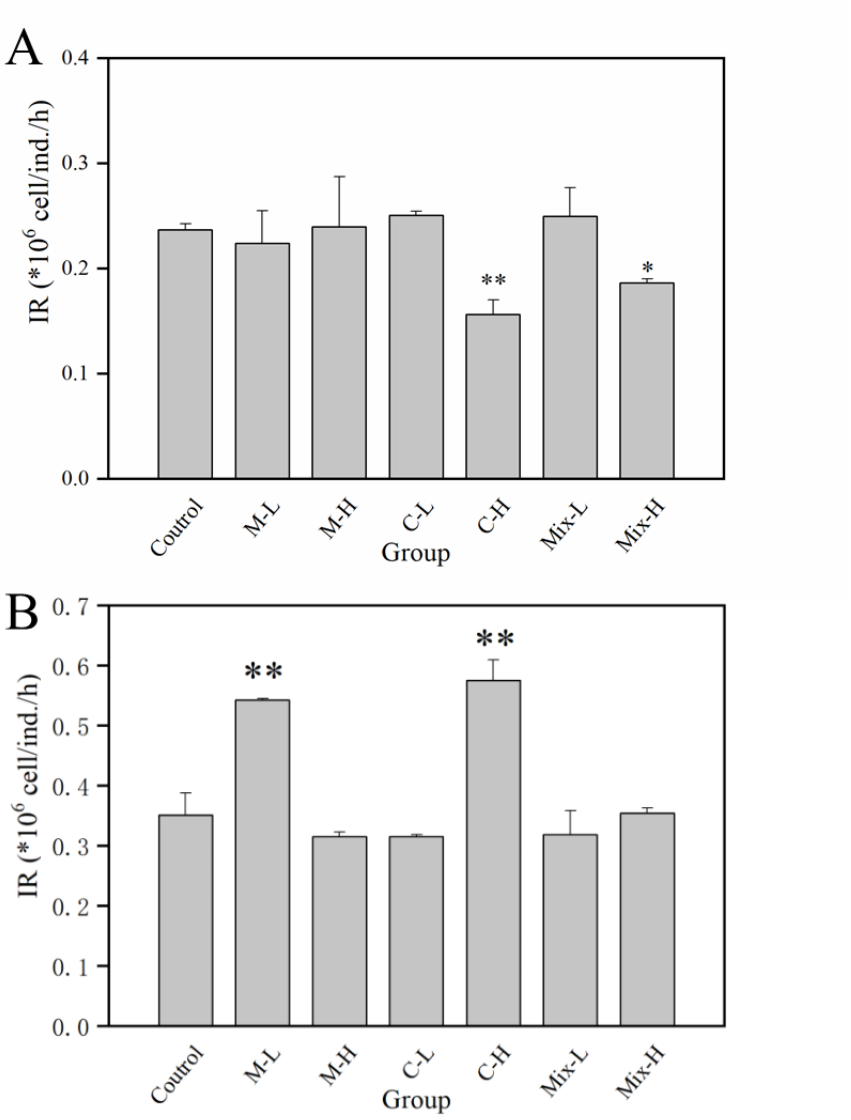

Figure 1

Effects of CTP and MTP on feeding behavior of D. magna after exposure period (A) and recovery period (B). *: significant difference between control group and treatment groups; **: extremely significant difference between control group and treatment groups.

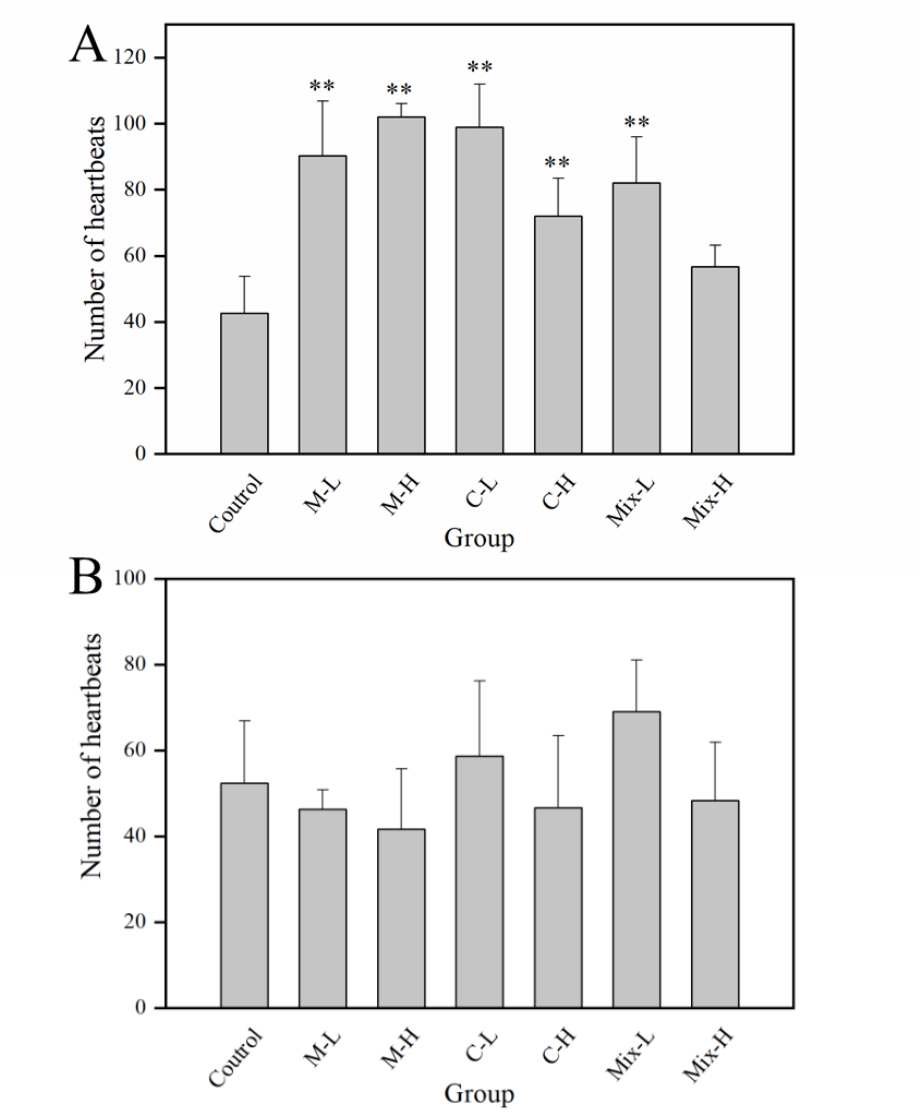

Figure 2

Effects of CTP and MTP on heartbeats of D. magna after exposure period (A) and recovery period (B). *: significant difference between control group and treatment groups; **: extremely significant difference between control group and treatment groups.

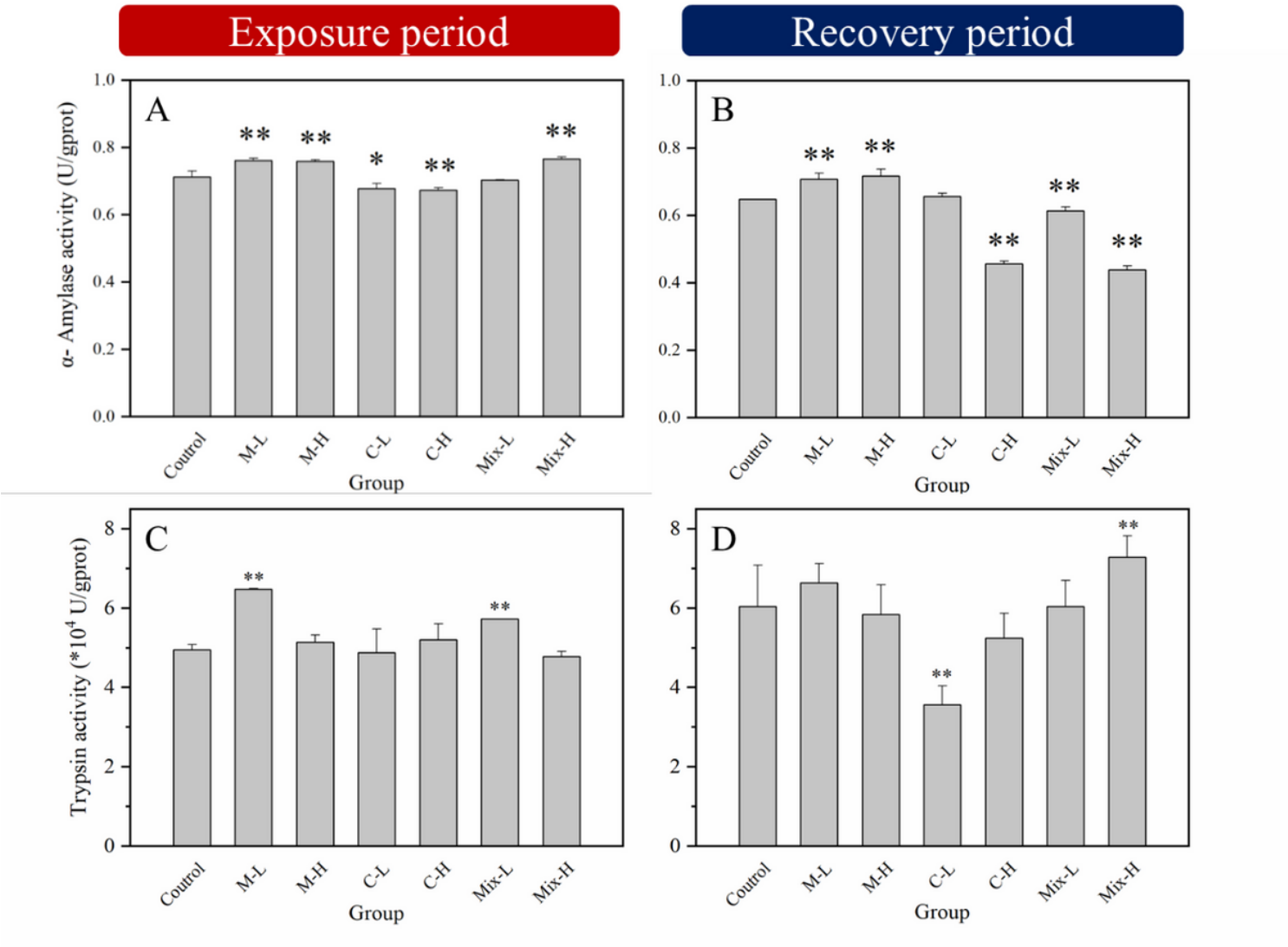

Figure 3

α-Amylase and trypsin activities of D. magna in different treatment groups after exposure period and recovery period. A: α-amylase activities after exposure period; B: α-amylase activities after recovery period; C: trypsin activities after exposure period; and D: trypsin activities after recovery period. *: significant difference between treatment groups and control group ($p < 0.05$); **: extremely significant difference between treatment groups and control group ($p < 0.01$).

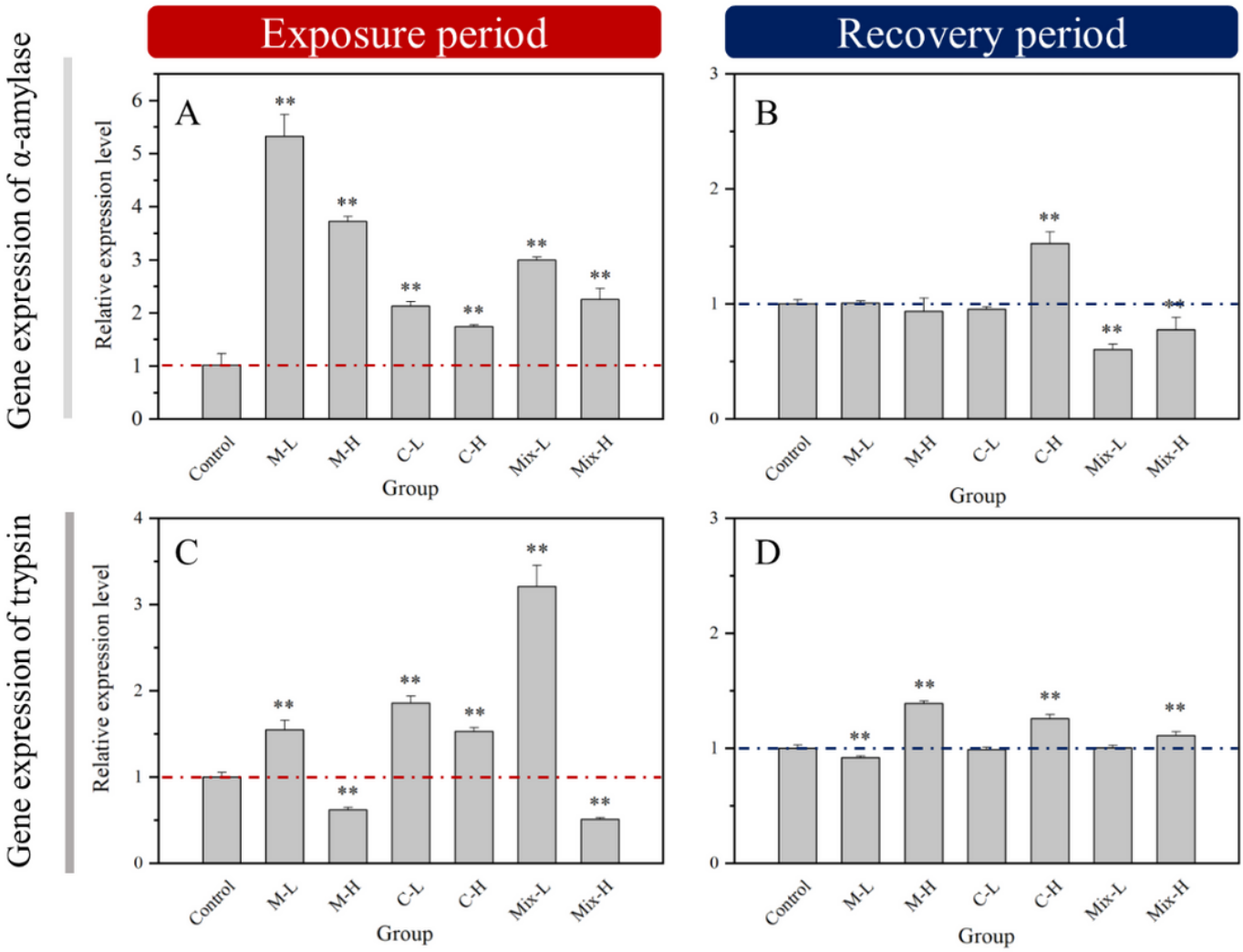

Figure 4

Relative gene expression of α-amylase and trypsin of D. magna in different treatment groups after exposure period and recovery period. A: α-amylase after exposure period; B: α-amylase after recovery period; C: trypsin after exposure period; and D: trypsin after recovery period. Gene transcription below 1 represented down-regulation and above 1 represented up-regulation. All data represented means ± SD (n = 6, three biological repeats and two technical repeats). *: significant difference between treatment groups and control group ($p < 0.05$); **: extremely significant difference between treatment groups and control group ($p < 0.01$).

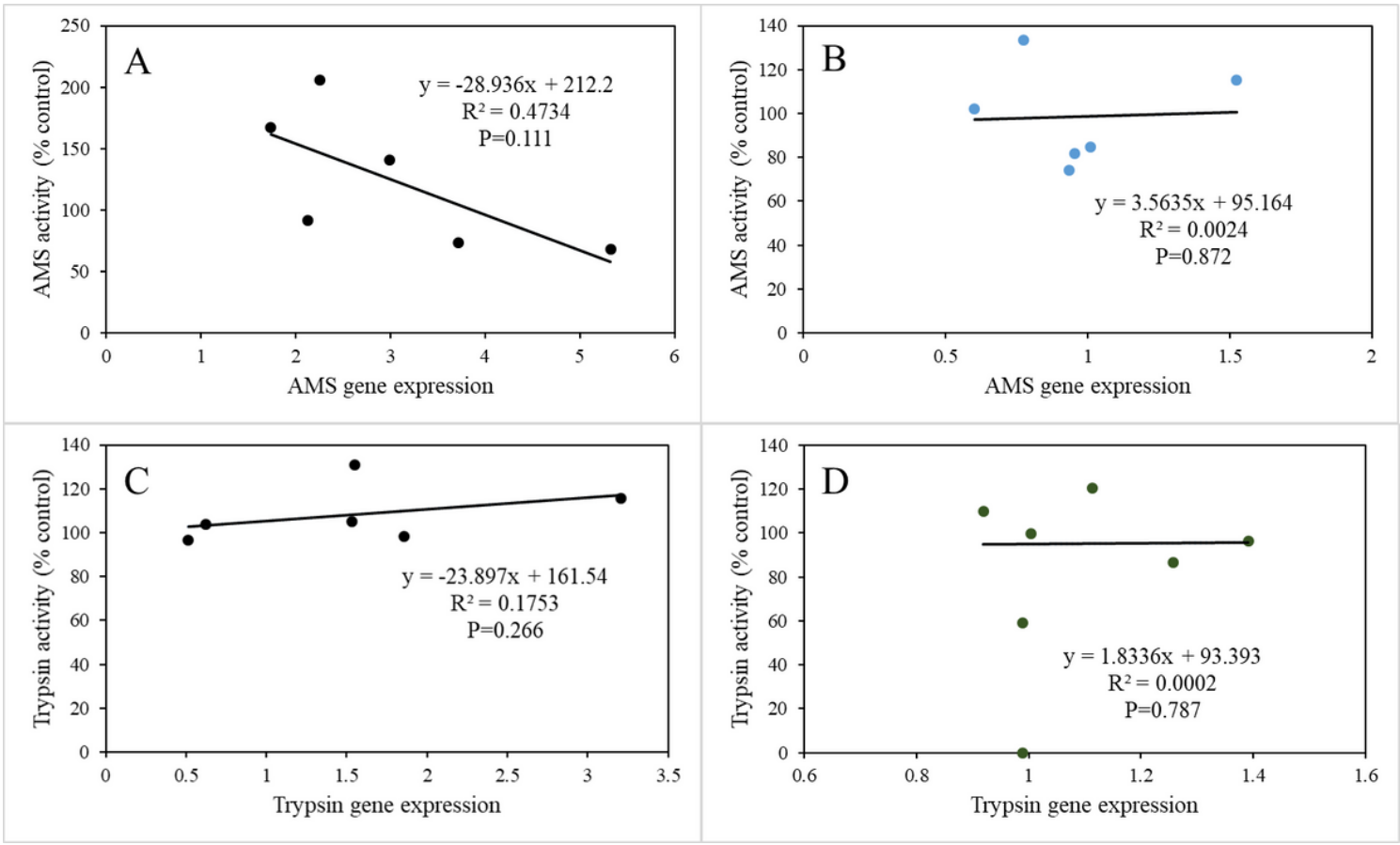

Figure 5

Relationship between gene expression and enzyme activity in exposure daphnia. A: AMS in exposure period; B: AMS in recovery period; C: trypsin in exposure period; and D: trypsin in recovery period. Gene expression and enzyme activity are listed in X-axis and Y-axis, respectively. Regression lines and R2 values are delineated. *: statistical significance of the correction ($p < 0.05$).

Supplementary Files

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