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Assessment of cytogenotoxic potential of Ethion in Zebrafish (*Danio rerio* F. Hamilton, 1822) by using micronucleus assay

Vinayan. K. V. and *R.V. Tijare

Institute of Science, Civil lines, R.T Road, Nagpur - 440001 (India)
*Corresponding author: rvtijare@gmail.com

Abstract

Ethion used in agriculture commonly reaches the non-target organisms like aquatic life and raises environmental concerns which has cautioned the use and many countries have put it under restricted use. Genotoxic impact of Ethion on the non-target aquatic life was assessed by exposing. Zebrafish (*Danio rerio*) to three sublethal doses, *i.e.* 0.340 mg/L, 0.476 mg/L and 0.612 mg/L at four-time intervals, viz., 24 hours, 48 hours, 72 hours and 96 hours. The study established that Ethion exerts a genotoxic effect on the erythrocytes of Zebrafish, causing the appearance of micronuclei in the erythrocytes after exposure in a manner that was both concentration-dependent and time-dependent. At higher concentrations the changes were frequent, and the exposure time had a direct correlation to the abnormal behaviour which is statistically significant.

Key words : Cytogenotoxic, Zebrafish, micronucleus, erythrocytes.

Pesticide use in Asia contributes to more than 50% of global use. Unlike global trends where herbicides dominate, insecticides are the most widely used category in Indian agriculture, followed by fungicides. In Asia, India stands 3rd after China in pesticide use and India shares only 1% of the global use of pesticide However, India shares only 1% of the global use of pesticides and 0.31 kg per hectare use as per data of 2017 [14]. Indiscriminate use of pesticides prevalent among the Indian farmers remains a concern leading to the high toxic residues in ecology, causing serious adverse implications for animals and humans. Due to improper handling of pesticides, farmers are always at high risk of direct exposure of these pesticides. The indiscriminate use of pesticides put the consumers at risk because of ingestion of foods with residue contamination. Chronic exposure is linked to various health issues. These pesticides are adversely impacting the biodiversity by contaminating soil, water bodies, and air, affecting non-target organisms like beneficial insects especially pollinators.

Only 0.1% of these pesticides used reaches the target[20] and are responsible for

adversely impacting the non-target organisms. The rapid degradation and continuous use of these pesticides become highly toxic for the aquatic organisms[19]. Over-reliance on a few chemical groups has led to pests developing resistance, necessitating even higher or more potent doses.

Fishes are non-target organisms, vulnerable to the contaminants like pesticides due their direct and constant exposure to these chemicals through gills, skin and indirectly through food chain. Hence fishes are the ideal bioindicators and are helpful to study the direct impact of these toxicants on aquatic life[7]. Zebrafish, (*Danio rerio*) has been extensively studied in many genotoxicity studies and considered as an ideal research model for studying the impact of the pesticides on freshwater fish because of low cost and easy to maintain[8]. *Danio rerio* is being extensively studied in toxicology as its adaptation to environmental changes is fast and can be breed easily and fast[9]. The significant fact that Zebrafish shows more than 80 per cent genetic homology with human beings, which makes it as an ideal research model for studying the risk to health caused by these pesticides which can be inferential on human health[22].

Organophosphates are preferred class of pesticides today in agriculture because of low levels of bioaccumulation and faster mode of action of neurotoxic and low cost[16]. Ethion is an organophosphate pesticide used on a variety of food, crops (Ministry of Agriculture & Farmers Welfare, Government of India, 2024) and is being absorbed by cell membranes because of its small size and lipophilic molecule. It readily converts into ethion monoxon and inhibits Acetylcholinesterase enzyme activity, which is required for facilitating the nerve impulse transmission[21]. The study of ethion genotoxicity in zebrafish, despite the availability of data for other organophosphate (OP) pesticides, is highly significant. It addresses a critical knowledge gap regarding a specific chemical, leveraging the zebrafish model to understand environmental and human health risks that cannot be reliably inferred from existing OP data alone.

Micronucleus (MN) Formation is the result of the fragments or whole chromosomes that fail to incorporate into the main daughter nuclei form a small, spherical, nucleus-like structure in the cytoplasm of the interphase cell, known as a Micronucleus. The frequency of micronucleate cells is directly proportional to the amount of irreversible DNA/chromosomal damage induced by the toxicant. Micronuclei analysis in peripheral blood samples is a basic technique widely used in fishes. Micronucleus assay (MN) has some noticeable advantages such as rapidity and simplicity[5]. This assay detects the chromosomal breakage during the cell division or chromosomal loss during the anaphase[23].

This comprehensive ecotoxicological study followed a strict ethics protocol approved by the Institutional Animal Ethics Committee (IAEC), documented under approval reference number NVC/IAEC/29/2022 (dated 27 Dec 2022).

The experiment utilized Zebrafish (*Danio rerio*), procured from an approved vendor. Upon arrival, the fish were briefly dipped in a 1% $KMnO_4$ solution to mitigate

any potential infections before being transferred to a 45 cm X 30 cm X 30 cm glass tank, which had also been cleaned with 1% $KMnO_4$. The fish underwent a 30-day acclimatisation period in this tank[12]. The aquarium was filled with tap water that was allowed to stand for 24 hours to ensure dechlorination. The fish were fed commercially available dry pellets, which were crushed to a smaller size suitable for *Danio rerio*. During acclimatisation, the natural mortality rate was acceptably low, less than 3% in the initial 48 hours, likely attributable to travel stress. Feeding was regular throughout this period but was stopped 24 hours prior to exposure to the test medium. Water levels were maintained against evaporation, and the tank was routinely cleaned using a syphon pipe to remove faeces and uneaten food, preventing fouling. The static renewal system incorporated aeration pumps for optimum oxygenation and maintained a precise 12-hour dark and 12-hour light photoperiod using an autotimer switch[12].

The selected fish of both sexes were 6–7 months old, with an average length of 4.0 cm (±0.5 cm) and an average weight of 0.35 g (±0.20 g). The physicochemical characteristics of the dechlorinated tap water were rigorously maintained according to the CCAC guidelines 2020 on Zebrafish. Specifically, controlled laboratory conditions ensured the water temperature remained at 25ºC, the pH at 7.4 ±0.3 and dissolved oxygen levels were kept above 6.0 ± 0.2mg/L. Salinity was held at 0.01% and Total Dissolved Solids (TDS) at 162± 2 ppm. A 12:12 h light-dark cycle was strictly upheld throughout the entire exposure period[12].

The study utilized Ethion 50% EC (54.5% w/w), branded as Tafethion, procured locally and manufactured by Rallis India Limited. The experimental design was divided into two core parts: first, the determination of the LC_{50} (Lethal Concentration 50) of ethion in Danio rerio, and second, the subsequent evaluation of the genotoxicity caused by sublethal dose.

Genotoxicity assessment :

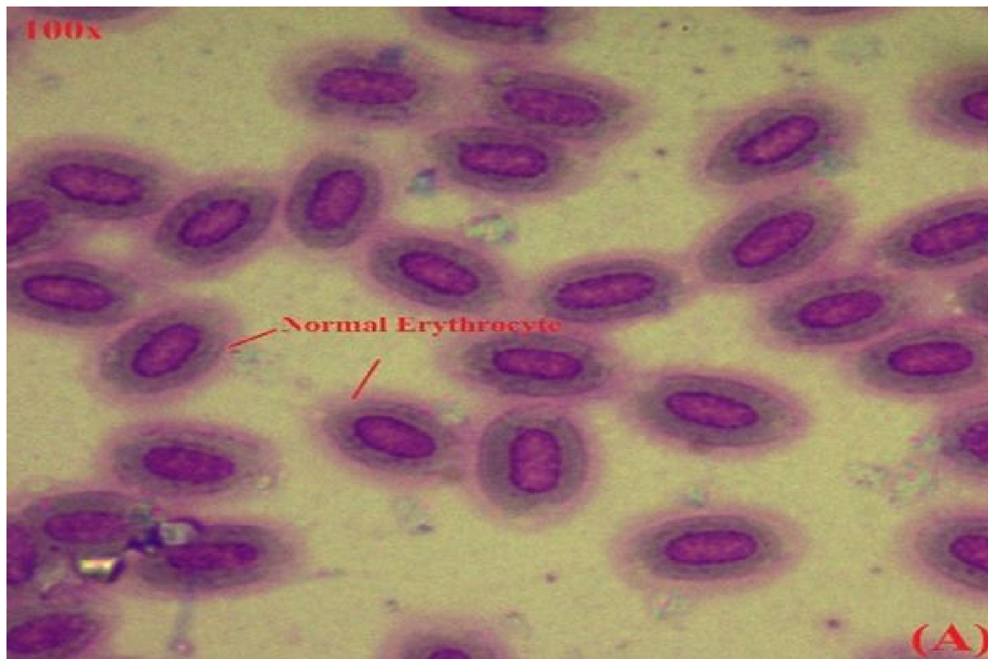
Based on the LC_{50} concentration, 3 sublethal concentrations of Ethion were identified as 0.340 mg/L, 0.476 mg/L, and 0.612 mg/L. Two fishes per group for 3 sublethal doses were subjected to the sublethal doses for 24 hours, 48 hours, 72 hours and 96 hours and one negative control which was not exposed to any dose of Ethion. Zebrafish were anaesthetised by immersing them in ice water until they lost their equilibrium completing the stipulated time intervals of exposure to respective doses. With the help of blotting paper, the water and slime were cleaned from the fish body. Peripheral blood samples were collected by severing the caudal fin using a scalpel and blood samples were collected following the method outlined by Babaei *et al.*[3]. Immediately after the blood collection, blood smears were prepared on a clean glass slide. 2 slides per fish for each dose and each time interval were prepared, totalling 4 slides per treatment per time interval. The slides were dried at room temperature for 10 minutes and then slides were stained with 4% Giemsa stain for 7 minutes[13]. Once stained, the slides were cleaned using running water and after air drying overnight the analysis of slides was performed under an optical microscope Karl Zeiss Primo star microscope at 1000x magni-

fication and photographed with Tucsen camera H series. A minimum 1000 cells were observed per slide for each time interval and each dose. The cells were considered as normal when the cells have an oval and round shaped nucleus maintained unique to species, while if the erythrocytes shown small intracytoplasmic body which is separated from the nucleus without any kind of binding bridge and with same staining colour as that of nucleus varying from 1/3 to 1/20 in size it can be categorised as micronucleus formation due to the DNA damage[24]. To calculate the micronucleus frequency first the mean was calculated for the MN cells found and then following formula was used to calculated the MN frequency[10].

$$MN\% = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

Statistical Analysis :

Descriptive statistics were calculated for each experimental group and time interval, with data expressed as mean ± standard deviation (MN/1000 cells). To assess overall group differences, the Kruskal–Wallis test was applied as a non parametric for comparing multiple independent groups. Additionally, Spearman's rank correlation analysis was conducted to evaluate the strength and direction of associations between treatment groups, particularly to explore dose dependent relationships. Further, a two way ANOVA was performed to examine the combined effects of Dose and Time on MN/1000. All statistical procedures were performed using SPSS software (version 30.0.0), with significance levels set at $\alpha = 0.05$.



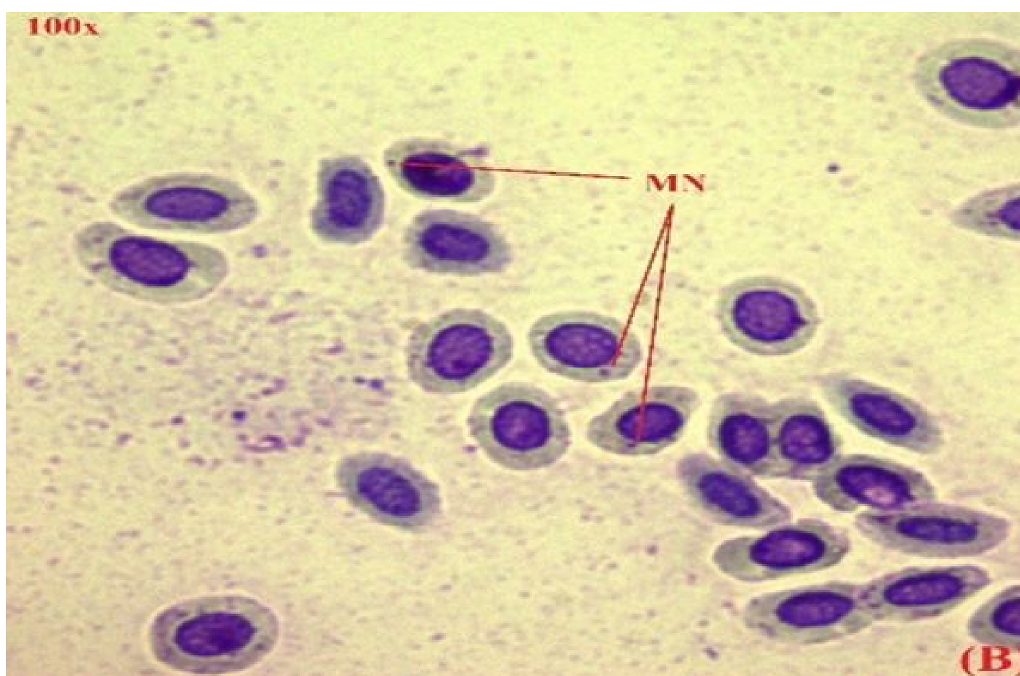
(A)Normal erythrocytes

Figure 1. Blood smear of *Danio rerio,* erythrocytes stained with 4% giemsa.

The mean frequency of micronucleus in erythrocytes of Zebrafish for 3 different sublethal concentrations and a negative control at 4 different time intervals are shown in Table 2. The 3 sub-lethal doses considered were 0.340 mg/L, 0.476 mg/L, and 0.612 mg/L and frequency of micronucleus appearance was ascertained in the erythrocytes at 24 hours, 48 hours, 72 hours and 96 hours of exposure. In the control group, the erythrocytes were oval and percentage of micronucleus was infrequent and less at baseline, while in the Ethion treatment group, there was an incremental effect in the frequency of micronucleus.

In the control group normal erythrocytes an oval and round shaped nucleus were seen mostly, while in the erythrocytes from the treated group, small intracytoplasmic body which is separated from the nucleus were observed with the same staining colour as that of nucleus categorised as micronucleus formation due to the DNA damage[6]. The frequency of micronucleus was higher in treated group and exposed for longer time interval. The erythrocytes with micronucleus shown in Figure 1.

The micronucleus frequency has shown an incremental effect depending on the dose and time of exposure. As the sublethal dose increased, it has produced more damage to DNA and as the time of exposure is prolonged there seems a cumulative effect to this damage The data is presented in Table 1 and the graph to this effect is presented in the Graph 1.

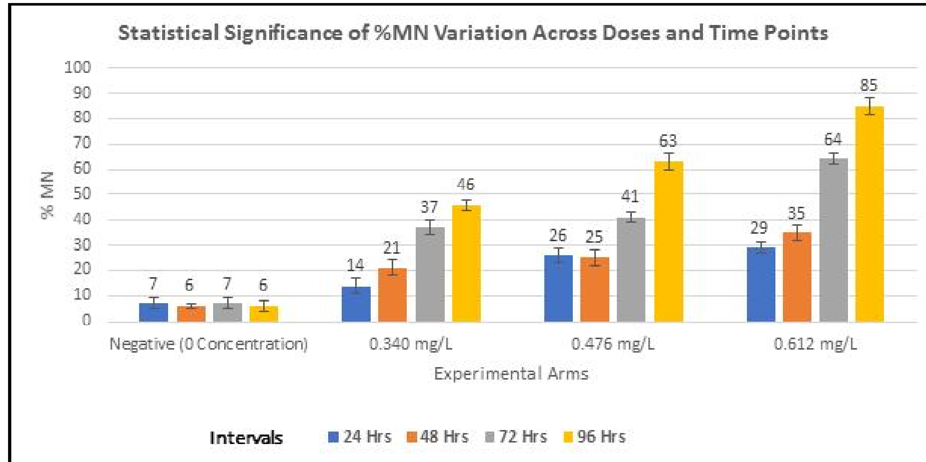


(B) Micronucleus shown in arrows

Figure 2: Blood smear of *Danio rerio,* erythrocytes stained with 4% giemsa.

Table-1. Mean frequency of erythrocytic micronucleus in *Danio rerio* exposed to three sub-lethal concentrations (0.34, 0.476 and, 0.612 mg/L) of Ethion and the negative controls at different time interval of exposure (24, 48, 72 & 96 hours).

Groups	MN/1000 Cell Frequencies			
	24 Hrs	48 Hrs	72 Hrs	96 Hrs
Control	7±2	6±1	7±2	6±2
0.340 mg/L	14±3	21±3	37±3	46±2
0.476 mg/L	26±3	25±3	41±2	63±3
0.612 mg/L	29±2	35±3	64±2	85±3



Graph : The table presents micronuclei (MN) formation per 1000 cells measured at 24, 48, 72, and 96 hours for control and three dose groups. Increase in MN formation depending on the dose and time of exposure increase.

Table-2. Kruskal–Wallis test to check the significant difference between the control and dose groups

Groups	Mean	Std. Deviation	Test Statistic	*P-value*
Control	6.63	1.455	40.322	0.000**
D1	28.19	14.451		
D2	37.56	16.46		
D3	50.56	22.624		

The Kruskal–Wallis test yielded a test statistic of 40.322 with a p value of 0.000,which is well below the 5% significance threshold (α = 0.05). The progressive increase in mean values from D1 (28.19) to D2 (37.56) and D3 (50.56), compared to the control (6.63), demonstrates a clear dose response relationship, suggesting that higher doses are consistently associated with greater biological activity.

Table-3. Two Way ANOVA: Effects of Dose and Time on MN/1000.

Dependent Variable: MN/1000					
Source	Sum of Squares	df	Mean Square	*F - Value*	*P- value*
Corrected Model	15574.969a	15	1038.331	261.627	0.000**
Intercept	30196.531	1	30196.531	7608.575	0.000**
Dose	8297.844	3	2765.948	696.932	0.000**
Time	5141.594	3	1713.865	431.84	0.000**
Dose * Time	2135.531	9	237.281	59.787	0.000**
Error	63.5	16	3.969		
Total	45835	32			
Corrected Total	15638.469	31			
R Squared = .996 (Adjusted R Squared = .992)					

The Pairwise comparisons between control and dose groups using Kruskal–Wallis post hoc analysis comparisons revealed statistically significant differences between the control and each dose group. Specifically, the test statistics were –24.562 (p = 0.001) for Control vs D1, –32.156 (p = 0.000) for Control vs D2, and –39.156 (p = 0.000) for Control vs D3. The increasingly negative test statistic values reflect the growing divergence of the dose groups from the control, consistent with the observed dose response trend. These results confirm that each administered dose produced a significantly greater effect than the control, with higher doses associated with progressively stronger biological activity.

A two-way analysis of variance (ANOVA) was conducted to examine the effects of Dose and Time on the dependent variable MN/1000. The overall model was highly significant, $F(15,16) = 261.63$, $p < .001$, with an $R^2 = 0.996$ (Adjusted $R^2 = .992$), indicating that the predictors explained nearly all of the variance in MN/1000. There was a significant main effect of Dose on MN/1000, $F(3,16) = 696.93$, $p < .001$. This suggests that different dose levels produced distinct changes in MN/1000 values. A significant main effect of Time was also observed, $F(3,16) = 431.84$, $p < .001$, indicating that MN/1000 varied across different time points. The interaction between Dose and Time was statistically significant, $F(9,16) = 59.79$, $p < .001$. This indicates that the effect of Dose on MN/1000 was dependent on the Time of measurement. In other words, the impact of dose levels was not consistent across time points, but varied depending on when the measurement was taken.

The use of organophosphates like ethion is now rampant in agricultural practice across globe and it's such indiscriminate use has started affecting adversely to all non-target organisms especially aquatic and terrestrial organisms and poses serious ecological concerns. The genotoxic assessment of these pesticides is important to know the effect on the genomic stability of the organisms which are exposed to these compounds. Micronucleus (MN) Test in zebrafish (*Danio rerio*) erythrocytes is a

sensitive and simple, economical yet crucial approach to evaluate the toxicity in non-target aquatic organisms[5]. Genotoxicity assessment is widely used as biomarkers to evaluate the mutagenic risk caused by toxicants especially in aquatic life. The aquatic organisms are in continuous and constant contact with the contaminants, results into many physiological damages to the organisms, but DNA damage is often the early signs of the biological adverse effect caused by the exposure to toxicants. It may cause genomic alterations like chromosome loss or breakage due to single or double stranded DNA breaks. (Avelyno H. D'Costa *et al.* 2018). The MN test helps to detect the clastogenicity of the compound[1] and Through Micronucleus test, these genomic alterations can be easily detected and can be used to detect the environmental stress caused by these toxicants like pesticides.[15] The various physiological changes in the body system of the aquatic organisms which are exposed to such contaminants are the manifestation of these genomic alterations.

The study of peripheral erythrocytes are excellent bioindicators as they are direct contact with the aquatic environment and this environmental stress brings changes in these cells which can be easily detected by simple experiments. The persistent exposure of toxicants to these organisms can cause a long-lasting impact on the physiological functioning of the organisms like fertility which in turn may affect the population size and biodiversity.[17]

This study fills that knowledge gap and ascertain the LC_{50} value of Ethion and genotoxicity in Zebrafish by using MN Assay. The LC_{50} value ascertained is 0.966 mg/L which is statistically significant. The LC_{50} value indicates that Ethion is a potential high toxic compound in aquatic organisms which is similar for Organophosphate class of pesticides. This has stressed the need for a careful and judicious use of Ethion when it is used in agricultural practice. These results clearly underline the potential hazard that this pesticide can cause to all who may get exposed to this especially to non-target organisms.

The study clearly established with 3 sublethal doses that the persistent levels of contamination in real world environmental conditions can caused serious genotoxicity to aquatic life. The results demonstrated a significant dose and time dependent increase in genotoxic effect. At sublethal concentrations, pose a significant risk to the genomic stability of the organisms like fishes. The study established that the genotoxicity caused by Ethion in Zebrafish is dose dependent and time dependent where at 4-time intervals 24, 48, 72 and 96 hours Ethion was exposed and the most significant result is that the interaction of dose and time where the effect of particular concentration of Ethion is not constant but it increases over the time period. So, at low and sublethal concentrations also, Ethion is potentially toxic as the exposure time is prolonged which is normally been seen in real world aquatic ecosystems.

The acute toxicity of many organophosphates is well documented, Organophosphate pesticides causes DNA damage by alkylating the DNA bases by protein alkylation which causes the DNA disintegration[4]. In organophosphates the phosphorous group may causes the phosphorylation of DNA which leads to the DNA damage[18]. This study has ascertained

the LC_{50} value for Ethion in Zebrafish (*Danio rerio*) and providing quantitative assessment of genotoxicity through MN Assay, clearly establishing the DNA damage and dose and time dependency which can mimic a real-world ecological condition. The genotoxicity may not cause lethal effect of these aquatic organisms but may cause serious ecological stress. The genomic alterations may lead to many physiological changes like low fertility, decreased immune response, changes in behavioural patterns and altered sensory functions. The accumulation of these genomic alterations may cause serious alterations in future generations affecting survival and raising biodiversity concerns. The results of LC_{50} value, sublethal concentrations can be helpful to define the water quality standards. These results clearly indicating the need for formulation and implementation of integrated pest management and to reduce the use of chemical pesticides and fertilizers with the biopesticides and biofertilizers. This can be helpful to reduce the environmental stress caused especially to water bodies and soil.

The study establishes the genotoxic potential of Ethion in acute toxicity test which can be scientific basis formulating a rational regulation for the use of these pesticides. This study has done the acute genotoxicity for four-time intervals maximum up to 96 hours and not beyond. Moreover, this study has not done any post recovery period assessment, whether the genotoxicity caused by Ethion was persistent or there was a negative change in the genotoxic effect when the fishes were kept in normal conditions.

As a conclusion, micronucleus test can be used as bioindicator to assess the early signs of ecological stress in fishes and is very effective yet the most economical tool. The Ethion causes the genotoxic effects in Zebrafish and it is at sublethal concentrations also poses serious genomic alteration risk. The LC_{50} value of 0.966 mg/L indicating Ethion is toxic to Zebrafish at such low level. The genotoxicity shown is significant at sublethal doses and is directly correlated with time of exposure and concentration. The interaction of time and concentration is direct and significant results in increasing the micronucleus frequency in the erythrocytes in Zebrafish.

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Conflict of interest :

The authors declare that they have no conflicts of interest.

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