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Original Article

# Environmentally relevant methylphenidate exposure impairs antioxidant and cholinesterase activities in Nile tilapia (*Oreochromis niloticus*)

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**Abstract**

Methylphenidate (MPH) is a widely prescribed psychostimulant whose release into aquatic environments raises concerns about its potential biochemical effects on non-target organisms. This study evaluated the effects of MPH at environmentally relevant concentrations (20, 100, and 200 ng $L^{-1}$) following a 5-day exposure in adult male Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758). The effects of MPH were assessed through biomarkers related to antioxidant defenses, biotransformation, lipid peroxidation, and esterase activity. All MPH-exposed groups exhibited reduced catalase (CAT) and glutathione peroxidase (GPx) activities in the gills compared with the control group. No significant alterations were observed in ethoxyresorufin-O-deethylase (EROD) or benzyloxyresorufin-O-dealkylase (BROD) activities in either the liver or gills. Acetylcholinesterase (AChE) activity decreased in the gills of fish exposed to 100 and 200 ng $L^{-1}$ MPH. Lipid peroxidation levels were significantly reduced in the liver of all MPH-treated groups, while no changes were detected in the gills. These findings indicate that environmentally relevant concentrations of MPH can impair antioxidant and esterase activities, particularly in gill tissue, without inducing lipid peroxidation, highlighting the potential biochemical effects of this pharmaceutical on non-target aquatic organisms.

**Keywords**: Biochemical biomarkers; oxidative stress; enzymatic inhibition; aquatic ecotoxicology; biotransformation; lipid peroxidation.

## INTRODUCTION

The increasing occurrence of emerging contaminants, particularly pharmaceuticals, in surface waters has become a significant environmental concern, as conventional wastewater treatment plants (WWTPs) may not effectively remove these compounds (Ahmed & Hameed, 2018; Hejna *et al*., 2022). Consequently, pharmaceutical residues can be discharged into freshwater and marine ecosystems, potentially affecting aquatic organisms and ecosystem functioning (Ortúzar *et al*., 2022). A global survey of pharmaceutical pollution in rivers revealed the widespread occurrence of these contaminants, with at least one pharmaceutical exceeding concentrations considered safe for aquatic organisms or thresholds of concern for antimicrobial resistance at 25.7% of the sampling sites (Wilkinson *et al.*, 2022). Several classes of pharmaceuticals, including anti-inflammatory drugs (Luo *et al*., 2014), antidepressants (Arnnok *et al*., 2017), and antibiotics (Danner *et al.*, 2019), have been detected in wastewater

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and aquatic environments. Their removal efficiency varies depending on the physicochemical properties of the compounds and the treatment processes employed (Hejna *et al.*, 2022).

The environmental occurrence of pharmaceutical residues can also be understood within the One Health framework, which recognizes the interdependence of human, animal, and environmental health. Wang *et al*. (2024) highlighted that the increasing diversity and environmental distribution of emerging contaminants require integrated approaches to assess their potential risks to ecosystems and human health. Focusing specifically on medicines and biocides, Anadón *et al*. (2025) emphasized the importance of balancing their benefits for human and animal health with their potential environmental impacts through integrated sustainability assessments.

Furthermore, Cristini *et al*. (2025) discussed the complementary roles of environmental monitoring and *in vivo* studies in evaluating the ecological consequences of pharmaceutical contamination, including alterations in the behavior of aquatic organisms. Together, these perspectives reinforce the relevance of investigating the biochemical responses of non-target aquatic species to environmentally relevant concentrations of pharmaceuticals.

Following administration, pharmaceuticals may be partially metabolized and subsequently excreted as metabolites or unchanged compounds, contributing to their release into wastewater systems (Bunch & Bernot, 2011). Continuous consumption and discharge can result in the recurrent introduction of pharmaceutical residues into aquatic environments, even when individual compounds exhibit limited environmental persistence (Halling-Sørensen *et al.*, 1998; Glassmeyer *et al*., 2005). These contaminants have been detected at concentrations ranging from ng $L^{-1}$ to µg $L^{-1}$ (Andreu *et al.*, 2016; Chen *et al*., 2011; Cortez *et al.*, 2018), which may be sufficient to induce biological responses in aquatic organisms, including endocrine, behavioral, and reproductive alterations (Gonzalez-Rey *et al.*, 2014).

Methylphenidate (MPH) is a widely prescribed psychostimulant whose environmental occurrence has raised concerns regarding its potential effects on non-target aquatic organisms. Its metabolite, ritalinic acid, has been detected in wastewater effluents, indicating the release of MPH-related residues into aquatic environments (Letzel *et al*., 2010). Conventional wastewater treatment processes may not completely eliminate pharmaceutical residues, including MPH-related compounds, highlighting the importance of investigating their potential environmental effects (Heberer, 2002). Although the pharmacological mechanisms of MPH in mammals are well characterized (Swanson & Volkow, 2002; Castellanos *et al.*, 1996; Volkow *et al.*, 2005), its biochemical effects on aquatic organisms remain insufficiently understood.

Following uptake, xenobiotics may undergo enzymatic biotransformation, which generally facilitates their elimination through the conversion of lipophilic compounds into more water-soluble metabolites (Livingstone, 2001). Biotransformation is commonly divided into phase I reactions, involving oxidation, reduction, or hydrolysis, and phase II reactions, involving conjugation with endogenous molecules (van der Oost *et al.*, 2003). Phase I biotransformation involves cytochrome P450-dependent monooxygenase activities, including ethoxyresorufin-O-deethylase (EROD) and benzyloxyresorufin-O-dealkylase (BROD), which are commonly used as biomarkers of xenobiotic metabolism in aquatic organisms (van der Oost *et al.,* 2003).

Xenobiotic exposure and biotransformation may alter cellular redox balance by promoting the generation of reactive oxygen species (ROS), potentially resulting in oxidative damage to cellular macromolecules (Dzul-Caamal *et al.*, 2014; Sakuragui *et al.*, 2013; Vioque-Fernández *et al.*, 2009). To counteract the potentially harmful effects of ROS, organisms possess enzymatic and non-enzymatic antioxidant defense systems, including catalase (CAT) and glutathione peroxidase (GPx), which contribute to the regulation of oxidative balance (Lushchak, 2011). Furthermore, acetylcholinesterase (AChE) activity has been widely used as a biomarker of neurotoxic effects in aquatic organisms, providing valuable information for assessing the biological effects of environmental contaminants on non-target species (Lionetto *et al.*, 2013; Nunes, 2010). AChE is responsible for the hydrolysis of acetylcholine, a neurotransmitter involved in cholinergic signaling. Inhibition of AChE may lead to acetylcholine accumulation and disruption of normal cholinergic function, potentially resulting in adverse physiological effects (De Lima *et al.*, 2013; Peakall, 1992).

*Oreochromis niloticus* is one of the most widely farmed freshwater fish species worldwide, particularly in tropical and subtropical regions. Its fast growth, adaptability, environmental tolerance, and economic importance make it a relevant model for ecotoxicological investigations involving aquatic contaminants (El-Sayed, 2006; FAO, 2020).

Previous studies have reported behavioral and neurochemical effects of MPH exposure in fish (Endres *et al.*, 2017; Levin *et al.*, 2011; De Serrano *et al.*, 2021;

Batalhão *et al.,* 2019). Specifically, Batalhão *et al*. (2019) demonstrated that exposure to 100 ng $L^{-1}$ MPH increased aggressive behavior and reduced brain dopamine (DA) and serotonin (5-HT) levels in male Nile tilapia. At this concentration, tyrosine hydroxylase (TH) transcript levels increased, whereas exposure to 20 ng $L^{-1}$ increased D5 dopamine receptor transcript levels. These findings indicate that MPH can alter behavioral responses and monoaminergic signaling in fish. However, the effects of MPH on antioxidant defenses, lipid peroxidation, cytochrome P450-related biotransformation activities, and acetylcholinesterase activity in the liver and gills remain insufficiently characterized. Investigating these biochemical endpoints may provide complementary information on the physiological responses of fish to environmentally relevant MPH exposure.

The present study used biological samples obtained from the same experimental exposure previously reported by Batalhão *et al.* (2019), involving the same animals and biological material. However, the present investigation addresses distinct biochemical endpoints that were not reported in that publication. Specifically, this study aimed to evaluate the effects of environmentally relevant concentrations of MPH on catalase (CAT), glutathione peroxidase (GPx), ethoxyresorufin-O-deethylase (EROD), benzyloxyresorufin-O-dealkylase (BROD), and acetylcholinesterase (AChE) activities, as well as lipid peroxidation assessed by thiobarbituric acid-reactive substances (TBARS), in the liver and gills of adult male *O. niloticus*. By examining these endpoints, the present study seeks to expand the understanding of biochemical responses to MPH exposure in non-target aquatic organisms while providing findings complementary to those previously published by Batalhão *et al.* (2019).

## MATERIALS AND METHODS

### Animals and Acclimation

Adult male *O. niloticus* (mean length: 12.9 ± 1.2 cm; mean weight: 61.4 ± 18.2 g) were sourced from the Aquaculture Center at São Paulo State University (UNESP), São José do Rio Preto, Brazil. Fish were randomly selected and acclimated for 30 days in 500 L tanks, maintaining a stocking density of 5 L per fish, with continuous aeration. As previously described, environmental conditions were set to a stable temperature of 27 °C and a photoperiod of 12L:12D. Fish were fed twice daily with commercial fish feed (Guabi-Pirá, Brazil) at 3% of biomass. This study was conducted following the guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Animal Research Ethics Committee (CEUA) at São Paulo State University (UNESP), São José do Rio Preto, Brazil (protocol nº. 109/2015).

Only adult males were used in order to minimize physiological variability associated with sex-specific differences, particularly those related to reproductive status, hormonal fluctuations, or metabolic differences that could influence antioxidant capacity and xenobiotic metabolism. Standardizing the sex and developmental stage of the animals increases experimental reliability and strengthens the interpretation of biochemical responses related to MPH exposure.

### Exposure to Methylphenidate

Methylphenidate (>98% purity, Sigma-Aldrich, CAS: 1219804-02-0) was dissolved in acetone (>98% purity, Sigma-Aldrich, CAS 67-64-1) and added to the exposure aquaria to achieve the desired concentrations, with a final acetone concentration of 0.001% (v/v; below the maximum concentrations of 0.01% recommended by the OECD for solvents in ecotoxicological tests; OECD, 2019). The control group was also exposed to the same acetone volume to minimize solvent effects.

After the acclimation period, the fish were divided into four groups of nine individuals (N = 9 per treatment) and individually placed in glass aquaria containing 17 L of dechlorinated water. Fish were exposed for five days to the following treatments: a solvent control group, with no addition of MPH but with acetone at same volume used for MPH solubilization (here named as the control group), and three experimental groups exposed to 20, 100, and 200 ng $L^{-1}$ of MPH. The five-day exposure period was established in the experimental design previously reported by Batalhão *et al.* (2019). This duration was used to investigate biochemical responses to environmentally relevant MPH concentrations rather than conventional acute toxicity endpoints, and also considering the half-life of MPH in water (~48 h, McCallum *et al.*, 2019).

The environmental relevance of the concentrations selected for this study is supported by reports of residues associated with MPH use in aquatic environments. Wastewater monitoring studies have documented MPH-related residues in different regions, including Sweden (Östman *et al.*, 2014). Its primary metabolite, ritalinic acid, has been detected in treated wastewater effluents in Germany at concentrations ranging from below 50 to 170 ng $L^{-1}$ (Letzel *et al.,*

2010). Furthermore, Burgard *et al*. (2013) reported ritalinic acid concentrations reaching 1,500 ng $L^{-1}$ in untreated wastewater. Although these measurements primarily concern the metabolite rather than the parent compound, they demonstrate the release of MPH-related residues into wastewater systems and provide environmental context for investigating the effects of MPH on aquatic organisms. Considering the similarity in per capita pharmaceutical loads among industrialized countries and the fact that approximately half of the wastewater produced in Brazil is discharged untreated into aquatic environments, concentrations of MPH used in this study were selected to simulate a condition similar to a realistic contamination scenario.

During the experimental period, fish were fed the same diet used during acclimation, corresponding to 3% of biomass, provided once daily. Photoperiod was maintained at 12L:12D, and no differences were observed in dissolved oxygen levels (5.3 ± 1.4 mg $L^{-1}$), pH (7.8 ± 1.1), temperature (25.8 ± 1.6 °C), or ammonia (0.09 ± 0.05 mg $L^{-1}$) across experimental groups throughout the five-day exposure period.

**Anesthesia, Euthanasia, and Tissue Collection**

At the end of the experimental exposure period and after the behavioral assessments, fish were anesthetized with benzocaine (9 mg $L^{-1}$) for blood collection and subsequently euthanized with a lethal dose of benzocaine (28 mg $L^{-1}$), as previously described by Batalhão *et al.* (2019). Liver tissue and gill tissue fragments were collected immediately after euthanasia and stored at −80 °C until subsequent biochemical analyses. The gill tissue fragments were subsequently subdivided into portions for the different biochemical analyses.

**Sample Preparation**

The liver and gills of the fish were homogenized at a ratio of 1:4 (w/v) in 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.15 M KCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged at 10,000 g for 20 minutes at 4 ºC. The resulting supernatant was collected and centrifuged at 50,000 g for one hour at 4 ºC.

The obtained supernatant was stored at -80 ºC for analyses of CAT and GPx activity, while the pellet was resuspended in 100 µL of 0.1 M Tris buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 0.1 M KCl, and 20% glycerol, and used for EROD and BROD analyses. For the determination of acetylcholinesterase (AChE) activities, the samples were homogenized at a ratio of 1:4 (w/v) in 0.1 M Tris-HCl buffer, pH 8.0, centrifuged at 10,000 g for 30 minutes at 4 ºC, and the supernatant was collected for enzyme activity quantification. Protein quantification was performed following the Bradford (1976) methodology.

**Enzymatic Assays**

Catalase (CAT) activity was evaluated using a Thermo Evolution 300 double-beam spectrophotometer, following the method described by Beutler (1975). This method is based on the decomposition of hydrogen peroxide by CAT, monitoring the decrease in absorbance at 240 nm at 30 ºC for 1 minute.

Glutathione peroxidase (GPx) activity was measured using a Victor™ X3 microplate reader (Perkin Elmer®). The evaluation of GPx involved monitoring the oxidation of NADPH, which is linked to the reduction of glutathione disulfide mediated by glutathione reductase, using t-butyl hydroperoxide as a substrate, as described by Chance *et al*. (1979). The activities of CAT and GPx were expressed in units per milligram of protein ($U.mg^{-1}$). EROD and BROD activities were determined fluorometrically in the microsomal fraction using 7-ethoxyresorufin and 7-benzyloxyresorufin as substrates, respectively, based on the approach described by Burke *et al.* (1985), with modifications. Resorufin formation was monitored at excitation and emission wavelengths of 537 and 583 nm, respectively, for 3 min at 30 °C using a Victor™ X3 microplate reader (PerkinElmer) in the presence of NADPH. Enzyme activities were expressed as pmol $min^{-1}$ $mg^{-1}$ protein.

Acetylcholinesterase (AChE) activity was evaluated following the methodology described by Ellman *et al.* (1961), monitoring the formation of the reaction product between thiocholine, generated by the enzymatic hydrolysis of acetylthiocholine, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 405 nm and 25 °C. Readings were performed using the Victor™ X3 microplate reader (Perkin Elmer), and the enzymatic activity was expressed in units per milligram of protein ($U.mg^{-1}$).

**Lipid Peroxidation**

The level of lipid peroxidation was assessed in the gills and liver of the fish following the protocol proposed by Esterbauer *et al*. (1991), with minor modifications. The tissues were homogenized in a 1:3 (w/v) ratio in 100 mM Tris–HCl buffer, pH 8.0. After homogenization, 300 µL of a 0.4% solution of 2-thiobarbituric acid (TBA) diluted in 0.2 M HCl was added to each sample.

The mixture was then incubated at added to each sample. The mixture was then incubated at 90 °C for 40 minutes. Following incubation, the samples were cooled on ice for five minutes to stop the reaction. A liquid-liquid extraction was then performed using 1 mL of n-butanol, followed by centrifugation at 2,800 × g for 5 minutes. Finally, the absorbance of the supernatant was measured at 535 nm using a Victor™ X3 microplate reader (Perkin Elmer). The MDA-TBA reaction product was quantified based on a calibration curve using malondialdehyde (MDA) obtained from the hydrolysis of tetramethoxypropane (TMP). Data were expressed as pmol MDA/mg tissue.

**Statistical Analyses**

Statistical analyses were conducted using Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA). The normality of the data was assessed using Normal Probability Plots of Residuals, while homoscedasticity was evaluated with Levene's test, assuming a significance level of 0.05. Experimental groups were compared using a General Linear Model (GLM) in a one-way ANOVA format (Quinn & Keough, 2002).When applicable, Fisher's *post hoc* test was applied to evaluate significant differences among groups. Graphs were generated using GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA), and data were expressed as mean ± standard deviation (SD). Statistical significance was considered at $P < 0.05$. Each fish was treated as an independent experimental unit.

## RESULTS

**CAT and GPx Activities and TBARS Levels**

The activity of the antioxidant enzyme catalase (CAT) in the liver showed no significant differences between the control group and those exposed to MPH (Fig. 1A). However, in the gills, CAT activity was significantly reduced at all tested MPH concentrations compared with the control group. Fish exposed to 20, 100, and 200 ng $L^{-1}$ of MPH displayed approximately 2.2-, 5.0-, and 4.1-fold decreases, respectively, compared to the control ($p < 0.05$). Glutathione peroxidase (GPx) activity was also evaluated, revealing no significant changes in hepatic GPx activity relative to the control group (Fig. 1B). In the gills, GPx activity followed a similar inhibition pattern as observed for CAT, with reductions of 3.2-, 2.7-, and 3.7-fold in fish exposed to 20, 100, and 200 ng $L^{-1}$ of MPH, respectively.

These findings indicate that MPH exposure reduced GPx activity in the gills at all tested concentrations. Lipid peroxidation levels, measured as TBARS (Fig. 1C), were significantly reduced in the liver of fish across all groups exposed to MPH, with decreases of 1.5, 1.5, and 1.7 times observed in the groups exposed to 20, 100, and 200 ng $L^{-1}$ MPH, respectively, compared to the control. In the gills, lipid peroxidation levels did not change significantly among the groups.

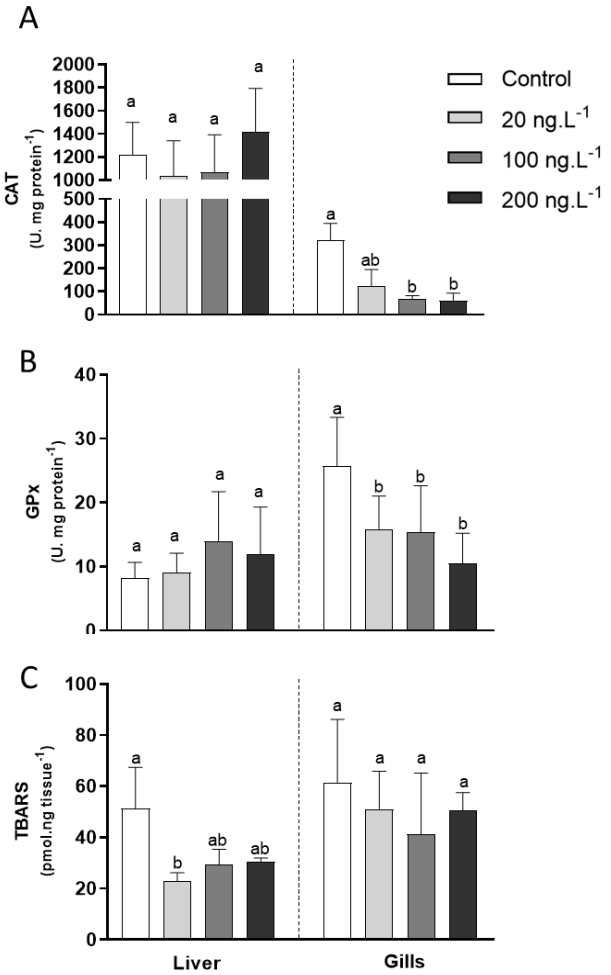


**Figure 1.** Antioxidant responses in the liver and gills of adult male Nile tilapia (*Oreochromis niloticus*) exposed to methylphenidate (MPH) for 5 days. (A) Catalase (CAT) activity; (B) Glutathione peroxidase (GPx) activity; and (C) Lipid peroxidation levels (TBARS) in fish exposed to 0, 20, 100, and 200 ng·$L^{-1}$ of MPH. Data are presented as mean ± standard deviation (n = 9). Different letters indicate significant differences among treatments within each tissue independently ($P < 0.05$). Treatments sharing the same letter are not significantly different.

**Activity of EROD and BROD**

The activities of the detoxification enzymes ethoxyresorufin-O-deethylase (EROD) and benzyloxyresorufin-O-dealkylase (BROD) were assessed in the liver and gills. No significant changes were observed in EROD (Fig. 2A) or BROD (Fig. 2B) activities in either organ at any tested concentration of

MPH.

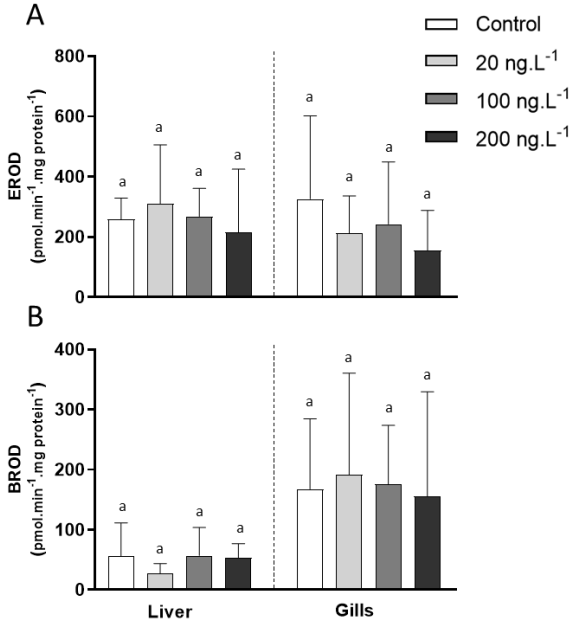


**Figure 2.** Biotransformation enzyme activity in the liver and gills of adult male Nile tilapia (*Oreochromis niloticus*) exposed to methylphenidate (MPH) for 5 days. (A) Ethoxyresorufin-O-deethylase (EROD) activity; and (B) Benzyloxyresorufin-O-dealkylase (BROD) activity in fish exposed to 0, 20, 100, and 200 ng·L⁻¹ of MPH. Data are presented as mean ± standard deviation (n = 9). No significant differences were observed among treatments within either tissue (P > 0.05).

**Activity of AChE**

Hepatic acetylcholinesterase (AChE) activity remained unaltered across all tested groups (Fig. 3A). In the gills, AChE activity showed a significant decrease in fish exposed to 100 and 200 ng $L^{-1}$ of MPH, with reductions of 1.6- and 1.4-fold, respectively, compared to the control group ($p < 0.01$). These results indicate that MPH exposure inhibited AChE activity in the gills at 100 and 200 ng $L^{-1}$.

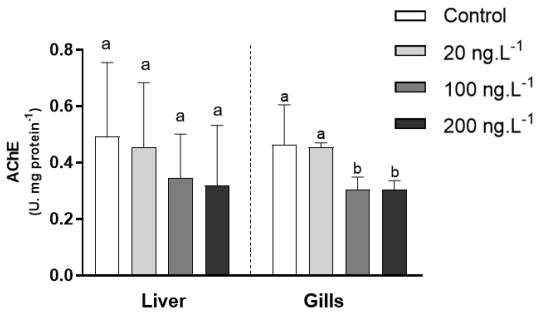


**Figure 3.** Acetylcholinesterase (AChE) activity in the liver (A) and gills (B) of adult male Nile tilapia (*Oreochromis niloticus*) exposed to methylphenidate (MPH) at concentrations of 0 (control), 20, 100, and 200 ng $L^{-1}$ for 5 days. Data are expressed as mean ± standard deviation (n = 9 per treatment). Different letters indicate statistically significant differences among treatments within the same tissue ($p < 0.05$). Treatments sharing the same letter do not differ significantly. No significant differences were observed among treatments in the liver.

## DISCUSSION

The present study demonstrated that exposure to MPH in *O. niloticu*s resulted in significant biochemical alterations, particularly in the gills. Previous investigations have demonstrated that MPH can affect behavioral and neurochemical responses in fish, including alterations in aggressive behavior, brain monoamine levels, and dopamine-related gene transcription in Nile tilapia (Batalhão *et al.*, 2019). However, the effects of environmentally relevant MPH concentrations on antioxidant defenses, lipid peroxidation, cytochrome P450-related biotransformation activities, and acetylcholinesterase activity in the liver and gills remain insufficiently characterized. By examining these complementary biochemical endpoints, the present study extends the understanding of tissue-specific responses to MPH exposure in fish.

Mechanistically, MPH inhibits the dopamine transporter, thereby increasing extracellular dopamine availability (Volkow *et al.*, 1998). Alterations in dopaminergic signaling may influence cellular redox homeostasis, as dopamine metabolism can contribute to the generation of reactive oxygen species (ROS) (Miyazaki & Asanuma, 2008). Previous studies have also reported oxidative alterations following MPH exposure (Martins *et al.*, 2006), while evidence from other pharmaceutical and stimulant exposures suggests potential interactions between oxidative stress and mitochondrial function (Virmani *et al.*, 2002; Cardoso-Vera *et al.*, 2024). However, these mechanisms were not directly investigated in the present study.

In this context, exposure to MPH may trigger compensatory physiological responses, including the activation of adaptive antioxidant mechanisms, as reported in other models exposed to psychostimulants (Freddo *et al.,* 2021). Based on these potential adaptive responses, MPH exposure at environmentally relevant concentrations could be expected to stimulate antioxidant defenses as a protective mechanism against oxidative damage. However, the direction and magnitude of these responses may depend on the tissue examined and the experimental conditions.

However, our results indicate an inhibitory effect of MPH on CAT and GPx activities in the gills but not in the liver, revealing a more pronounced biochemical response in gill tissue. This differential effect between the gills and the liver may be associated

with the direct exposure of gill tissue to waterborne contaminants, potentially contributing to tissue-specific biochemical responses (Ben Ameur *et al.,* 2015). Previous studies have also reported alterations in oxidative stress-related biomarkers following MPH exposure in rodents (Comim *et al.*, 2014), suggesting that antioxidant responses to this compound may vary according to the tissue and experimental conditions. The inhibition of CAT and GPx observed in the gills of Nile tilapia suggests that MPH may compromise antioxidant defenses, potentially increasing susceptibility to oxidative damage under environmental exposure conditions (e.g., Parida & Sahoo, 2024). These findings highlight the sensitivity of gill tissue to MPH exposure and reinforce its relevance for assessing biochemical responses to aquatic contaminants.

Cytochrome P450-dependent enzymes play important roles in the biotransformation of xenobiotics, including pharmaceuticals, in aquatic organisms. In the present study, no significant changes were observed in EROD and BROD activities in the liver or gills of Nile tilapia exposed to MPH. These findings suggest that the evaluated cytochrome P450-related enzymatic activities were not significantly modulated under the experimental conditions. However, the absence of changes in these enzymatic biomarkers does not exclude the involvement of other biotransformation pathways in MPH metabolism.

Acetylcholinesterase (AChE) inhibition is widely recognized as a biomarker of neurotoxicity in aquatic ecotoxicology, commonly applied in studies with fish and invertebrates. For instance, Freddo *et al.* (2026) reported a significant reduction in brain AChE activity in zebrafish (*Danio rerio*) following repeated exposure to a high concentration of MPH, accompanied by behavioral alterations involving locomotion, anxiety-related responses, and memory. These findings suggest that MPH exposure can affect cholinergic activity alongside behavioral responses in fish. Although AChE inhibition was observed in the gills rather than the brain in the present study, the findings are consistent with the sensitivity of this enzyme to MPH exposure across different fish species and tissues. Together, these observations highlight the relevance of investigating cholinergic biomarkers alongside behavioral endpoints to better understand the potential neurobehavioral effects of MPH in aquatic organisms. AChE plays a key role in regulating cholinergic neurotransmission by hydrolyzing acetylcholine in the synaptic cleft (Zimmerman & Soreq, 2006).

In the present study, MPH exposure significantly inhibited AChE activity in the gills at 100 and 200 ng $L^{-1}$, suggesting that cholinergic-related biochemical processes may be affected in this tissue. This finding is consistent with observations by Solé *et al.* (2010), who reported AChE inhibition in the gills of mussels (*Mytilus galloprovincialis*) exposed to acetaminophen. Additionally, Arunagiri and Balamurugan (2016) reported reduced brain AChE activity in mice following repeated MPH administration. Although these studies involved different species, tissues, and exposure conditions, they provide complementary evidence that AChE activity can be affected by pharmaceutical exposure, including MPH.

In the present study, we assessed lipid peroxidation in *O. niloticus* following exposure to MPH. No significant changes in TBARS levels were observed in the gills; however, a consistent decrease in lipid peroxidation was detected in the liver across all exposed groups compared with the control. Reductions in lipid peroxidation following pollutant exposure may be associated with changes in antioxidant defenses or the metabolism of lipid peroxidation products. Garcia *et al*. (2020) reported decreased malondialdehyde levels in *Astyanax altiparanae* exposed to diesel, suggesting the involvement of hepatic aldehyde metabolism. The reduction in hepatic TBARS levels may similarly reflect alterations in the metabolism of lipid peroxidation products rather than an increase in antioxidant enzyme activities. However, as aldehyde dehydrogenase activity was not evaluated, this mechanism remains a hypothesis requiring further investigation.

## CONCLUSION

Exposure to MPH at environmentally relevant concentrations induced tissue-specific biochemical alterations in adult male *Oreochromis niloticus*, with more pronounced effects in the gills than in the liver. Significant reductions in catalase (CAT) and glutathione peroxidase (GPx) activities were observed in the gills, indicating alterations in antioxidant defenses.

Acetylcholinesterase (AChE) activity was also reduced at 100 and 200 ng $L^{-1}$ MPH, suggesting that cholinergic-related biochemical processes may be affected in this tissue. Despite the inhibition of antioxidant enzymes, no significant increase in lipid peroxidation was detected in the gills, whereas hepatic TBARS levels decreased across all exposed groups.

EROD and BROD activities remained unchanged in both tissues under the experimental conditions. These findings provide additional evidence of the biochemical effects of environmentally relevant MPH exposure in Nile tilapia, complementing

previously reported behavioral and neurochemical responses. Further studies are needed to elucidate the mechanisms underlying these tissue-specific alterations and their potential physiological and ecological consequences.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

**IGB**: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft Preparation. **DL**: Methodology, Investigation, Laboratory Analyses, Data Curation, Writing – Review & Editing. **CNPB**: Conceptualization, Investigation, Writing – Review & Editing. **DGHS**: Formal Analysis, Visualization, Writing – Review & Editing. **EAA**: Conceptualization, Supervision, Resources, Funding Acquisition, Project Administration, Writing – Review & Editing.

## REFERENCES

Ahmed, M. J., & Hameed, B. H. (2018). Removal of emerging pharmaceutical contaminants by adsorption in a fixed-bed column: A review. Ecotoxicology and Environmental Safety, 149, 257–266.

Anadón, A., Bridges, J., & Tarazona, J. V. (2025). Sustainability of medicines and biocides. A One Health approach. Environment International, 203, 109768. https://doi.org/10.1016/j.envint.2025.109768

Andreu, V., Gimeno-García, E., Pascual, J. A., Vazquez-Roig, P., & Picó, Y. (2016). Presence of pharmaceuticals and heavy metals in the waters of a Mediterranean coastal wetland: Potential interactions and the influence of the environment. Science of the Total Environment, 540, 278–286.

Arnnok, P., Singh, R. R., Burakham, R., Pérez-Fuentetaja, A., & Aga, D. S. (2017). Selective Uptake and Bioaccumulation of Antidepressants in Fish from Effluent-Impacted Niagara River. Environmental science & technology, 51(18), 10652–10662. https://doi.org/10.1021/acs.est.7b02912

Arunagiri, P., & Balamurugan, E. (2016). Omega-3 fatty acids combined with aripiprazole and lithium modulates activity of mitochondrial enzymes and acetylcholinesterase in methylphenidate-induced animal model of mania. PharmaNutrition, 4(2), 54–61.

Batalhão, I. G., Lima, D., Russi, A. P. M., Boscolo, C. N. P., Silva, D. G. H., Pereira, T. S. B., Bainy, A. C. D., & de Almeida, E. A. (2019). Effects of methylphenidate on the aggressive behavior, serotonin and dopamine levels, and dopamine-related gene transcription in brain of male Nile tilapia (*Oreochromis niloticus*). Fish Physiology and Biochemistry, 45(4), 1377–1391. https://doi.org/10.1007/s10695-019-00645-2

Ben Ameur, W., El Megdiche, Y., de Lapuente, J., Barhoumi, B., Trabelsi, S., Ennaceur, S., Camps, L., Serret, J., Ramos-López, D., Gonzalez-Linares, J., Touil, S., Driss, M. R., & Borràs, M. (2015). Oxidative stress, genotoxicity and histopathology biomarker responses in Mugil cephalus and Dicentrarchus labrax gill exposed to persistent pollutants. A field study in the Bizerte Lagoon: Tunisia. Chemosphere, 135, 67–74. https://doi.org/10.1016/j.chemosphere.2015.02.050

Beutler, E. (1975). Red cell metabolism: A manual of biochemical methods (2nd ed.). Grune & Stratton.

Bunch, A. R., & Bernot, M. J. (2011). Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. Ecotoxicology, 20(1), 97–109. https://doi.org/10.1007/s10646-010-0560-6

Burgard, D. A., Fuller, R., Becker, B., Ferrell, R., & Dinglasan-Panlilio, M. J. (2013). Potential trends in attention deficit hyperactivity disorder (ADHD) drug use on a college campus: Wastewater analysis of amphetamine and ritalinic acid. Science of the Total Environment, 450–451, 242–249. https://doi.org/10.1016/j.scitotenv.2013.02.020

Burke, M. D., Thompson, S., Elcombe, C. R., Halpert, J., Haaparanta, T., & Mayer, R. T. (1985). Ethoxy-, pentoxy- and benzyloxyphenoxazones and

homologues: A series of substrates to distinguish between different induced cytochromes P-450. Biochemical Pharmacology, 34(18), 3337–3345. https://doi.org/10.1016/0006-2952(85)90355-7

Castellanos, F. X., Elia, J., Kruesi, M. J. P., Marsh, W. L., Gulotta, C. S., Potter, W. Z., Ritchie, G. F., Hamburger, S. D., & Rapoport, J. L. (1996). Cerebrospinal fluid homovanillic acid predicts behavioral response to stimulants in 45 boys with attention deficit/hyperactivity disorder. Neuropsychopharmacology, 14(2), 125–137. https://doi.org/10.1016/0893-133X(95)00077-Q

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. Physiological Reviews, 59(3), 527–605. https://doi.org/10.1152/physrev.1979.59.3.527

Chen, F., Ying, G.-G., Kong, L.-X., Wang, L., Zhao, J.-L., Zhou, L.-J., & Zhang, L.-J. (2011). Distribution and accumulation of endocrine-disrupting chemicals and pharmaceuticals in wastewater irrigated soils in Hebei, China. Environmental Pollution, 159(6), 1490–1498. https://doi.org/10.1016/j.envpol.2011.03.016

Comim, C. M., Gomes, K. M., Réus, G. Z., Petronilho, F., Ferreira, G. K., Streck, E. L., Dal-Pizzol, F., & Quevedo, J. (2014). Methylphenidate treatment causes oxidative stress and alters energetic metabolism in an animal model of attention-deficit hyperactivity disorder. Acta Neuropsychiatrica, 26(2), 96–103.

Cortez, F. S., Souza, L. S., Guimarães, L. L., Almeida, J. E., Pusceddu, F. H., Maranho, L. A., Mota, L. G., Nobre, C. R., Moreno, B. B., Abessa, D. M. S., Cesar, A., Santos, A. R., & Pereira, C. D. S. (2018). Ecotoxicological effects of losartan on the brown mussel Perna perna and its occurrence in seawater from Santos Bay (Brazil). Science of the Total Environment, 637–638, 1363–1371. https://doi.org/10.1016/j.scitotenv.2018.05.069

Cristini, I., Poluzzi, E., Soardo, F., Crisafulli, S., Polesello, S., Kirchmayer, U., Milani, M., Russo, F., Nicoletti, M., Scroccaro, G., Deambrosis, P., Tavakolian Haghighi, S., Grisotto, J., Moretti, U., Paolone, G., & Trifirò, G. (2025). A narrative review on the environmental impact of medicines: From water analysis and in vivo studies to prescribing appropriateness, based on European and Italian legal frameworks. Frontiers in Drug Safety and Regulation, 5, Article 1681648. https://doi.org/10.3389/fdsfr.2025.1681648

Danner, M.-C., Robertson, A., Behrends, V., & Reiss, J. (2019). Antibiotic pollution in surface fresh waters: Occurrence and effects. Science of the Total Environment, 664, 793-804.

de Lima, D., Roque, G. M., & de Almeida, E. A. (2013). In vitro and in vivo inhibition of acetylcholinesterase and carboxylesterase by metals in zebrafish (*Danio rerio)*. Marine Environmental Research, 91, 45–51. https://doi.org/10.1016/j.marenvres.2012.11.005

De Serrano, A. R., Hughes, K. A., & Rodd, F. H. (2021). Paternal exposure to a common pharmaceutical (Ritalin) has transgenerational effects on the behaviour of Trinidadian guppies. Scientific Reports, 11, Article 3985. https://doi.org/10.1038/s41598-021-83448-x

Dzul-Caamal, R., Domínguez-López, M. L., Olivares-Rubio, H. F., García-Latorre, E., & Vega-López, A. (2014). The relationship between the bioactivation and detoxification of diazinon and chlorpyrifos, and the inhibition of acetylcholinesterase activity in Chirostoma jordani from three lakes with low to high organophosphate pesticides contamination. Ecotoxicology, 23(5), 779–790. https://doi.org/10.1007/s10646-014-1216-8

Ellman, G. L., Courtney, K. D., Andres, V., Jr., & Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7(2), 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

El-Sayed, A.-F. M. (2006). Tilapia culture. CABI.

Endres, H. C., da Rosa, J. G., Kalala, C. K., Barcellos, H. H., Bertol, C. D., Barcellos, L. J. G., & Rossato-Grando, L. G. (2017). First evidence that waterborne methylphenidate alters endocrine and behavioral stress responses in zebrafish. Neuroscience Letters, 650, 114–117. https://doi.org/10.1016/j.neulet.2017.04.039

Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Biology and Medicine, 11(1), 81–128. https://doi.org/10.1016/0891-5849(91)90192-6

Food and Agriculture Organization of the United Nations. (2020). The state of world fisheries and aquaculture 2020: Sustainability in action. https://doi.org/10.4060/ca9229en

Freddo, N., Soares, S. M., Fortuna, M., Pompermaier, A., Varela, A. C. C., Maffi, V. C., Mozzato, M.

T., de Alcantara Barcellos, H. H., Koakoski, G., Barcellos, L. J. G., & Rossato-Grando, L. G. (2021). Stimulants cocktail: Methylphenidate plus caffeine impairs memory and cognition and alters mitochondrial and oxidative status. Progress in neuro-psychopharmacology & biological psychiatry, 106, 110069. https://doi.org/10.1016/j.pnpbp.2020.110069

Freddo, N., Nardi, J., Tamagno, W. A., Soares, S. M., Fortuna, M., Cole Varela, A. C., Siqueira, L., Pompermaier, A., do Prado, L., Berton, N., Amaral, F. U. I., de Oliveira, A. P., Biazus, I. C., Barcellos, L. J. G., & Rossato-Grando, L. G. (2026). Chronic methylphenidate abuse: Effects on behaviour, redox homeostasis, and cholinergic system. European Journal of Pharmacology, 1010, 178259. https://doi.org/10.1016/j.ejphar.2025.178259

Garcia, D., Lima, D., da Silva, D. G. H., & de Almeida, E. A. (2020). Decreased malondialdehyde levels in fish (Astyanax altiparanae) exposed to diesel: Evidence of metabolism by aldehyde dehydrogenase in the liver and excretion in water. Ecotoxicology and Environmental Safety, 190, 110107. https://doi.org/10.1016/j.ecoenv.2019.110107

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Cahill, J. D., Zaugg, S. D., Werner, S. L., Meyer, M. T., & Kryak, D. D. (2005). Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination. Environmental Science & Technology, 39(14), 5157–5169. https://doi.org/10.1021/es048120k

Gonzalez-Rey, M., Mattos, J. J., Piazza, C. E., Bainy, A. C., & Bebianno, M. J. (2014). Effects of active pharmaceutical ingredients mixtures in mussel Mytilus galloprovincialis. Aquatic Toxicology, 153, 12-26.

Halling-Sørensen, B., Nielson, S. N., Lützhøft, H. C. H., & Jørgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment. Chemosphere, 36(2), 357–393.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. Toxicology Letters, 131(1–2), 5–17.

Hejna, M., Kapuścińska, D., & Aksmann, A. (2022). Pharmaceuticals in the aquatic environment: A review on ecotoxicology and the remediation potential of algae. International Journal of Environmental Research and Public Health, 19(13), 7717.

Letzel, M., Weiss, K., Schüssler, W., & Sengl, M. (2010). Occurrence and fate of the human pharmaceutical metabolite ritalinic acid in the aquatic system. Chemosphere, 81(11), 1416–1422. https://doi.org/10.1016/j.chemosphere.2010.09.018

Levin, E. D., Sledge, D., Roach, S., Petro, A., Donerly, S., & Linney, E. (2011). Persistent behavioral impairment caused by embryonic methylphenidate exposure in zebrafish. Neurotoxicology and Teratology, 33(6), 668–673. https://doi.org/10.1016/j.ntt.2011.06.004

Lionetto, M. G., Caricato, R., Calisi, A., Giordano, M. E., & Schettino, T. (2013). Acetylcholinesterase as a biomarker in environmental and occupational medicine: New insights and future perspectives. BioMed Research International, 2013, Article 321213. https://doi.org/10.1155/2013/321213

Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin, 42(8), 656–666. https://doi.org/10.1016/S0025-326X(01)00060-1

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S., & Wang, X. C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of the Total Environment, 473–474, 619–641.

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology, 101(1), 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Martins, M. R., Reinke, A., Petronilho, F. C., Gomes, K. M., Dal-Pizzol, F., & Quevedo, J. (2006). Methylphenidate treatment induces oxidative stress in young rat brain. Brain Research, 1078(1), 189–197. https://doi.org/10.1016/j.brainres.2006.01.004

McCallum, E. S., Lindberg, R.H., Andersson, P.L., Brodin, T. (2019) Stability and uptake of methylphenidate and ritalinic acid in nine-spine stickleback (Pungitius pungitius) and water louse (Asellus aquaticus). Environmental Science and Pollution Research, 26(9), 9371-9378. doi: 10.1007/s11356-019-04557-9.

Miyazaki, I., & Asanuma, M. (2008). Dopaminergic neuron-specific oxidative stress caused by

dopamine itself. Acta Medica Okayama, 62(3), 141–150. https://doi.org/10.18926/AMO/30942

Nunes, B. (2010). Fármacos no ambiente: implicações ecotoxicológicas. Revista Captar: Ciência e Ambiente para Todos, v. 2, n. 1, p. 9–20, 2010.

OECD, Organisation for Economic Co-operation and Development (2019) Guidance doccument on aqueous-phase aquatic toxicity testing of difficult test chemicals. Series on testing and assessment No. 23 (Second Edition), 81 p.

Ortúzar, M., Esterhuizen, M., Olicón-Hernández, D. R., González-López, J., & Aranda, E. (2022). Pharmaceutical Pollution in Aquatic Environments: A Concise Review of Environmental Impacts and Bioremediation Systems. Frontiers in Microbiology, 13, Article 793234.

Östman, M., Fick, J., Näsström, E., & Lindberg, R. H. (2014). A snapshot of illicit drug use in Sweden acquired through sewage water analysis. Science of The Total Environment, 472, 862–871.

Parida, S., & Sahoo, P. K. (2024). Antioxidant defence in Labeo rohita to biotic and abiotic stress: Insight from mRNA expression, molecular characterization and recombinant protein-based ELISA of catalase, glutathione peroxidase, CuZn superoxide dismutase, and glutathione S-transferase. Antioxidants, 13(1), Article 18. https://doi.org/10.3390/antiox13010018

Peakall, D. B. (1992). Biomarkers of the nervous system. In Animal biomarkers as pollution indicators (pp. 29–48). Chapman & Hall. https://doi.org/10.1007/978-94-011-2346-4_2

Quinn, G. P., & Keough, M. J. (2002). Experimental design and data analysis for biologists. Cambridge University Press.

Sakuragui, M. M., Paulino, M. G., Pereira, C. D. S., Carvalho, C. S., Sadauskas-Henrique, H., & Fernandes, M. N. (2013). Integrated use of antioxidant enzymes and oxidative damage in two fish species to assess pollution in man-made hydroelectric reservoirs. Environmental Pollution, 178, 41–51.

Solé, M., Shaw, J. P., Frickers, P. E., Readman, J. W., & Hutchinson, T. H. (2010). Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. Analytical and Bioanalytical Chemistry, 396(2), 649–656. https://doi.org/10.1007/s00216-009-3182-1

Swanson, J. M., & Volkow, N. D. (2002). Pharmacokinetic and pharmacodynamic properties of stimulants: Implications for the design of new treatments for ADHD. Behavioural Brain Research, 130(1–2), 73–78. https://doi.org/10.1016/S0166-4328(01)00433-8

van der Oost, R., Beyer, J., & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environmental Toxicology and Pharmacology, 13(2), 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Vioque-Fernández, A., Almeida, E.A., López-Barea, J. (2009). Assessment of Doñana National Park contamination in Procambarus clarkii: Integration of conventional biomarkers and proteomic approaches. Science of the Total Environment, v. 407, n. 5, p. 1784–1797.

Virmani, A., Gaetani, F., Imam, S., Binienda, Z., & Ali, S. (2002). The protective role of L-carnitine against neurotoxicity evoked by drug of abuse, methamphetamine, could be related to mitochondrial dysfunction. Annals of the New York Academy of Sciences, 965(1), 225–232.

Volkow, N. D., Wang, G.-J., Fowler, J. S., & Ding, Y.-S. (2005). Imaging the effects of methylphenidate on brain dopamine: new model on its therapeutic actions for attention-deficit/hyperactivity disorder. Biological Psychiatry, 57(11), 1410–1415. https://doi.org/10.1016/j.biopsych.2004.11.006

Volkow, N. D., Wang, G. J., Fowler, J. S., Gatley, S. J., Logan, J., Ding, Y. S., Hitzemann, R., & Pappas, N. (1998). Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. American Journal of Psychiatry, 155(10), 1325–1331. https://doi.org/10.1176/ajp.155.10.1325

Wang, F., Xiang, L., Leung, K. S.-Y., Elsner, M., Zhang, Y., Guo, Y., Pan, B., Sun, H., An, T., Ying, G., Brooks, B. W., Hou, D., Helbling, D. E., Sun, J., Qiu, H., Vogel, T. M., Zhang, W., Gao, Y., Simpson, M. J., … Tiedje, J. M. (2024). Emerging contaminants: A One Health perspective. The Innovation, 5(4), Article 100612. https://doi.org/10.1016/j.xinn.2024.100612

Wilkinson, J. L., Boxall, A. B. A., Kolpin, D. W., Leung, K. M. Y., Lai, R. W. S., Galbán-Malagón, C., Adell, A. D., Mondon, J., Metian, M., Marchant, R. A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moermond, M., Luarte, T., ... Teta, C. (2022). Pharmaceutical pollution of the

world's rivers. Proceedings of the National Academy of Sciences, 119(8), e2113947119. https://doi.org/10.1073/pnas.2113947119

Zimmerman, G., & Soreq, H. (2006). Termination and beyond: Acetylcholinesterase as a modulator of synaptic transmission. Cell and Tissue Research, 326(2), 655–669. https://doi.org/10.1007/s00441-006-0239-8

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