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

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Abstract

Citalopram (CTP) and mirtazapine (MTP) are two typical psychoactive drugs used for the depression treatment. As emerging pollutants, CTP and MTP has been given widely concern because they are active substances for organisms. Therefore, the ecotoxicological risks of aquatic organisms should be paid more attention to. In this study, the effects of CTP and MTP on the feeding behavior, heartbeat, nutritional enzymes and related gene transcriptions 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-hexposure, the ingestion rate decreased by 34.2% and 21.5%, in the group of C-H and Mix-H respectively. After 24-h recovery, the feeding behavior of *D. magna* was stimulated by compensatory stimulation. In exposure period, the heartbeat rate of *D. magna* increased by 132.3%, 69%, 111.9%, 139.4%, and 92.4%, in the group of C-L, C-H, M-L, M-H and Mix-L respectively, and was recovered during the recovery period. The activity of α-amylase (AMS) and trypsin were significantly changed in most of the exposed daphnia, both in the exposure period and recovery period. CTP/MTP exposure stimulated transcription of the α-amylase gene. M-H and Mix-H exposure inhibited transcription of the trypsin gene and other stimulated transcriptions. After 24-h recovery, the stimulative or inhibitory effects were alleviated. There were different responses between gene transcription and enzyme activity. In conclusion, our results highlighted the toxic effects of single and mixed pollution of CTP and MTP on feeding, heartbeat, enzymes and genes of *D. magna.*

Highlights:

1. The feeding behavior of *D. magna* was inhibited by citalopram (CTP).
2. A form of overcompensation of the feeding behavior occurred after recovery.
3. The heartbeats of *D. magna* increased after exposed to CTP and mirtazapine (MTP).
4. The exposure of CTP and MTP disturbed the digestive system in *D. magna*.
5. The gene expression of α-amylase and trypsin was changed after exposure.

1. Introduction

The use of antidepressants has been increasing considerably since 2000, and they were inevitably released into the environments and caused serious environmental pollution (Sehonova et al., 2018). As emerging contaminants, CTP and MTP are not target pollutants in the waste water treatment plant (WWTP). The average CTP removal efficiency in WWTP was 25% (Gros et al., 2020), therefore the effluent concentration of CTP was still considered high (Kuzmanovic et al., 2015; Petrie et al., 2015). CTP and MTP are typical antidepressant drugs, widely used for the depression treatment (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 and 6–20% of MTP was excreted into the aquatic environments (Bergheim et al., 2012; Gundlach et al., 2021).

Recently, the use of CTP and MTP continues to increase, both drugs can be found in most environment compartments, such as sediments, surface water, groundwater, etc. (Silva et al., 2015; Proctor et al., 2021). 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 (Styrishave et al., 2011). Antidepressants maybe highly dangerous for aquatic ecosystems (Minguez et al., 2016). The risks of disturbances in behavior and endocrine system may be expected in fish exposed to 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 received more attentions. 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 Acetyl cholinesterase (ACHE) activity was 73% 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, many pollutants usually exist simultaneously in the aquatic environments. When aquatic organisms were exposed to a mixture of pollutants simultaneously, 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 may cause significant decrease in swimming activity of zebrafish during dark conditions when compared with individuals CTP and tramadol (Bachour et al., 2020). In the depression treatment, CTP and MTP were often used in combination (Zhuang and Hospital, 2019), and appeared together in the environment. Therefore, the combined toxicity of CTP and MTP was worthy of more attentions.

Because of the characteristics of easy cultivation, short life cycle and high sensitivity to pollutants, *D. magna* is a good model organism for the evaluation of aquatic environment pollution (He et al., 2019; Tkaczyk et al., 2021). *D. magna* has been used to assess the acute toxic effects of MTP or CTP (Yang et al., 2017), while many adverse effects are not well characterized. Toxicology studies have suggested that the feeding behavior and heartbeat of *D. magna* were used to assess the sub-lethal effects of pollutants. Assessment of effects on feeding activity is a

valuable tool for determination of early effects induced by bioactive substances. The daphnid heart responded to sublethal levels of various environmental stressors. Heart may be considered as a promising sensor of effects induced by stressful factors in the aquatic environment and as a model for testing cardioactive drugs (Bownik, 2017, 2020). The pollutant concentration of in the environment is constantly changing. However, few studies have analyzed the recovery effects after exposed to CTP/MTP of *D. magna*. In previous studies, Yan et al. (2018) revealed that after 7-day exposed-recovery to sulfamethazine (SMZ), the activities of SOD and MDA of zebrafish were reversed. However, after 1-week 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 deepen the understanding of potential toxicity of CTP and MTP, the individual and combined toxicity on the feeding behavior, heart rate, nutritional enzymes, and related gene transcription of *D. magna* were thoroughly studied in this study during exposure and recovery periods. There were three research objectives 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 feasible to evaluate the potential risks 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 purchased 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 contains methanol and water with the ratio of 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. As an inorganic salt, BG-11 was the mineral salt medium provided to the microalgae and there was no external carbon source provided to the microalgae. Microalgae grew autotrophically under light conditions. Before the experiment began, *C. pyrenoidosa* were harvested by centrifuging at 4000 rpm for 10 min. *D. magna* was cultured in water with medium hardness 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* under exposure and recovery for 24 h. Acute toxicity tests are described in SI-S1. 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 single exposure 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. Microalgae was usually cultured to logarithmic growth stage were fed (1×10^6 cells/mL) to *D. magna* after centrifugal cleaning. To avoid the growth of microalgae, all groups were conducted in the dark condition. The solution before and after 24-h exposure and 24-h recovery in each beaker were shaken fiercely to resuspended the *C. pyrenoidosa* cells. The algal density was detected by 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.

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, 245 *D. magna* were cultured at the same time for each group. 200 of them were used for the enzyme response experiment and the rest was for gene transcription. In general, samples were collected after 24 h of exposure and 24 h of recovery for further enzyme gene analyze. After 24 h of exposure and 24 h of recovery, *D. magna* was collected and mixed with 0.9% sodium chloride solution 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 at a temperature of 4℃. 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 animals to detect gene transcription under different stress conditions, three replicates were set for each group. 15 *D. magna* were involved in each replicate for detecting the gene transcription of AMS and trypsin. After 24-h exposure and 24-h recovery, *D. magna* was 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 (under the 4°C). Nanodrop 2000 was performed to detect the concentration of total RNA (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 instructions.

The gene transcription 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 two 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 transcriptions 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 transcription values were calculated by the 2 - △ CT method (Livak and Schmittgen, 2001). Three independent biological repeats were performed in these experiments.

2.6 Data analyze

Statistical analyses were performed by SPSS statistics 26.0 software. The difference between the experimental group and the control group were performed by an ANOVA. The correlation between activity of digestive enzymes and their corresponding genes were 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* were used to study the single or joint toxicity of CTP/MTP to aquatic organisms during exposure and recovery period, and the results are shown in Fig. 1A and 1B, respectively. After 24 h of exposure, the ingestion rate decreased by 34.2% and

21.5%, in the group of C-H and Mix-H respectively. There was no significant decrease in feeding rate in the other exposure groups ($p > 0.05$). Food consumption of *D. magna* decreased with increasing CTP concentration, which indicated a concentration-response relationship (Lari et al., 2017). Compared with the control, there were no obvious differences in feeding behavior when exposed to MTP for 24 h, which showed that *D. magna* was more susceptible to CTP than MTP. In the binary mixture toxicity experiment, the feeding rate decreased significantly in the group of Mix-H, but it was higher than the group of C-H. Considering the groups the concentrations of CPT Mix-H group, the results can be interpreted as follows. It remains to be further verified whether the two compounds in terms of additivity, synergistic or antagonistic. CTP can cause oxidative stress in *D. magna*. There were several studies that showed that a significant increase of ROS generation, accompanied by increased T-AOC and MDA of *D. magna*, was induced by CTP exposure (Yang et al., 2018). The activity of Superoxide dismutase (SOD) of *D. magna* significantly increased, while ACHE activity was inhibited (Yang et al., 2017). The neurons and muscle fibers of *D. magna* were induced to change by toxic oxygen free radicals, which induced swimming behavior disorder (Bownik et al., 2020).The *D. magna* needs the coordination of nervous system to complete food filtering and feeding behavior. The neuroactive substance (chlorpromazine) can cause the lack of coordination and inhibition of feeding behavior of *D. magna* (de Alkimin et al., 2020). The addition of SSRI might enhance the movement behavior of *D. magna* (Campos et al., 2012a). When exposed to high concentrations of CTP, the ingestion rate was significantly reduced. It may be related to the loss of coordination caused by the stimulation of neuroactive substances to the motion of the *D. magna* and oxidative damage.

After 24 h of recovery, the ingestion rate increased by 34.2% and 21.5%, in the group of M-L and C-H respectively. This may be due to 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). Liu et al. (2019) found that *D. magna* enhanced its self-protection mechanism by eating more food to better adapt to the new environment when exposed to Bisphenol analogues (BPs).

3.2 Heart rate

CTP is a selective 5-hydroxytryptamine (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, the heartbeats of *D. magna* significantly increased in the exposed groups ($p < 0.01$) except Group Mix-H. For MTP, the heartbeats of *D. magna* increased with the increase of pollutant concentration, showing a concentration-dependent mode. 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 suggesting that *D. magna* may be damaged which was consistent with the findings of Liang et al. (2017), in which heartbeat was significantly stimulated by low-concentration perfluorooctane sulfonate (PFOS) and inhibited by high-concentration PFOS (the change from a low-dose stimulation to a high-dose inhibition). The heartbeats in the mix groups were lower than the single treatment groups. And the trend was from low dose stimulation to high dose inhibition, it was suggested that the mixed toxicity of the compounds revealed a synergistic toxicity occur.

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 further studies.

3.3 Enzyme activity

Digestive tract has primary sites of toxicant uptake in *D. magna*. Trypsin and α-amylase were synthesized in the digestive F-cell of crustaceans found in the alimentary tract (Lehnert and Johnson, 2002). The effects of CTP and MTP on the digestive enzymes (AMS and trypsin) of *D. magna* were investigated to identify the potential mechanisms 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 ($p > 0.05$), which 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 ($p < 0.05$). There was no significant change in Group C-L ($p > 0.05$). It 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). Stimulating 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 concentrations 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*

It was found that the pollutants could affect the feeding and nutrition-related enzyme activities of *D. magna*. The nutritional enzyme gene was used as exploratory data to further analyze the possible cause of damage to *D. magna* caused by pollutants. Chronic exposure to hexachlorocyclopendiene (HCCDP), Houde et al. (2013) used transcriptome tools to identify five significant differential in genomic transcription related to metabolic function, including amylase and trypsin. Therefore, gene transcription involved in digestive enzyme synthesis was selected for evaluation based on the changes of amylase and trypsin in order to further explain the possible mode of action of pollutants to *D. magna*. Figure 4A shows that the up-regulated significantly of transcription of AMS genes in all the exposed groups ($p < 0.01$). Moreover, the up-regulated of the Mix groups were smaller than the one of MTP group. This indicated that CTP, MTP and their mixture stimulated the transcription of AMS genes during exposure period and the mixed toxicity of the compounds revealed a synergistic toxicity. It has been reported that SSRI increases the oxygen consumption rate and aerobic catabolism of *D. magna*, and decreases the carbohydrate level of adult Daphnia magna (Campos et al., 2012b). The upregulation of digestive genes may be regarded as a compensation mechanism for reduced carbohydrate reserves (Soetaert et al., 2007). This was consistent with the results of the Houde et al. (2013), in which the transcription of AMS genes was stimulated in the groups added with hexachlorocyclopentadiene (HCCPD). However, this phenomenon was contrary to the results of Zhao et al. (2019), in which the transcription 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 transcription of AMS gene may be related to different pollutants. After 24 h of recovery, the damage of the target chemicals on *D. magna* was alleviated, but it requires further study to validate whether this damage can be completely removed.

In Fig. 3A, the activities of AMS increased significantly in MTP exposed daphnia after 24 h of exposure, and the gene transcription of AMS was up-regulated significantly (Fig. 4A). However, there was no significant correlation between gene transcription 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 transcription 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 both ($R^2 = 0.0002$, $P = 0.787$, Fig. 5B).And no significant correlation between gene transcription level and enzyme activity in trypsin. Houde et al. (2013), showed that the activity of AMS was decreased significantly while *D. magna* exposed to HCCPD, and the gene transcription levels of AMS were decreased significantly. Schwarzenberger and Fink (2018) found that there was significant correlation between enzyme activity and gene transcription. These results suggested that enzyme activity and gene transcription may exhibit different responses to various environmental stress. The Study of gene transcription can help understand the possible modes of action of pollutant stressors to aquatic organisms. Gene transcription is the basis of organism's response to external pressure. However, the relationship between gene transcription and related enzyme activity is complex. The lack of correlation between enzyme activity and gene transcription may be related to the time required for gene transcription and many different factors involved in gene transcription process, which increased the uncertainty of the transcription process (Ashouri and Farshbaf Pourabad, 2021).

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. 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 transcription 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 research is needed to determine the toxic pattern of those compound contaminations and investigate the potential mechanisms of their toxicity.

5. 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 & Dong Xu: carried out experiments, analyzed experimental results and wrote the paper; 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

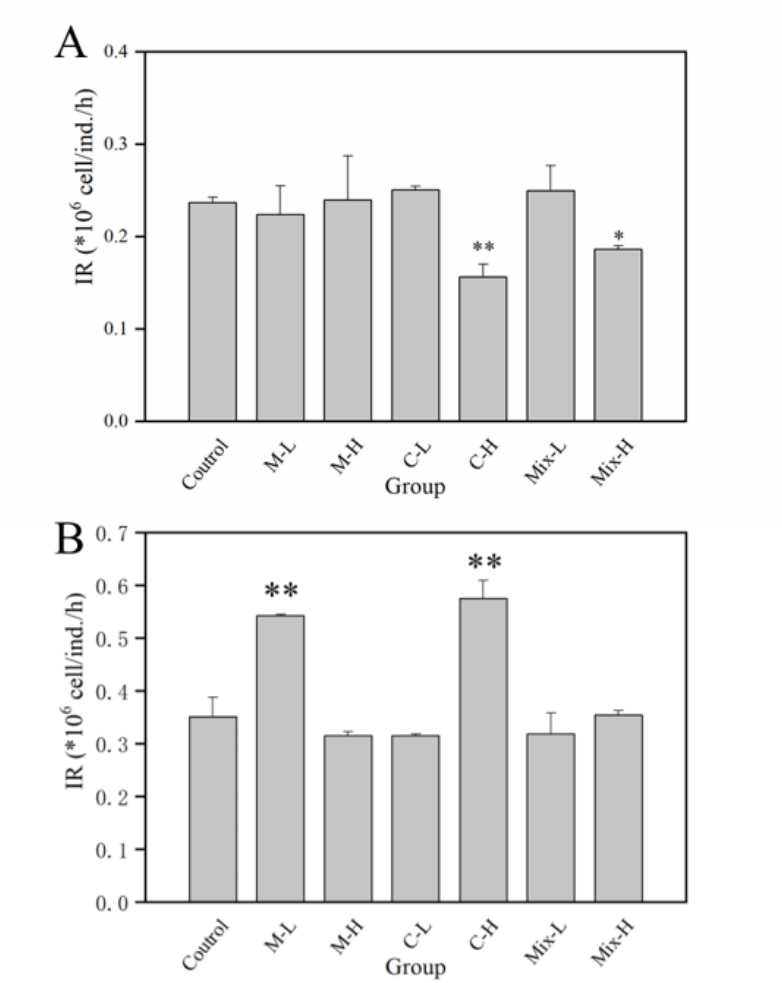

Figure 1

Effects of CTP and MTP on feeding behavior of D. magna after exposure period (A) and recovery period (B). Abbreviations: M-L, mirtazapine- low concentration; M-H, mirtazapine- high concentration; C-L, citalopram- low concentration; C-H, citalopram- high concentration; Mix-L, mixture- low concentration; Mix-H, mixture- high concentration; *: statistical significance of the correction ($p < 0.05$); **: statistical significance of the correction ($p < 0.01$).

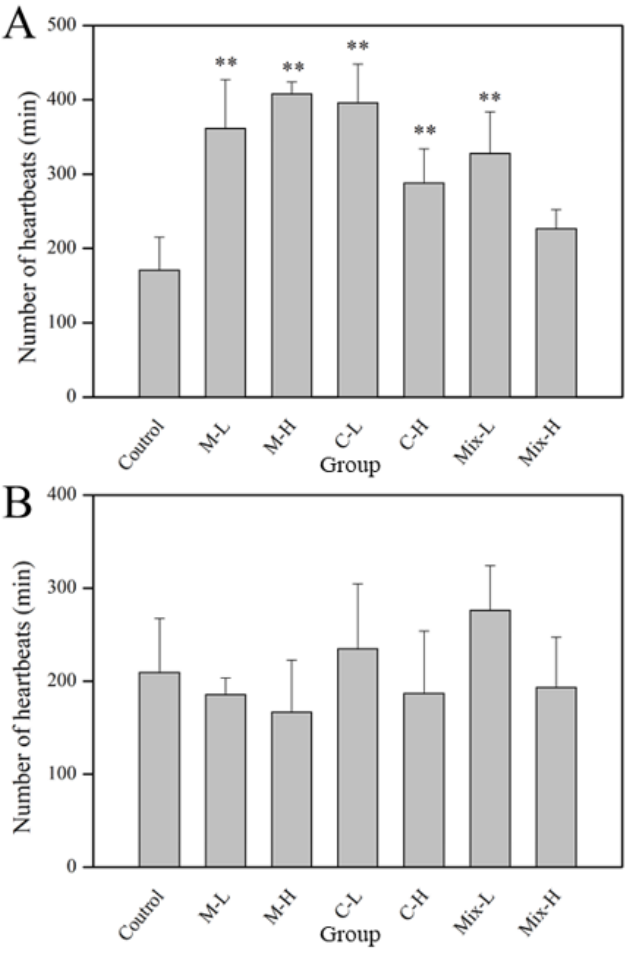

Figure 2

Effects of CTP and MTP on heartbeats of D. magna after exposure period (A) and recovery period (B). Abbreviations: M-L, mirtazapine- low concentration; M-H, mirtazapine- high concentration; C-L, citalopram- low concentration; C-H, citalopram- high concentration; Mix-L, citalopram and mirtazapine- low concentration; Mix-H, citalopram and mirtazapine- high concentration; *: statistical significance of the correction ($p < 0.05$); **: statistical significance of the correction ($p < 0.01$).

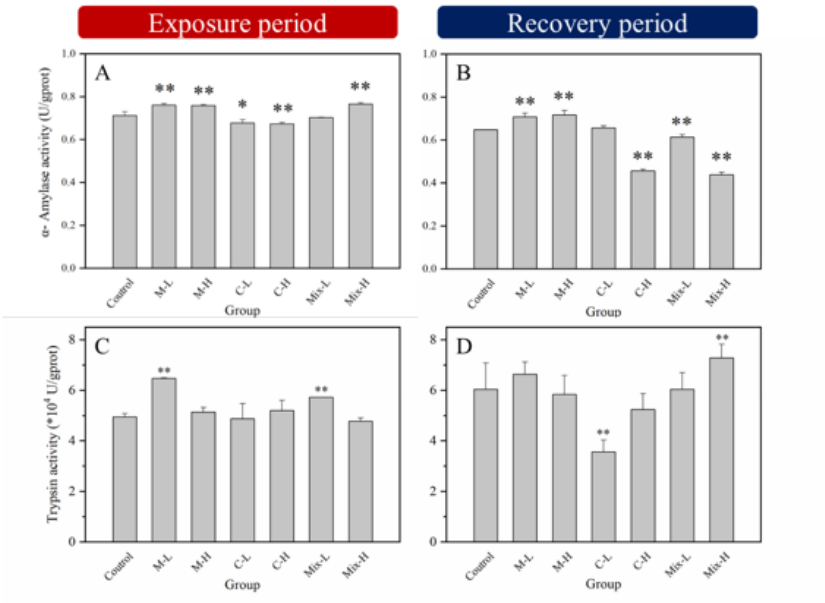

Figure 3

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. Abbreviations: M-L, mirtazapine- low concentration; M-H, mirtazapine- high concentration; C-L, citalopram- low concentration; C-H, citalopram- high concentration; Mix-L, citalopram and mirtazapine- low concentration; Mix-H, citalopram and mirtazapine- high concentration; *: statistical significance of the correction ($p < 0.05$); **: statistical significance of the correction ($p < 0.01$).

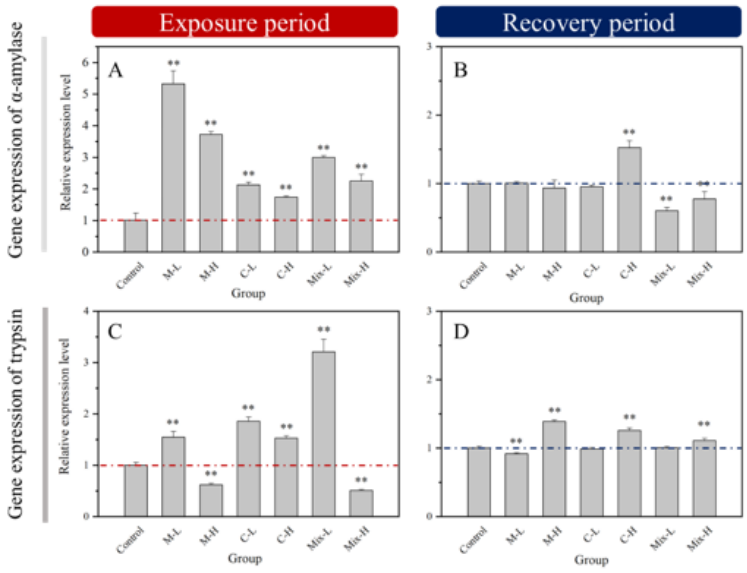

Figure 4

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). Abbreviations: M-L, mirtazapine- low concentration; M-H, mirtazapine- high concentration; C-L, citalopram- low concentration; C-H, citalopram- high concentration; Mix-L, citalopram and mirtazapine- low concentration; Mix-H, citalopram and mirtazapine- high concentration; *: statistical significance of the correction ($p < 0.05$); **: statistical significance of the correction ($p < 0.01$).

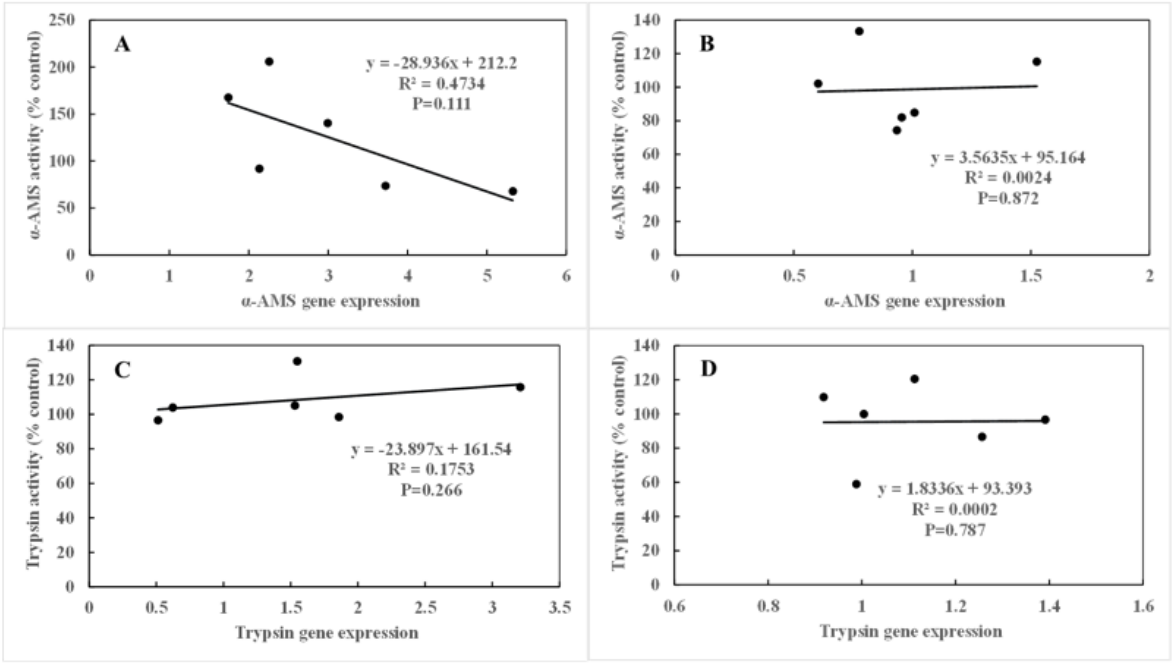

Figure 5

Relationship between gene expression and enzyme activity in exposure daphnia. A: 24 h-AMS; B: 48 h-AMS; C: 24 h-trypsin and D: 48 h-trypsin. Gene expression and enzyme activity are listed in X-axis and Y-axis, respectively. Regression lines and R2 values are delineated.

Supplementary Files

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