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Preliminary study on the behavioral response of zebrafish to the presence of methionine and polypropylene residues in water

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Abstract
When it comes to pollutants in the aquatic environment, there is a wide variety of products, from fibers and plastics to food and medicinal compounds and their by-products. Pollutants have been an area of interest for decades and are becoming more popular with each passing year, whether their presence and effects are studied separately by class or in combination. Among all, polymeric materials are in the center of attention, and their presence in the environment has long been established with studies on their harmful effects. Improper disposal of pharmaceutical materials is just one of many ways that lead to their accumulation in water and soil. The worldwide spread in the environment of polymeric materials, as well as pharmaceutical pollutants is described by the literature, but also the effects of these two materials separately in terms of the behavior of zebrafish (*Danio rerio*). In this paper, we studied the "joint effect" of one polymeric material (polypropylene) and one pharmaceutical product (methionine - one of the essential aminoacids of the body, with antioxidant properties and multiple uses in the pharmaceutical field). Although we did not find many significant differences in terms of behavior, one aspect worth noting was the anxiolytic-like effect of microplastic on the social, and stress related behavior of zebrafish.

Keywords: methionine, microplastics, residues, zebrafish.

1. INTRODUCTION

Polypropylene, according to a multitude of well-established studies, is a persistent threat pollutant in the environment, especially in aquatic environments [1]. Polypropylene is large-scale used in different industries, from packaging, textile materials and fibres to construction materials, thus its easy access and reckless unreasonable dumping into the environment is facilitated [2]. On the other hand, methionine is one of the essential aminoacids of the body [3], due to its involvement in angiogenesis and its antioxidant properties. Nowadays, it is also used as a treatment for copper and acetaminophen poisoning [4], liver disorders and viral infections amongst other things [5]. Increased levels of methionine may lead to oxidative lesions, apoptosis and oxidative stress [6]. Unproper discarding of medicines or just natural excretion of the active substances can lead to their presence in water in higher or lower concentrations. Both substances have been suggested to be linked to behavioral changes as well as tissular damage and unbalanced oxidative status.

The present study explored the possibility of these two polluting agents to act in synergy and worsen the symptoms. For this purpose, we have used a well-known experimental model, zebrafish. From a genetic point of view, zebrafish has been shown to share over 70% of the genes with humans [7]. Moreover, it has been shown that zebrafish is able to exhibit complex behaviors such as anxiety, depression, shoaling preferences and others which makes them the perfect animal model for toxicological studies focused on the behavior [8].

2. EXPERIMENTAL

All the animals were maintained and treated under the EU Commission Recommendation (2007), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 norms, referring to the guidelines for accommodation, care and protection of animals used for experimental and other scientific purposes. The experiment was approved by the Ethics Committee of the Faculty of Biology, "*Alexandru Ioan Cuza*" University, of Iasi.

Our experiment was performed using a fish species, *Danio rerio*, as animal model, and consisted of 4 experimental groups (n = 10 fishes/group, females/males ratio being 5:5). To ensure that the treatments are administered to each specimen, the fishes were housed in individual containers made of glass with a volume of 800 ml, water at a temperature of 25°C and a light/dark period of 10/12 h. The animals were exposed to the two contaminants for a period of 7 days in accordance to the findings in literature about similar experiments with single exposure [9]. The four groups were distinguished based on the treatment as follows: 1 control group, 1 methionine (6.0 mM), 1 polypropylene in form of microfibers with dimensions ranged between 1-4 mm (2 mg/L) and 1 methionine (6.0 mM) + polypropylene (2 mg/L). Water was changed daily, and the parameters reassessed. At the end of the exposure period, we performed behavioural tests (novel tank test, social preference, aggression) using EthoVision XT software and the data were analysed statistically by ANOVA analysis.

3. RESULTS AND DISCUSSION

Zebrafishes are capable of responding to external stimuli starting from the early stages of development [10]. Anxiety is a trait characterized in multiple affective and psychiatric disorders. In the case of zebrafish, anxiety can be assessed by using the Novel Tank Test which is an already validated test as it is based on the natural tendency of zebrafish to sit at the bottom of a new tank and slowly starting to explore as they accommodate to the new environment [11].

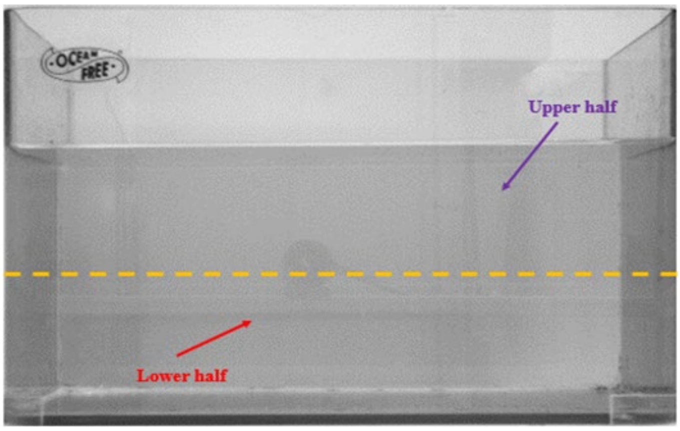

Figure 1 The experimental apparatus for the Novel Tank Test for measuring anxiety levels in zebrafish.

In our experimental setup, at the end of the administration period, each fish was placed in the novel tank (Figure 1) and recorded for a period of 6 minutes. The parameters measured for assessing the anxiety level were freezing, time spent in the upper half, circling clockwise and latency to reach the upper half.

The results can be observed in Figure 2. Freezing episodes are assessed as immobility periods longer than 1 second, and an increased number of episodes indicates anxiety that is generally higher in the stressed fishes. As it can be observed in Fig. 2A, although the number of freezing episodes was smaller in the groups MPs and Met + MPs compared to the control group, the only significant difference was found between the Met group and combined group suggesting that MPs might have an influence on the stress and anxiety levels. Interestingly, when it came to the latency to reach the upper half for the first time there were no significant differences between groups; however, for all the treated groups, it took longer to start exploring the upper half compared to the control. In terms of the time spent in the upper half, there were no significant differences, but the Met group did spend less time in the top portion of the tank. On top of that, the significant differences between the Control and MPs, as well as between Met and Met + MPs, suggest that MPs might have an anxiolytic effect since groups exposed to MPs froze less than the Control and Met, it took less time to reach upper half compared to Met, and even spend more time in the upper half.

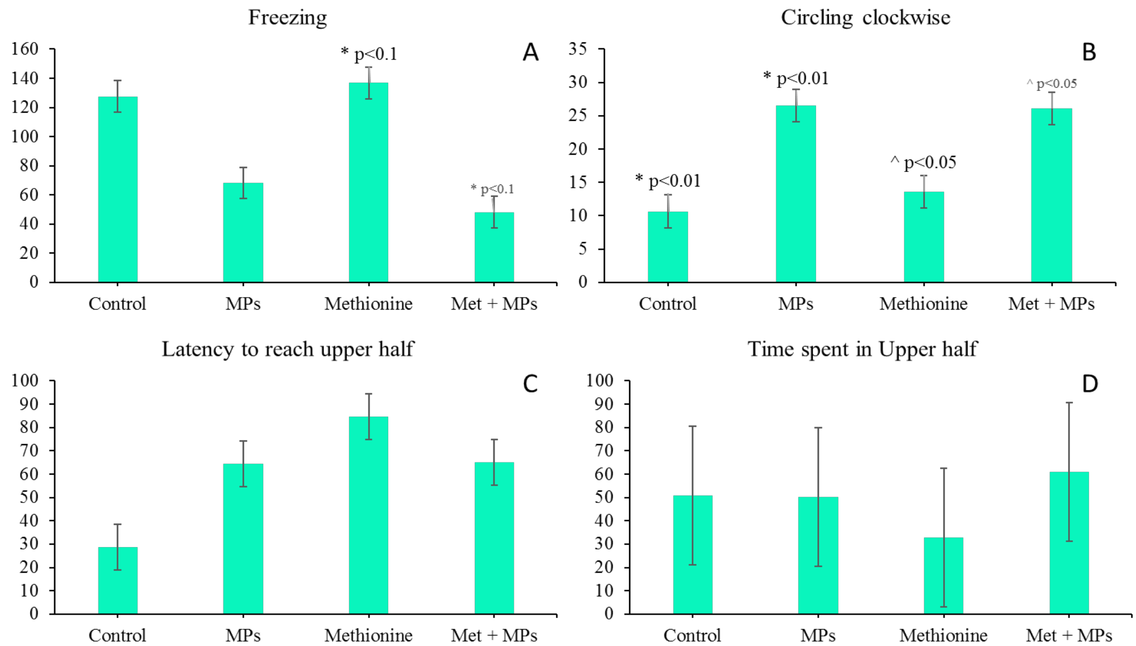

Figure 2 Novel Tank Test results based on the following parameters: Freezing, circling clockwise, latency to reach upper half and time spent in upper half.

In Figure 3, there are representative tracks and heatmaps of each of the 4 groups, as to better illustrate their behavior following treatments in terms of anxiety and exploration behavior.

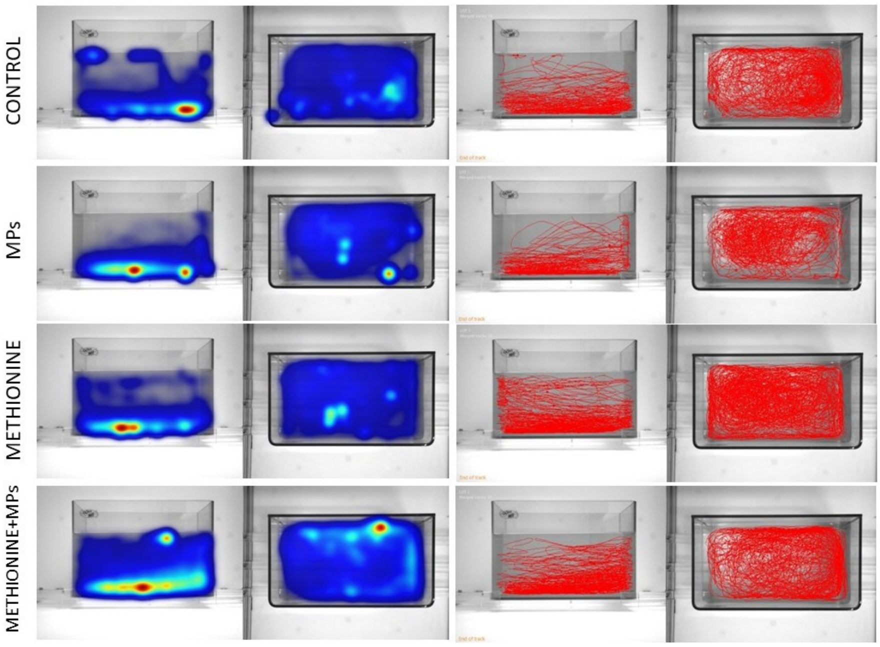

Figure 3. Representative tracks and heatmaps illustrating the average behavior of each group following treatment. Each group has exhibited different levels of anxiety with the Met group displaying the most the exploration tendency.

Zebrafish have an innate social nature and a tendency towards shoaling over being alone which makes them the perfect candidates for assessing the effects of neurotoxic substances on social behavior and social cues. The tests used for measuring these parameters are the aggressivity test which uses a mirror and it is based on the zebrafish inability to distinguish between real conspecifics and its own reflection and it follows their behavior towards the image in the mirror [12,13] and the social preference test where they have to choose between an empty arm and the one that has conspecifics [14]. The experimental apparatus for both tests can be observed in Figure 4.

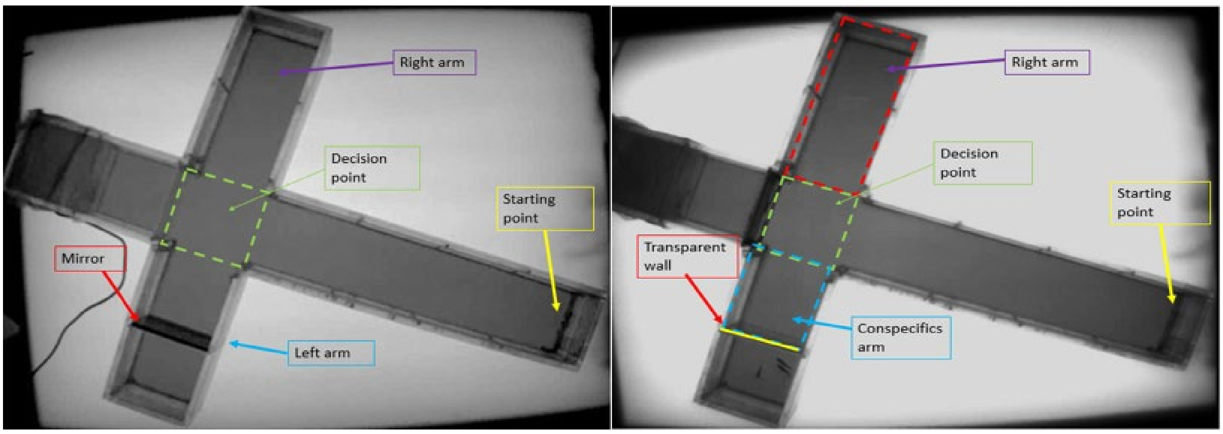

Figure 4. Experimental apparatus for the aggressivity test (left) and the social preference test (right).

The results can be observed in Figure 5. We represented the time spent in the right arm alongside latency to reach the right arm, as in the case of our experiments the fishes exhibited avoidance behavior and preferred the empty arm over their own reflection/conspecifics or they remained in the starting arm. As it can be observed, the fishes from the treatments group took a longer time to start exploring and entering the right arm in both the aggressivity and the social preference test; however, there were no significant differences between the groups.

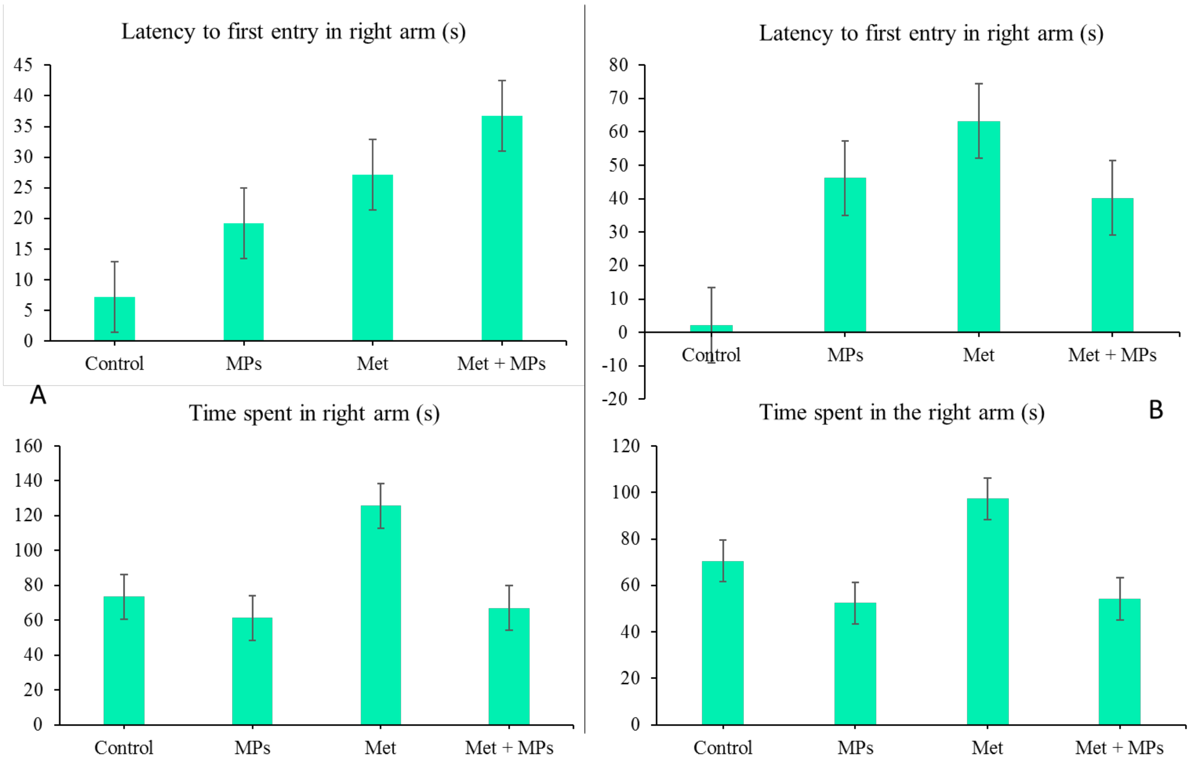

Figure 5 Results of the four experimental groups to the aggressivity test and social preference test with focus on the right arm as the animals exhibited social avoidance and fear-related behavior.

In figure 6, we analyzed anxiety-related behavior measured by freezing episodes and their duration during the social preference test, as well as swim bursts as a parameter for aggressivity and agitation during the aggressivity test. As it can be observed, there was a significant decrease in terms of the duration of the freezing episodes between the methionine group and the combined one, as well as significant differences between the control and the methionine group in terms of the number of episodes. Based on the results, the idea that exposure to MPs might have anxiolytic effect is further reinforced. There were no significant differences in terms of number of swims bursts and duration; however, the duration was lower for the MPs and Met groups.

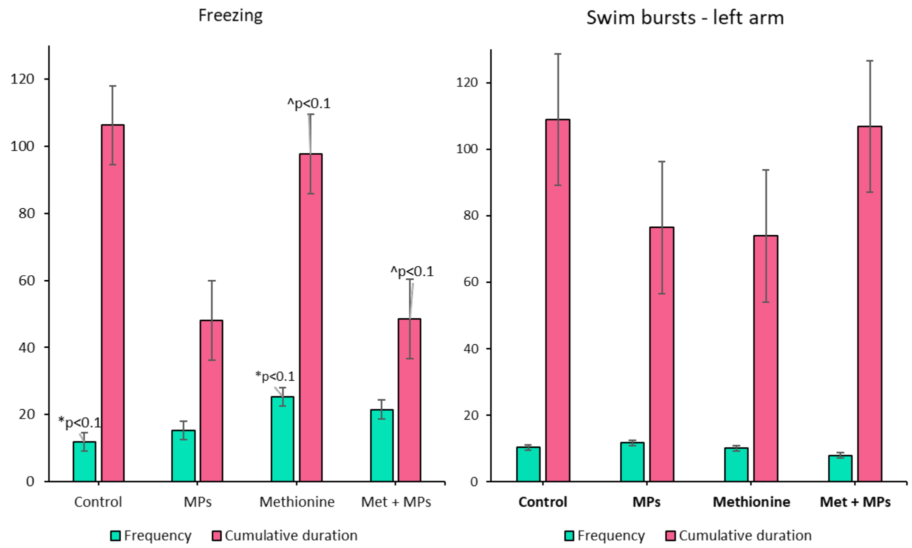

Figure 6 Anxious behavior measured through the duration and frequency of freezing episodes (A) and aggressive behavior displayed as swim bursts measured through their duration and frequency results (B).

In Figure 7, there are illustrated the average tracks and heatmaps of each group following treatment. Each group has presented different behavior and preferences.

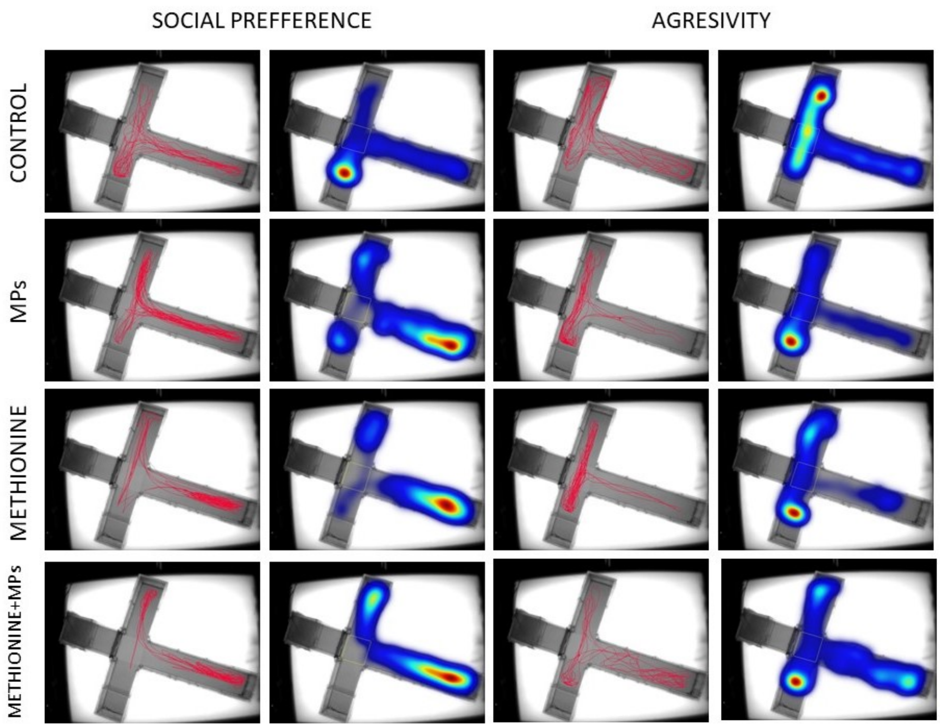

Figure 7 Average tracks and heatmaps of each group following treatment. First 2 columns – social preference test, last 2 columns – aggressivity test.

4. CONCLUSIONS

Our preliminary experiments have shown that there are no significant differences between the toxic effects of methionine and microplastics to social isolation. However, literature supports social isolation as a reliable way to obtain animal models for depression. These two aspects suggest that the neurotoxicity induced by the two studied substances are similar to depression.

Another observation is that our data suggests that MPs act through a mechanism that makes them capable of blocking methionine toxic effects, for example in the novel tank test and in the social preference one, it can be seen that the groups exposed to both Met and MPs exhibited significantly decreased anxiety level.

However, all groups have shown increased anxiety and increased aggressivity. Our future studies should and will focus on discovering whether these effects are augmented due to the social isolation factor or are as pronounced in groups kept and grown together too. Future studies should also focus on the exact mechanism behind the MPs anxiolytic-like effect.

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