Published in final edited form as:

Food Chem Toxicol. 2017 November ; 109(Pt 1): 727–734. doi:10.1016/j.fct.2017.08.041.

Atrazine exposure decreases the activity of DNMTs, global DNA methylation levels, and *dnmt* expression

Sara E. Wirbisky-Hershberger[†,#], **Oscar F. Sanchez**[‡,#], **Katharine A. Horzmann**[†], **Devang Thanki**[†], **Chongli Yuan**[‡,*], and **Jennifer L. Freeman**[†,*]

[†]School of Health Sciences, West Lafayette, IN, 47907

[‡]School of Chemical Engineering, Purdue University, West Lafayette, IN, 47907

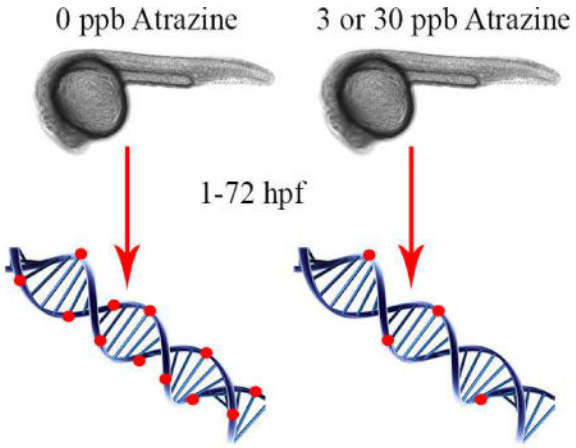
Abstract

Atrazine, a herbicide used on agricultural crops is widely applied in the Midwestern United States as well as other areas of the globe. Atrazine frequently contaminates potable water supplies and is a suspected endocrine disrupting chemical. Previous studies have reported morphological, hormonal, and molecular alterations due to developmental and adulthood atrazine exposure; however, studies examining epigenetic alterations are limited. In this study, the effects of atrazine exposure on DNA methyltransferase (DNMT) activity and kinetics were evaluated. Global DNA methylation levels and *dnmt* expression in zebrafish larvae exposed to 0, 3, or 30 parts per billion (ppb) atrazine throughout embryogenesis was then assessed. Results indicate that atrazine significantly decreased the activity of maintenance DNMTs and that the inhibition mechanism can be described using non-competitive Michaelis-Menten kinetics. Furthermore, results show that an embryonic atrazine exposure decreases global methylation levels and the expression of *dnmt4* and *dnmt5*. These findings indicate that atrazine exposure can decrease the expression and activity of DNMTs, leading to decreased DNA methylation levels.

Graphical Abstract

Corresponding authors: Jennifer L. Freeman, School of Health Sciences, 550 Stadium Mall Dr., West Lafayette, IN 47907 USA, Phone: (765) 494-1408, Fax: (765) 496-1377, jfreema@purdue.edu and Chongli Yuan, School of Chemical Engineering, 480 Stadium Mall Dr., West Lafayette, IN 47907 USA, Phone: (765) 494-5824, cyuan@purdue.edu.
[#]These authors contributed equally to the manuscript.

Competing Interests: The authors declare that they have no competing interests.



Keywords

atrazine; dnmt; epigenetics; methylation; zebrafish

1. Introduction

Epigenetic programming plays an important role in an organism's reaction to environmental stressors during development. Numerous definitions have been proposed to define epigenetics, the most encompassing representation is that epigenetics is heritable, reversible, and self-perpetuating phenotypic alterations elicited by gene expression changes resulting from structural adaptations of chromosomal regions that take place without any alteration of the integral DNA sequence where these modifications occur.[1–5] Three types of known epigenetic mechanisms include DNA methylation, histone modifications, and small non-coding RNAs that regulate gene expression.[6] Of these, the alteration of DNA methylation patterns and subsequent reprogramming of developmental processes by changing transcriptional gene expression has received most attention, especially as it pertains to the heritability of these epigenetic alterations.[7–12]

DNA methylation occurs with high frequency at CpG islands, which typically have greater than 50% GC content with the CpG ratio of at least 60%.[13–15] DNA methylation is critical for normal cellular development because of its role in regulating gene transcription, maintaining transposon inactivation, X-chromosome inactivation, and genomic imprinting. DNA methylation is mediated by the family of DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to cytosine of DNA. Mammals possess five DNMTs (DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L); however, only DNMT1, DNMT3A, and DNMT3B possess methyltransferase activity. DNMT1 functions to maintain proper methylation maintenance, while DNMT3A and DNMT3B contribute to *de novo* methylation during development.[14]

Endocrine disrupting chemicals (EDCs) are exogenous agents that disrupt endogenous hormone signaling pathways. These chemicals are diverse in structure and are found in numerous products such as plasticizers, pharmaceuticals, and pesticides, making human

exposure to these chemicals a likely event.[16] Studies show that EDCs can alter tissue formation, reproduction, and play a role in the onset of obesity and cancer.[16–23] Furthermore, studies implicate that exposure to EDCs can alter different levels of epigenetic processes which can act transgenerationally.[9–13, 24–27]

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a pre-emergent herbicide that is applied throughout the Midwestern United States and other parts of the globe. Atrazine is applied on a variety of agricultural crops including corn, sorghum, sugar cane, and wheat.[28–30] Due to atrazine's water solubility, mobility in soil, and long half-life, atrazine frequently contaminates potable water supplies and reaches levels above the maximum contaminant level (MCL) as set by the U. S. Environmental Protection Agency (EPA) of 3 parts per billion (ppb; μg/L).[31–32] The greatest risk of drinking water source contamination occurs following rainfall events after agricultural field application in spring and summer with recent monitoring data measuring over 300 ppb in the Midwestern US.[31] Widespread water contamination issues led the European Union to ban the use of atrazine in 2003.[33–34] Epidemiological studies show several potential adverse health effects associated with maternal atrazine exposure including an increased risk of babies born small for their gestational age (SGA), intrauterine growth retardation (IUGR), and birth defects.[35–38] Laboratory studies utilizing various animal models report numerous hormonal and molecular alterations caused by atrazine exposure.[20, 39–43] Many of these studies report adverse effects on the neuroendocrine system through the hypothalamus-pituitary-gonadal axis. For example, atrazine exposure disrupted the hypothalamic control of pituitary-ovarian function with additional studies reporting robust alterations in reproductive hormones including gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH).[20, 39–43] However, understanding the epigenetic alterations contributing to atrazine toxicity is still under investigation.[44–47]

We have previously reported that an embryonic atrazine exposure [0.3, 3, or 30 ppb throughout embryogenesis, 1–72 hours post fertilization (hpf)] resulted in alterations to the transcriptome of zebrafish larvae in genes associated with reproductive system function and development, cell cycle regulation, and cancer.[48] In addition, morphological alterations in head size of the zebrafish larvae exposed to 0.3, 3, or 30 ppb atrazine during embryogenesis was observed.[48] Furthermore, a decrease in spawning was observed in adult zebrafish exposed to atrazine only during embryogenesis with persistent morphological alterations in their offspring.[49] These adult females also had an increase in ovarian progesterone and follicular atresia, alterations in levels of 5-hydroxyindoleacetic acid (5-HIAA, a serotonin metabolite) and serotonin turnover in brain tissue, and transcriptome changes in brain and ovarian tissue supporting neuroendocrine alterations similar to what has been reported in other animal studies.[49–50] In a previous study, we also first began to investigate epigenetic mechanisms behind the observed alterations in larvae and adult zebrafish by evaluating changes in microRNA (miRNA) expression following the same atrazine exposure regimen (i.e., an embryonic atrazine exposure at 0.3, 3, or 30 ppb throughout embryogenesis, 1–72 hpf).[47] Results from that study revealed alterations in miRNAs that were associated with angiogenesis, cancer, and neurodevelopment, but there are still questions on additional epigenetic mechanisms of atrazine toxicity including DNA methylation.[47]

In order to investigate DNA methylation parameters associated with atrazine exposure, in the current study we evaluated the effects of atrazine on DNMT activity and kinetics. Next, methylation levels and expression of *dnmts* was quantified in zebrafish that were exposed to 0, 3, or 30 ppb atrazine throughout embryogenesis (1–72 hpf) to evaluate the current U.S. EPA MCL and a concentration 10X higher. The zebrafish is a strong complementary vertebrate model when used in investigating epigenetic dysfunction and developmental toxicity due to its *ex utero* fertilization and development, short developmental periods, relatively short life span, and high genetic homology to humans.[51–52] While there are some differences between DNA methylation reprogramming between zebrafish and mammals, all DNA methyl transferases identified in mammals are present in zebrafish.[53–58] In addition, in contrast to mammals, which have three active DNMTs (DNMT1, DNMT3A, and DNMT3B); the zebrafish genome contains multiple paralogs.[53–58] Zebrafish express *dnmt1* (homolog to mammalian DNMT1) and six DNMT3 paralogs, classified as *dnmt3–8*.[53–57] The DNMT3A orthologs of zebrafish are *dnmt6* and *dnmt8*; while DNMT3B orthologs are *dnmt3, dnmt4, dnmt5*, and *dnmt7*.[58]

2. Materials and Methods

2.1 Evaluation of DNMT activity using enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was performed to assess enzyme activity of human methyltransferases (DNMT1, DNMT3A, DNMT3B) and bacterial methyltransferase (*M. SssI*) in the presence and absence of atrazine. All methyltransferases were obtained commercially (NEB and Epigentek, NY). The reaction was performed using a commercial ELISA kit (DNMT1 and DNMT3A: EpiQuik DNMT activity assay kit (Colorimetric), Epigentek, NY; and DNMT3B: DNMT3B Inhibitor Screening Assay Kit, Abcam, MA). Atrazine concentrations of 0, 3, and 30 parts per billion (μg/L; ppb) were tested (CAS #1912-24-9; Chem Service, 98% purity). The specific enzyme activity (min^{-1} ng^{-1}) was determined following Eq.1.

$$\text{Enzyme Activity} = \frac{\text{Absolute Singnal}}{(\text{Reaction Time})(\text{Enzyme Amount})} \quad \text{(Eq. 1)}$$

To evaluate the effect of atrazine on DNMT activity, we reported the relative activity of DNMTs as the ratio of the enzyme activity in presence and absence of atrazine.

2.2 The effect of atrazine on DNMT kinetics

Kinetic assays were conducted using DNA fragments with defined sequence content (Supplementary Table 1) at varying DNA concentrations (0.15 – 0.75 μM). Atrazine was added to a reaction buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$ and 1 mM DTT) at concentrations of 0, 3, and 30 ppb. The reaction was carried out using excessive amounts of Ado-Met (SAM, 160 μM), initiated by the addition of bacterial methyltransferase (*M.SssI*, ~ 20 nM) and incubated at 37°C. *M.SssI* resembles maintenance DNA methyltransferase activity and has a much higher biochemical activity. As such, *M.SssI* was used as model enzyme to determine atrazine effects on maintenance DNMTs.

The reaction was stopped at selected time points by heating the reaction mixture at 65°C for 20 minutes to deactivate the methyltransferase. Then, the reacted DNA fragments were digested with a restriction enzyme, *HpaII*. The endonuclease activity of *HpaII* is completely blocked by ^{me}CG. *HpaII* is thus typically used in methylation-sensitive restriction enzyme assays to evaluate the methylation level of DNA fragments.[59] *HpaII* digestion products were analyzed using polyacrylamide gel electrophoresis (PAGE). The relative band intensities of unmethylated and methylated DNA (quantified using Image J), which are correlated with the digested (107 bp) and undigested (147 bp) DNA bands, respectively, were used to determine the methylation level of DNA fragments. The ^{me}CG concentration was calculated following Eq.2.

$$[^{\mathrm{me}}\mathrm{CG}]=\frac{I_U/147}{I_U/147+I_D/107}\times N_{\mathrm{CG}}\times[\mathrm{DNA}] \quad \text{(Eq. 2)}$$

where, I_U and I_D are the intensity of the undigested and digested band, respectively, and N_{CG} is the number of CG sites per DNA strand. The characterized [^{me}CG] was then used to determine the initial methyltransferase rate (V) under different reaction conditions. Apparent kinetics parameters, i.e., K_m and V_{max}, were obtained by fitting the kinetic data using a Michaelis-Menten model (Eq. 3).

$$\nu=\frac{V_{\max}[\mathrm{DNA}]}{K_m+[\mathrm{DNA}]} \quad \text{(Eq. 3)}$$

Control experiments were performed to test if the addition of atrazine or phenol may affect the endonuclease activity of HpaII. Typical PAGE results are shown in Supplementary Figure 1. Under the selected digestion condition, the presence of neither atrazine nor phenol affects the digestion results.

2.3 Zebrafish husbandry and embryonic atrazine exposure

Zebrafish (wild-type AB strain) were housed in a Z-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 hour light:dark cycle and maintained at 28 ± 1°C with a pH of 7.0–7.2 and conductivity range of 470–520 μS. Adult zebrafish aged 5–7 months were bred in groups in cages and embryos were collected, staged, and rinsed with system water as described previously for experimental use.[47–50] Zebrafish embryos were exposed to 0, 3, or 30 ppb atrazine (CAS #1912-24-9; Chem Service, 98% purity) from 1–72 hours post fertilization (hpf) as previously described.[47–50] Atrazine water sample concentrations were verified using a U.S. EPA approved immunoassay kit for atrazine (Abraxis Atrazine ELISA kit, Warminster, PA) as previously described.[47–50] Similar to our past studies no differences in mortality rates or increases in gross malformations were observed in the atrazine exposed groups compared to the controls with no atrazine treatment.[47–50] All animal protocols were approved and performed in accordance with Purdue University's Institutional Animal Care and Use Committee Guidelines.

2.4 Genomic DNA isolation and measurement of global methylation levels

Following atrazine exposure, zebrafish larvae (72 hpf) were rinsed and genomic DNA was extracted following a standard protocol.[60] Briefly, zebrafish embryos were homogenized in 0.5 mL Proteinase K lysis buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS, 100 mg/mL Proteinase K) and incubated at 55°C overnight. Following incubation, samples were transferred to Phase Lock Gel (PLG) light 15 mL tubes (5 Prime, Hilden, Germany) and phenol (Phenol-Tris saturated, pH 8) (Roche, Indianapolis, IN) was added. Next, 1 mL chloroform:isoamyl alcohol (24:1) (American Bioanalytical, Natick, MA) was added and samples were centrifuged at room temperature for 5 minutes at 1,500 rcf. The upper aqueous phase was placed into a second PLG tube. The addition of phenol and chloroform:isoamyl alcohol (24:1) and centrifugation was repeated. The aqueous phase was then placed into a 15 mL tube. 0.1× the volume of 3M sodium acetate and 1× isopropanol was added. Samples were inverted until DNA began to aggregate. Next, samples were incubated at room temperature for 20 minutes and then centrifuged at 4°C for 10 minutes at 800 rcf. Excess liquid was removed and the DNA pellet was washed in 70% ethanol and centrifuged at 4°C for 5 minutes at 800 rcf. The DNA pellet was rehydrated in 1× TLE buffer and incubated overnight at 55°C. The DNA concentration was tested on a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The DNA was then sonicated into fragments of 100 – 300 bp in length following an established procedure.[61] Fragmented DNA samples were analyzed using a 1% Agarose gel as shown in (Supplementary Figure 2). DNA fragments (~ 500 nM) were then mixed with a fluorescein-labeled DNA methylation probe that was created by our group to determine the methylation level of gDNA following our established approach.[62,63] This probe can quantify the total amount of methylated CG dinucleotides above 20 nM independent of DNA sequence contexts.

2.5 Quantitative polymerase chain reaction (qPCR) of dnmt expression in zebrafish larvae

Zebrafish embryos were exposed to 0, 3, or 30 ppb atrazine from 1–72 hpf as described above. Following the exposure period, 50 embryos were pooled per sample (n=9–10), homogenized in Trizol® Reagent (Life Technologies, Carlsbad, CA), and flash frozen in liquid nitrogen. Total RNA was isolated and purified following similar methods described in Freeman and Peterson using Trizol Reagent followed by RNA clean-up with Qiagen RNEasy Mini Kit.[64] The RNA samples were quality and quantity checked using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and further converted to cDNA with the Super Script® First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA). qPCR was performed on all seven DNMT genes found in zebrafish (*dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7, dnmt8*) using the BioRad iQ SYBR Green Supermix kit according to manufacturer's recommendations. Primers specific to target genes were designed using the Primer3 website (Table 1). qPCR was performed following a similar method as previously described following MIQE guidelines.[47–50, 65] Similar to previous studies in our laboratory, several genes were assessed to determine the best reference gene to be used for this data set (data not shown).[47–50] *β-actin* was found to be the most consistent, not altered by atrazine exposure, and least variable for this analysis. Experimental samples were run in triplicate (technical replicates), quantitative copies calculated using a standard curve, and gene expression normalized to *β-actin*. Efficiency and specificity were checked with melting and dilution curve analysis and no-template controls.

2.6 Statistical analysis

Statistical analysis was completed using OriginPro (Version 2015, Origin Lab Corp, North Hampton, MA) or SAS (Version 9.4) statistical software. ELISA, DNMT kinetics, and global DNA methylation levels were analyzed by a one-way analysis of variance (ANOVA) followed by a Duncan's multiple range post-hoc test when a significant ANOVA was observed ($\alpha < 0.05$). qPCR data was analyzed with an ANOVA and a post hoc least significant difference (LSD) test when a significant ANOVA was observed ($\alpha < 0.05$).

3. Results

3.1 Effects of atrazine on DNMT activity

The kinetics of DNMT1 was significantly reduced in a dose-dependent manner by 13% and 38% following an atrazine exposure of 3 and 30 ppb, respectively. No significant alterations in kinetic activity were observed in DNMT3A or DNMT3B. *M.Sss1* activity was significantly reduced in the 3 and 30 ppb atrazine treatment groups by approximately 25 and 50%, respectively ($p<0.05$; Figure 1).

3.2 Atrazine is a non-competitive inhibitor for DNA methyltransferase reactions

M.SssI was used as a model enzyme to determine atrazine effects on maintenance DNMT. Initial results demonstrated methylation levels at various time points (0, 2, 4, 6, 8, and 10 minutes) at 0, 3, or 30 ppb atrazine examined using a methylation-sensitive restriction enzyme digestion assay (Figure 2). The initial reaction rate was determined in order to calculate the kinetic parameters (V_{max} and K_m). Results show that an atrazine exposure of 3 or 30 ppb significantly decreases methylation rate ($p<0.05$; Figure 3A) at various DNA substrate concentrations. The kinetic data were fitted using a Michaelis-Menten model, and the parameters V_{max} and K_m were summarized (Table 2). The addition of atrazine significantly reduces the V_{max} value while no significant difference was observed for K_m. To validate the non-competitive inhibition, a Lineweaver-Burke analysis was completed (Figure 3B). The linearized kinetic data exhibit distinctive intercept (V_{max}), but similar Km depicted by almost identical abscissa intercepts. Both are characteristic features of non-competitive inhibitors.

3.3 Embryonic atrazine exposure in zebrafish decreased CpG methylation

In agreement with our past studies using a similar atrazine exposure regimen, no differences in mortality, hatching rate, or gross malformations were observed following the embryonic exposure. Developmental exposure to atrazine did cause a significant dose-dependent reduction in the average methylation level of the genomic DNA (gDNA). The relative methylation level of gDNA with respect to the control (0 ppb atrazine) was reduced by 36 ± 5% and 56 ± 3% in zebrafish embryos exposed to 3 ppb and 30 ppb atrazine, respectively ($p<0.05$; Figure 4 and Supplementary Figure 3).

3.4 Embryonic atrazine exposure in zebrafish altered dnmt expression

A significant decrease in gene expression in the 30 ppb atrazine treatment was observed for *dnmt4* and *dnmt5* (p=0.0083 and p=0.0419, respectively). No significant differences in gene

expression was observed for any other *dnmts* tested (*dnmt1:* p=0.8128; *dnmt3:* p=0.9055; *dnmt6:* p=0.0744; *dnmt7*: p=0.6016; *dnmt8*: p=0.4802) (Figure 5).

4. Discussion

Studies have begun investigating DNA methylation disruption in response to EDCs in numerous animal models with varying reports of alterations.[26,27,66] For example, exposure to bisphenol A in mice or phthalates in rats results in DNA methylation changes, but in contrast a maternal exposure to the anti-androgenic compounds vinclozolin, flutamide, or procymodine in rats had no effects on DNA methylation in male offspring.[26,27,66] There is currently very limited knowledge on epigenetic mechanisms of atrazine toxicity with only a couple published studies focused on DNA methylation, miRNA deregulation, or histone modifications.[44–47] Based on previous findings from our laboratory identifying immediate and later-in-life neurological and reproductive system impacts of an embryonic atrazine exposure in zebrafish and persistent morphological alterations in their subsequent generation[48–50] combined with rodent models also reporting later-in-life alterations from an embryonic atrazine exposure,[67–70] it is important to identify the effects of atrazine on DNA methylation parameters as a potential mode of action.

In the current study, the effects of atrazine on DNA methyltransferase activity *in vitro* was assessed and an observed decrease in the activity of DNMT1 and *M.SssI* was noted. This similar response can be explained by the conserved sequence motifs in the catalytic and the Ado-met-binding site of both enzymes.[71] In addition, *M.SssI* is the only prokaryotic methyltransferase that can methylate cytosine of a CG similar to DNMT1.[72] Meanwhile, the activity of the *de novo* methyltransferase DNMT3A and DNMT3B remained unaffected by atrazine under the selected concentrations. As our results indicated that atrazine alters maintenance DNMTs *in vitro*, it was necessary to identify the potential mechanism of inhibition caused by atrazine exposure. To accomplish this task, *M.SssI* was chosen as the model enzyme due to its large *in vitro* activities and similarity to DNMT1.[72] The decrease in methylation and initial methylation reaction rate following atrazine exposure that were observed suggest that atrazine acts as a noncompetitive inhibitor for *M.SssI*. To further support this hypothesis, validation through a Lineweaver-Burke analysis showed linearized kinetic data that exhibited a distinctive intercept (V_{max}), but similar K_m values depicted by almost identical abscissa intercepts, which are characteristic kinetic features of non-competitive inhibitors.[73] This noncompetitive inhibition mechanism suggests that atrazine can bind to the enzyme (either in a free or an enzyme-substrate complex form) at a site different from the catalytic center. The binding can result in reduced activities with respect to the DNA substrate.

Exposure to chemicals during critical developmental periods can induce heritable and stable alterations in DNA methylation, therefore potentially altering gene regulation and contributing to disease onset. In this study, the effect of an embryonic atrazine exposure on global methylation in zebrafish larvae was investigated. Assessment during this period is ideal as the establishment of methylation patterns can take up to 96 hpf to complete in the zebrafish;[74] therefore, providing a snapshot into the effects of atrazine during primary methylation events. A decrease in global methylation in zebrafish larvae were observed in

both the 3 and 30 ppb treatments. To our knowledge, only one other set of studies has evaluated DNA methylation changes following atrazine exposure *in vivo*. These two recent studies investigated the effects of atrazine on DNA methylation in various tissues of the common carp.[44–45] Atrazine concentrations as low as 4.28 ppb were found to cause global hypomethylation patterns in the liver, kidney, gill, gonad, and brain tissue of juvenile carp.[44–45] In those studies, reduction in global DNA methylation ranged from 10 to 45% depending on the analyzed tissue. Although these studies were completed with various tissues in juvenile carp, we report a similar global hypomethylation pattern in zebrafish exposed to 3 or 30 ppb atrazine during embryogenesis.

We then assessed expression patterns of zebrafish *dnmts* following an embryonic atrazine exposure at the same concentrations. Multiple studies have characterized the developmental expression of *dnmts* in zebrafish embryos, larvae, and adults.[57–58,74–76] In addition, studies have also begun investigating EDCs and their effects on global methylation and *dnmt* expression with the zebrafish. For example, Aluru et al. reported altered *dnmt* expression in zebrafish with an exposure to dioxin observing upregulation of some *dnmts* and downregulation of others, but they did not observe global methylation changes, while Fang et al. reported decreased global methylation with exposure to benzo[a]pyrene.[77–78] Following an embryonic atrazine exposure, we report a significant decrease at 30 ppb in gene expression of *dnmt4* and *dnmt5* (both are orthologs of human DNMT3B). Currently, the developmental characterization of *dnmt4* and *dnmt5* has been identified in the zebrafish. By 72 hpf, *dnmt4* is located in the ciliary marginal zone, pharyngeal arches, auditory capsule, pectoral fin buds, intestine, pancreas, and liver. In addition, *dnmt4* is also expressed in the tectum, dorsal and ventral thalamus, and in the pallium region.[75] *dnmt5* expression has also been identified throughout the anterior zebrafish embryo with expression localizing to the ciliary marginal zone of the retina by 72 hpf.[76]

Our *dnmt* expression results are similar with the previous studies conducted in juvenile common carp (*Cyprinus carpio L.*), which reported a consistent reduction in the expression of *dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7,* and *dnmt8* following a chronic 40 day atrazine exposure at concentrations ranging from 4.2–42.8 ppb in brain, gonad, liver, kidney, and gill tissue.[44–45] Although no significant gene expression alterations were identified in the remaining *dnmts* (*dnmt1, dnmt3, dnmt6, dnmt 7, dnmt8*) in our study, a decreasing trend was noted, especially within the 30 ppb atrazine treatment group. The discrepancy between these studies regarding the expression levels of *dnmt1, 3, 6, 7*, and *8* may be due to the differences between embryonic and juvenile exposure, dosing periods, and atrazine concentrations or are based on the specific function of the genes.

Understanding the toxicological implications of *dnmt* alterations by EDCs, their impact on later-in-life function, and impacts to future generations requires further investigation. In addition, studies are needed to further compare *in vitro* and *in vivo* alterations in maintenance and *de novo* methyltransferases and gene-specific changes. Further studies are also needed to define the relationship between downregulation of *dnmt4* and *dnmt5* and adverse effects observed in other studies of atrazine toxicity.[47–50]

5. Conclusions

Overall in this study, an atrazine exposure was shown to elicit epigenetic dysfunction through decreasing maintenance DNMT activities *in vitro* and decreased global methylation and *dnmt* expression in developing zebrafish. The results from this and a previous study conducted from our laboratory support that atrazine elicits developmental toxicity at the epigenetic level therefore, providing a potential mode of action behind the molecular and morphological alterations.[47] Further studies are warranted in order to determine specific genes that have altered methylation status. In addition, future studies are needed to determine if epigenetic dysfunction persists into adulthood, and if so, does it contribute to previously identified genomic and functional alterations.[49–50] In addition, it will be important to determine if epigenetic alterations are present in future generations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health, National Institute of Environmental Health Sciences (R15 ES019137 to J.L.F.), a Bilsland Dissertation Fellowship (S.E.W. and J.L.F), the US Army Medical Research (Award Number: W81XWH-14-1-0012 to C.Y.), the School of Chemical Engineering, Purdue University (C.Y.), and the Administrative Department of Science, Technology and Innovation (COLCIENCIAS) from Colombia and Fulbright (Grant 529) (O.F.S.).

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Highlights

- Atrazine exposure reduces activity of maintenance DNMTs
- Atrazine inhibition mechanism is described using non-competitive Michaelis-Menten kinetics
- Zebrafish embryonic atrazine exposure decreases global methylation
- Zebrafish embryonic atrazine exposure decreases *dnmt4* and *dnmt5* expression

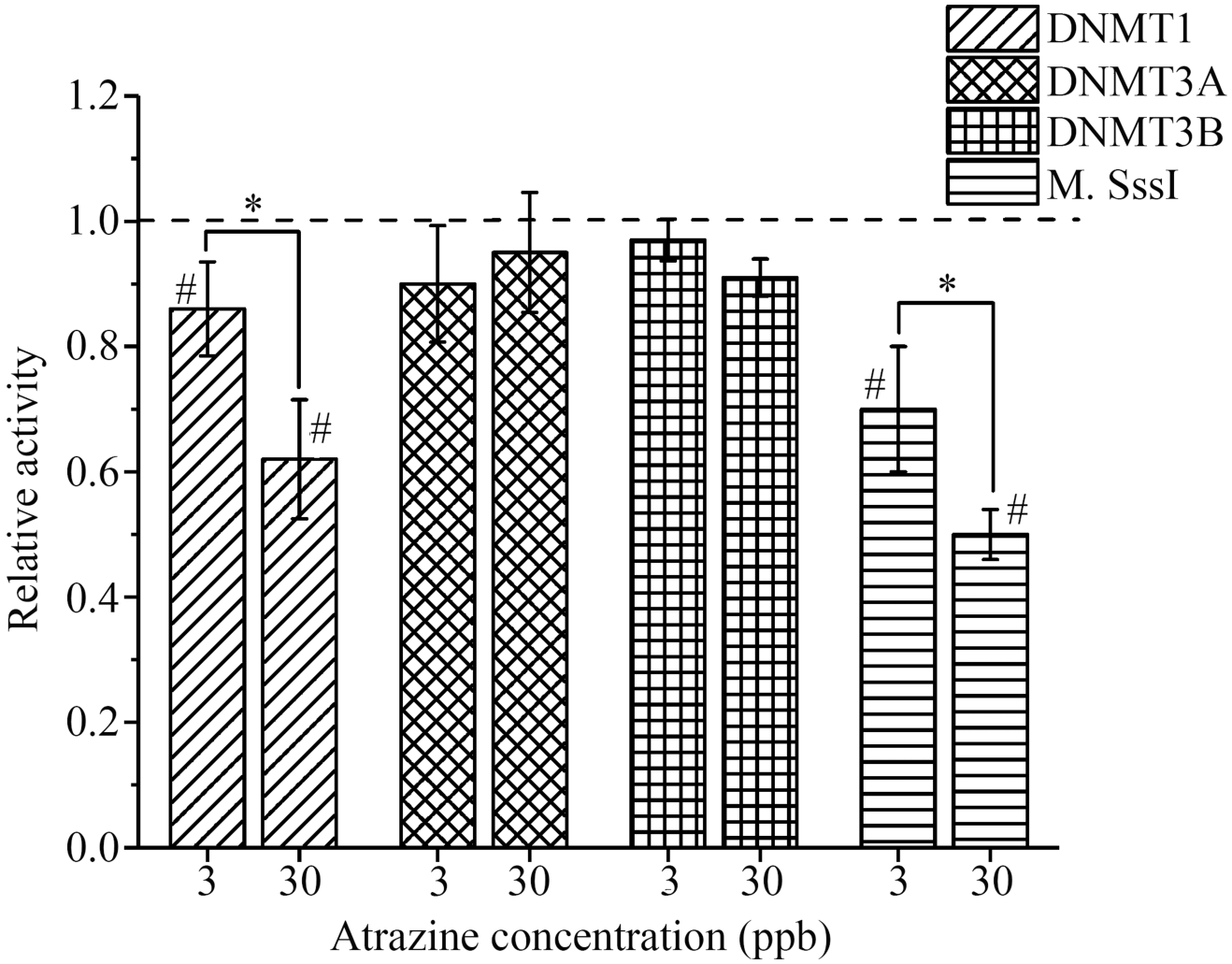

Figure 1. Atrazine altered DNMT1 and *M.SssI* activity

Relative activity of DNMT1, DNMT3A, DNMT3B, and *M.SssI* following an atrazine exposure of 0, 3, or 30 ppb was assessed. The activity of DNMT1 and *M.SssI* were significantly decreased following atrazine exposure compared to the control ($^{\#}p<0.05$). No significant changes were observed in the activity of DNMT3A or DNMT3B. Data is expressed as mean ± standard deviation (n≥3; *indicates a significant difference ($p<0.05$) between the 3 and 30 ppb samples)

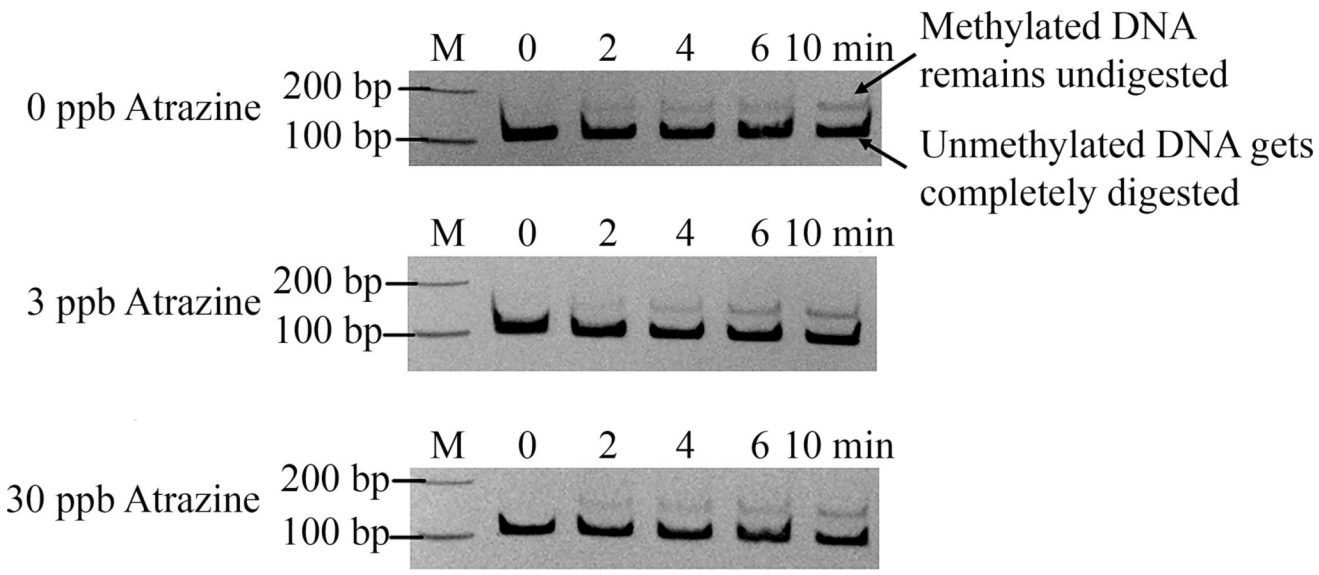

Figure 2. Effect of atrazine on the methylation activity of *M.SssI*

Changes in methylation levels due to an atrazine exposure of 0, 3, or 30 ppb at 2, 4, 6, 8, and 10 minutes. Reactions were run with 0.15 μM of DNA.

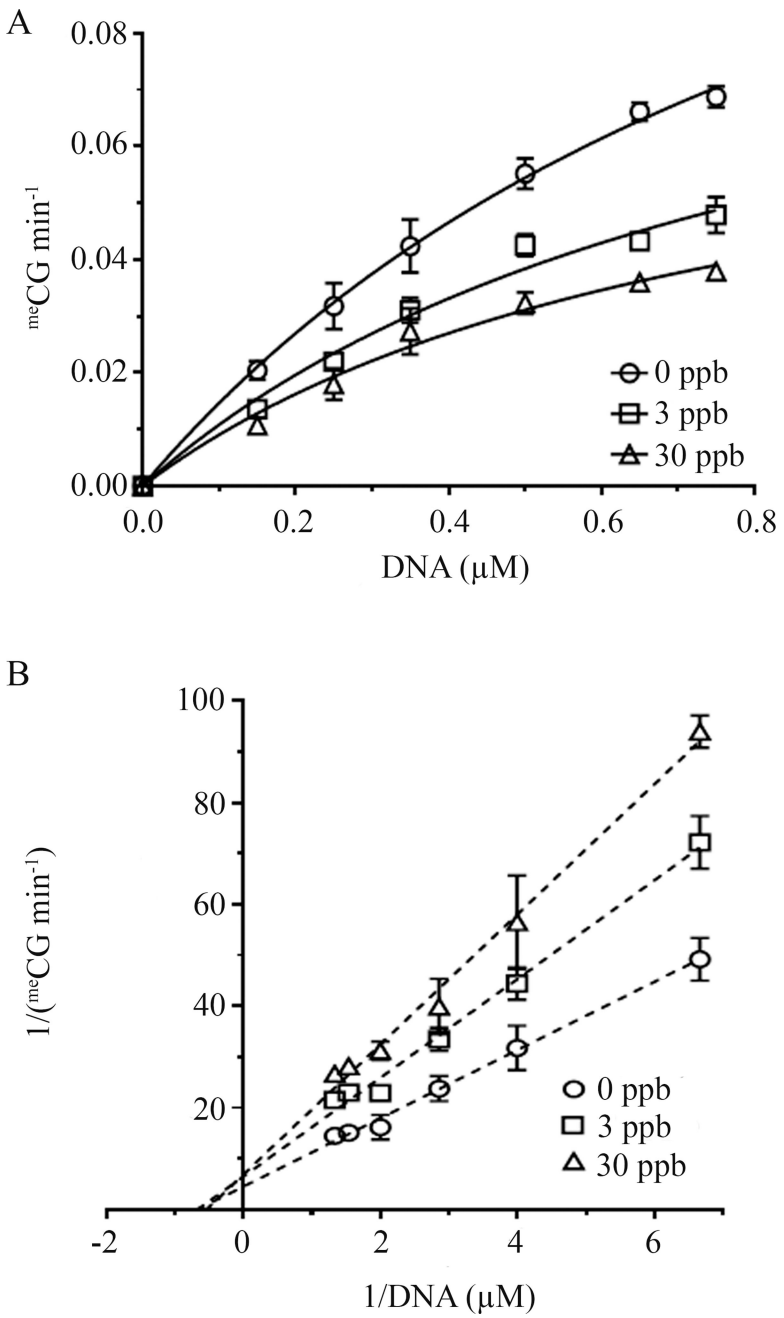

Figure 3. Atrazine inhibited DNA methyltransferase reactions

Results show that an atrazine exposure of 3 or 30 ppb significantly decreased methylation rate (A). A Lineweaver-Burke analysis of kinetic data obtained under varying atrazine concentrations (B). ($n \geq 3$; *$p < 0.05$).

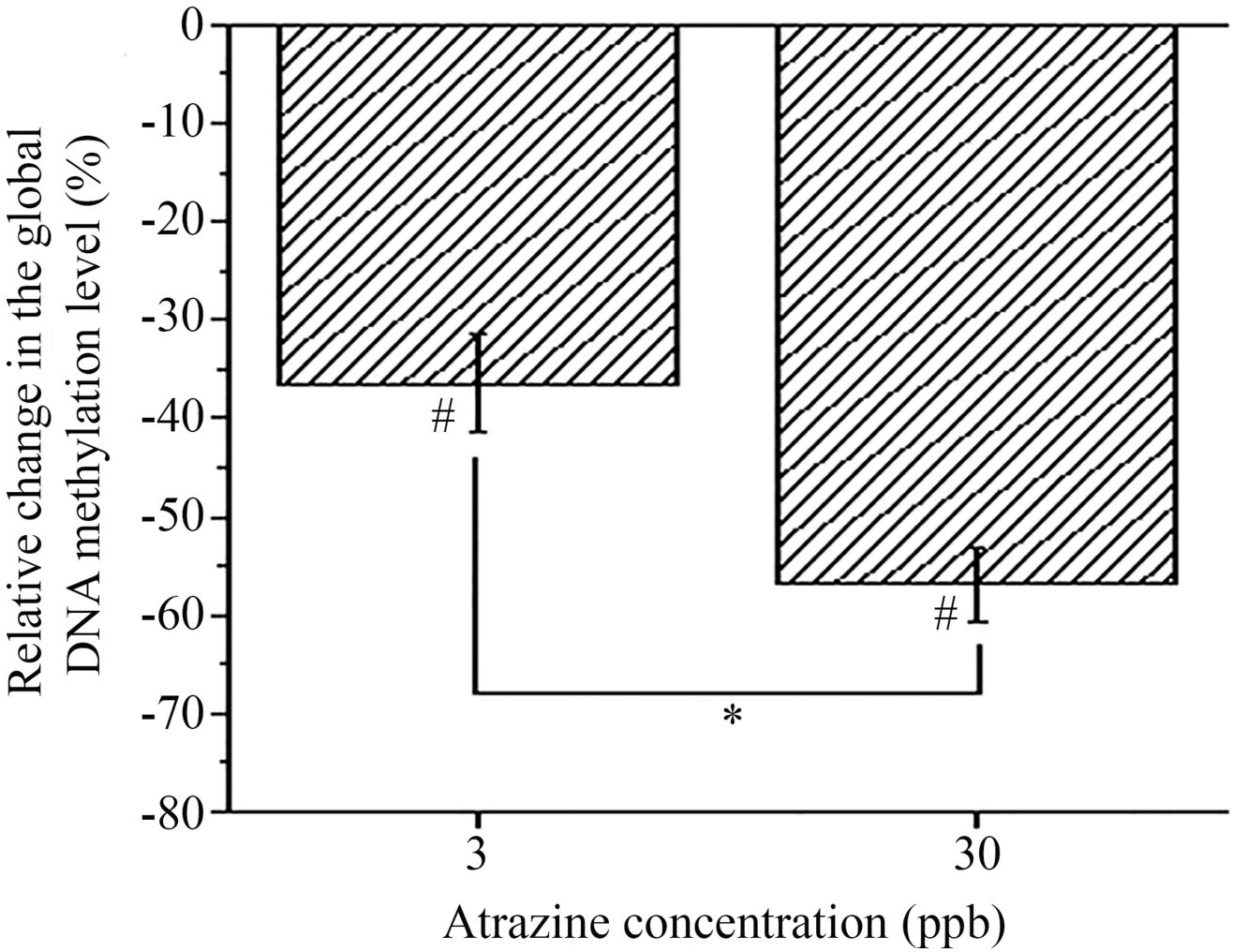

Figure 4. Developmental atrazine exposure decreased CpG methylation

Zebrafish were treated with 0, 3, or 30 ppb atrazine from 1–72 hpf and gDNA was isolated at 72 hpf. Results indicated a significant reduction in the relative global methylation in both the 3 and 30 ppb atrazine treatments compared with the 0 ppb control ($^{\#}p<0.05$). Data is expressed as mean ± standard deviation ($n\geq5$; *indicates a significant difference ($p<0.05$) between the 3 and 30 ppb samples)

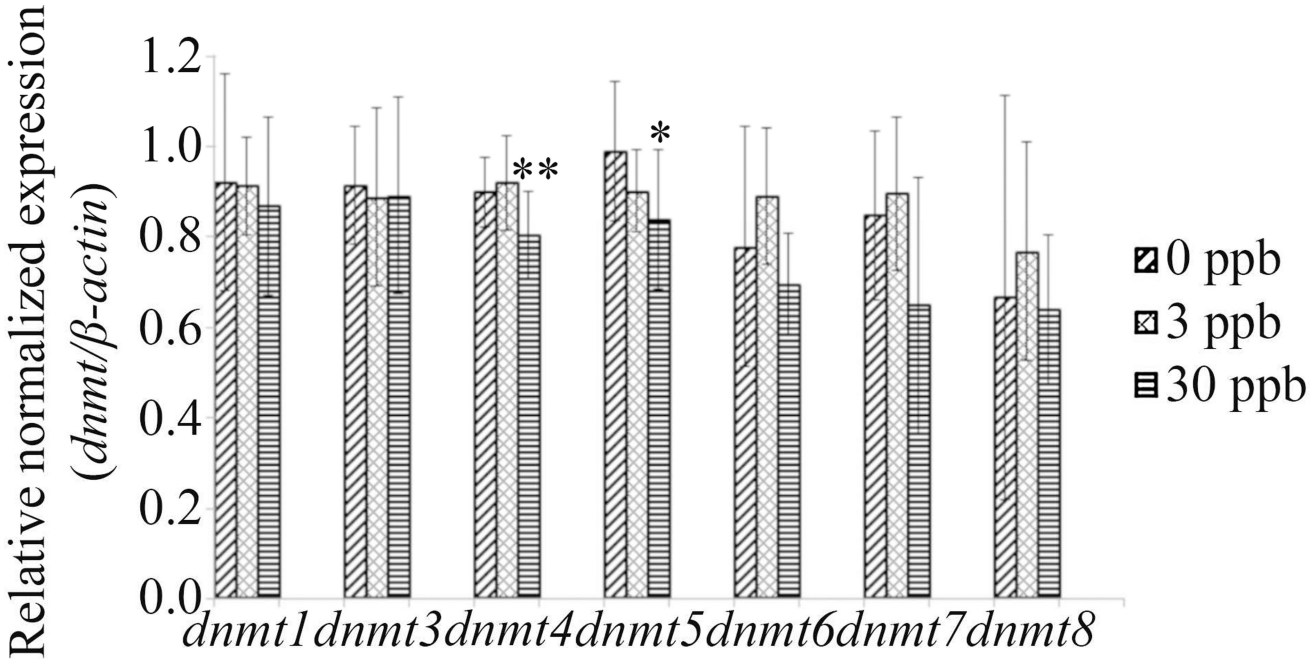

Figure 5. Developmental atrazine exposure alters *dnmt* expression

Zebrafish were exposed to 0, 3, or 30 ppb atrazine from 1–72 hpf. Following the exposure period, qPCR was performed to obtain expression levels of *dnmts*. A significant decrease in the 30 ppb atrazine treatment was observed in *dnmt4* and *dnmt5*. No alterations were reported for the remaining *dnmt*s tested in any of the atrazine concentrations ($p>0.05$). Data is expressed as mean ± standard deviation (n=9–10; *$p<0.05$; **$p<0.01$).

Table 1

qPCR primer list for *dnmt* analysis in zebrafish larvae

NCBI Accession Number	Gene Name	Primer Sequence
NM_131189.2	*dnmt1*	Forward: GAGGAGGATGTGTTGCCAGTTA
		Reverse: CCTCATTTTCCACACGCACTTT
NM_131386.1	*dnmt3*	Forward: TAGAGTCATGTTGAACTGGGCC
		Reverse: TCAGGTCCAGAGATTCAGGGAT
NM_001025450.1	*dnmt4*	Forward: AAGATTTACCCTGCAGTCCCAG
		Reverse: CTCGCATACTTCTGACGCAAT G
NM_001020479.1	*dnmt5*	Forward: TTATCCACCCACTGTTCGAAGG
		Reverse: ATGACCACACAGAATGACCTCC
NM_001018140.1	*dnmt6*	Forward: GTGTGGGGAAAGTTACGAGGAT
		Reverse: TGCTTATTGTAGGTTGGCTGGT
NM_001020746.1	*dnmt7*	Forward: AGGCAGCTTTTCGGGATTTAGA
		Reverse: CGATTTCTTGACCATCACGAGC
NM_001018134.1	*dnmt8*	Forward: CTTTGCCTGTTAATGAAGCCCC
		Reverse: TGTGAAGTGTCCTGTGGTTGAA
NM_181601	*β-actin*	Forward: CTAAAAACTGGAACGGTGAAGG
		Reverse: AGGCAAATAAGTTTCGGAACAA

Table 2

Kinetic parameters of DNA methyltransferase reactions following atrazine exposure catalyzed by *M.SssI.*

	0 ppb	3 ppb	30 ppb
V_{max}(^{me}CGμM min^{-1})	0.170±0.018	0.106±0.013 *	0.080±0.010 *
K_M (μM)	1.06±0.16	0.88±0.18	0.78±0.17
K_{Cat} (min^{-1})	7.31±0.75	4.56±0.57 *	3.43±0.45 *

* $p<0.05$