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The synergistic effect of microplastic and malathion exposure on fiddler crab *Minuca ecuadoriensis* microplastic bioaccumulation and survival

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ABSTRACT

We assessed the combined effects of polyethylene microplastic (MP) and malathion (MLT) on the survival of the fiddler crab *Minuca ecuadoriensis*, and MP tissue bioaccumulation in four treatments following 120 h exposure: T1) Control; T2) MLT 50 mg L^{-1}; T3) MP 200 mg L^{-1}; and T4) MLT (50 mg L^{-1}) + MP (200 mg L^{-1}). The highest mortality (80%) was in T4, followed by T2 (28%) and no mortality was in T3. Higher MP bioaccumulation was observed in T4 (572 items g $tissue^{-1}$) followed by T3 (70 items g $tissue^{-1}$). Our findings indicate that the synergistic effect of MLT and MP increased *M. ecuadoriensis* bioaccumulative capacity and decreases survival. Thus, as MP contamination in aquatic environments is ubiquitous, our study raises a warning on the synergistic effects of MP with other environmental contaminants and serves as a baseline for further studies.

Microplastic (MP) pollution is a global concern due to its omnipresence in all ecosystems (Andrady, 2011; de Souza Machado et al., 2018; Rochman et al., 2019). The increasing abundance of MP in aquatic ecosystems results in the ingestion of these contaminants by aquatic biota (Brennecke, 2015; Cole et al., 2013; Villegas et al., 2021; Yuan et al., 2019), causing effects that range from the subcellular to the ecosystem level (Anbumani and Kakkar, 2018; Guzzetti et al., 2018). The bioaccumulation of MP can cause internal and external injuries, ulcers, digestive tract blockage, among other lethal and sublethal effects (Islam et al., 2021). These contaminants also act as a cocktail for other contaminants, including plastic additives used during the manufacturing process, and environmentally persistent organic pollutants (POPs), such as organophosphate pesticides, among others (Islam et al., 2021; Rochman et al., 2019; F. Wang et al., 2020a). However, little is known about the effects caused by the combined effects of MP with other pollutants in aquatic organisms. Complex contaminant mixtures can influence both bioaccumulation and toxicity, which can trigger additive or synergistic effects (Barata et al., 2006; Coors and Frische, 2011; Deneer, 2000).

Pesticides and plastic litter pose major threats to estuarine ecosystem functionality and biodiversity (Syberg et al., 2015). Malathion is a broad-spectrum organophosphate pesticide (OPs) and one of the most widely used pesticides for both agricultural and non-agricultural purposes (Atwood and Paisley-Jones, 2017). Although it is a rapidly degrading compound with little bioaccumulative capacity, malathion (MLT) can cause physiological, biochemical and genotoxicological damage (Ullah et al., 2018). The main MLT mechanism of action is the inhibition of acetylcholinesterase (AChE), the enzyme responsible for nerve synapses. This inhibition causes uncontrollable movements, spasms, convulsion, slow reflexes, paralysis and even death (Correia and Smee, 2018; Schroeder-Spain et al., 2018; Wendel and Smee, 2009). Despite its negative biota effects, MLT is allowed to be used with risk mitigation, and is considered a moderately hazardous substance by the Sustainable Agriculture Network and by the Ecuadorian regulation (INEN, 2008; Sustainable Agriculture Network, 2017).

The mixture of both MP and OPs is of concern, as MP can act as a vector for these pollutants. It is important to evaluate the synergistic effects of MP combined with OPs in key ecosystem health species, such as fiddler crabs (Capparelli et al., 2019, 2016). These crabs play a key role in several ecosystem functions, such as biogeochemical cycles and nutrient recycling, stimulating mangrove microbial activity (Gribsholt et al., 2003; Zeil and Hemmi, 2006). A previous assessment reported high OP concentrations, including MLT, and MP in water and sediments collected from *Minuca ecuadoriensis* fiddler crab burrows and tissues

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(Villegas et al., 2021). However, how contaminants bioaccumulate in *M. ecuadoriensis* and how they affect the survival of the organisms remains yet to be unraveled.

Thus, to understand the effects of the MP and OP mixture under controlled laboratory exposure settings, we evaluated the effects of waterborne exposure to high-density polyethylene and malathion (25% Proficol®) in *Minuca ecuadoriensis* fiddler crab MP bioaccumulation and survival. We hypothesized that this MP and MLT mixture will act either in a synergistic or antagonic manner when compared to the effects of the isolated compounds.

One hundred adult *M. ecuadoriensis* specimens of both sexes were randomly collected from Isla Santay (2°13′04°S, 79°52′40°W), a Ramsar site located in the Guayas River estuary in Ecuador. The individuals were transported in pre-cleaned 1500 cm^3 (15 cm × 15 cm × 6 cm) plastic boxes containing sponge cubes moistened with water and a thin layer of sediment from their habitat to the "Laboratorio Nacional de Referencia del Agua", at the Universidad Regional Amazónica Ikiam. Only non-ovigerous, intermolt crabs displaying carapace width greater than 10 mm were used in the experiments. All crabs were maintained for 3 days under a 12 h photoperiod at room temperature for acclimatation to laboratory conditions prior to the experiment.

An MP solution was prepared by mixing 200 mg of High-Density Polyethylene (HDPE) particles smaller than 250 μm to 1 L of filtered Milli-Q water. The solution was then shaken vigorously until homogenization. A stock MLT 50 mg L^{-1} solution was prepared using commercial MLT 25% Proficol® and Milli-Q water. For the mixed solution, MP + MLT, 200 mg of HDPE MP were added to 1 L of the MLT stock solution. Milli-Q water used to prepare all solutions. Salinity was adjusted by 5‰S by adding Instant Ocean® seawater salts to the solutions. The amount of MP was based on a previous study (Brennecke, 2015) where 200 mg L^{-1} of MP did not cause mortality in another fiddler crab species. A higher than environmental dose of malathion (50 mg L^{-1}) was used to ensure exposure to acute exposure doses, as fiddler crabs were not exposed to total submersion and were not in direct contact with the contaminants during the assay. It is important to note that complete *M. ecuadoriensis* submersion leads to submersion stress (Capparelli et al., 2021) due to the semi-terrestrial habitat of this species.

The ability of MP particles to adsorb MLT was determined after 120 h. To determine the MLT partition between MP and water, we adapted the methodology applied by Garrido et al. (2019). Briefly, MPs were separated from the water by filtration using a 0.45 μm pore diameter microfiber filter. Next, each filter was cut into 4–6 pieces, which were placed in a tube containing 15 mL of dichloromethane, used as the extraction solvent. Then, MLT was extracted by sonication using an ultrasonic bath for 30 min. Sonication was repeated twice, changing the dichloromethane solution. The MLT in MP extracts and stock solutions were analyzed by gas chromatography with a nitrogen-phosphorus detector (GC-NPD) using a GC-2014 Shimadzu (Kyoto, Japan) equipped with an AOC-20i Shimadzu (Kyoto, Japan) autosampler. Compound identification was carried out by comparing retention times with a standard solution (99% purity). MLT concentrations were calculated from the calibration curves (RESKET, CT 32278).

MP adsorption depends on polymer type, molecule composition, structure, and chemical characteristics (Wang et al., 2020b). Thus, we calculated the sorption partitioning coefficient (Kd) as $Kd = MLTMP/MLTW$, where *MLTMP* is the MLT concentration adhered to the MP, expressed as mg kg^{-1}, and *MLTW* is the MLT concentration in water, expressed as mg L^{-1}. After 120 h, the MLT adsorbed to the MP particles was of 2.7 mg g^{-1}. Around 5% of the MLT was absorbed onto the MP surfaces, resulting in a partitioning coefficient of Kd = 54 L kg^{-1}.

One hundred crabs were divided into groups of 25 individuals (replicates) into four treatments: 1) Control, comprising Milli-Q water at 5‰ of salinity; 2) MLT 50 mg L^{-1} 3) MP200 mg L^{-1}; and 4) MP (200 mg L^{-1}) + MLT (50 mg L^{-1}). Each crab was put into an individual glass container (12 cm high and 73.2 mm in diameter), semi-submerged in 10 mL of each of the prepared solutions in each treatment. Water changes and feeding were performed every 48 h during the 5 day exposure period. Mortality was recorded every 24 h. After 5 days, the crabs were cryo-anesthetized in crushed ice for 10 min, after which all gill pairs and the digestive tract and the hepatopancreas (DT + H) were dissected for MP quantification.

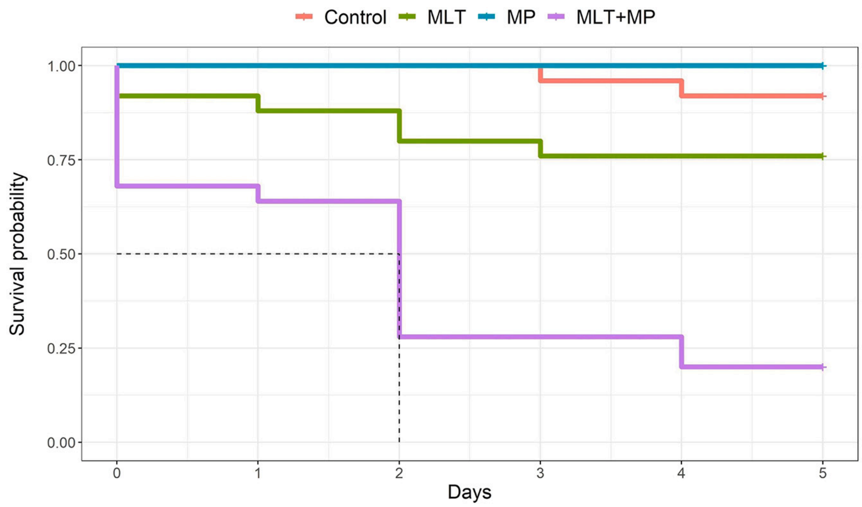
The *M. ecuadoriensis* tissues were pooled (3–5 crabs per replicate). Chemical tissue digestion was performed using H_2O_2 (30%, 200 mL/5 g of tissue) in an oscillation incubator at 60 °C at 100 rpm for 48 h–72 h. The solution was then maintained at room temperature (25 °C) for 48 h, followed by vacuum filtration (Li et al., 2015; Masura et al., 2015; Waite et al., 2018). The filters were stored in capped glass Petri dishes for further visual identification. Precautions were taken to avoid background MP contamination during sample treatment and analytical steps. All laboratory materials were first rinsed with Milli-Q water and then with ethanol prior to use. Clean filter papers were placed in Petri dishes and exposed to the air in the laboratory during the processing period to account for atmospheric contamination. The blank filters were then analyzed visually, and the detected MP was subtracted from the total samples. Filters were analyzed using an Amscope stereomicroscope as 20× magnification, equipped with a 10 megapixel digital camera employing the AmScope software. To evaluate toxicity effects on *M. ecuadoriensis* survival, a survival analysis was performed comparing treatment survival curves against the control using a log-rank test. A minimum significance level of $p = 0.05$ was employed for all procedures.

The highest mortality (80%, Fig. 1) after 120 h of contaminant exposure was observed in treatment 4 (MLT + MP). Two mortality peaks were observed, one after 24 h (32%) and the other after 72 h (36%) of exposure to MLT + MP. Survival probability was high in the other treatments, as follows: MLT (76%), MP (100%), and Control (92%). Evidence of survival probability differences were observed between the controls and MLT + MP ($p < 0.001$), MLT and MP ($p < 0.01$), and MLT and MLT + MP ($p < 0.001$) treatments.

MP alone did not cause mortality in *M. ecuadoriensis* following 5 days of exposure. Our results are similar to those reported by Brennecke (2015), where no mortality was observed after exposing the fiddler crab *Minuca rapax* to high levels of MP. Although no mortality was observed in both cases, MP exposure may still cause other damage, such as increased oxidative stress (Wang et al., 2021) following long-term exposure (Torn, 2020), and these mechanisms still require investigated.

When exposed to the MLT 50 mg L^{-1} treatment, a 28% *M. ecuadoriensis* mortality was observed after 5 days. *M. ecuadoriensis* seems to be more tolerant to MLT compared to other crustaceans. The survival of the blue crab, *Callinectes sapidus*, for example, decreased 33% after exposure to MLT (0.1 mg L^{-1}) (Schroeder-Spain et al., 2018). Furthermore, the mortality of half of the individuals of the white shrimp, *Litopenaeus vannamei* (LC_{50}-96 h) was reached at a concentration 60% below the one employed herein (Bautista-Covarrubias et al., 2020). However, the different effects of MLT exposure may be influenced by different habitats, as *M. ecuadoriensis* is a semi-terrestrial crab that does not spend all of its time submerged in water, being only partially exposed to MLT through water, whereas the blue crab (*C. sapidus*) and the white shrimp (*L. vannamei*) are both aquatic crustaceans. In addition to mortality, MLT exposure has been reported as affecting molting behavior and growth patterns, causing histopathological gill damage and increasing oxidative stress (Knapik and Ramsdorf, 2020; Silva de Souza et al., 2020; Singh et al., 2021).

M. ecuadoriensis individuals exposed to the MLT + MP mixture were exposed to MLT through MP uptake. The ingestion of MLT-contaminated MP may contribute to the leaching of MLT inside fiddler crab tissues, causing the observed mortality. Felten et al. (2020) reported decreased freshwater flea *Daphnia magna* survival due to combined exposure to MP and deltamethrin. The effects of MP and chlorpyrifos exposure in the marine copepod *Acartia tonsa* were 4–25 times more toxic than isolated chlorpyrifos exposure (Bellas and Gil, 2020). Thus, MP may represent an alternative exposure route to MLT and other OPs of semi-terrestrial and



Number at risk: n (%)

	0	1	2	3	4	5
Control	25 (100)	25 (100)	25 (100)	25 (100)	24 (96)	23 (92)
MLT	25 (100)	23 (92)	22 (88)	20 (80)	19 (76)	19 (76)
MP	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)
MLT+MP	25 (100)	17 (68)	16 (64)	7 (28)	7 (28)	5 (20)

Days

Fig. 1. Survival probability curve for the Control (orange), MLT (green), MP (blue) and MLT + MP (purple) *M. ecuadoriensis* treatments. The table below the graph informs on the number of individuals (n) in each treatment and the survival (%) from the beginning (0 days) to the end of the experiment (5 days). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aquatic organisms.

Fiddler crabs are known for their high tolerance to high concentrations of various contaminants (Callahan and Weis, 1983, Devi, 1987, Weis et al., 2011, Capparelli et al., 2016; Capparelli et al., 2017; Brennecke, 2015). Thus, a high MLT dose (50 mg L^{-1}) was employed in order to ensure acute exposure doses, as these organisms are not exposed to water the entire time due to their semi-terrestrial habitat. Toxicity results concerning the MP mixture with other contaminants are still controversial and the mechanism behind the observed toxicity requires investigation. On the one hand, decreased toxicity has been reported for compound mixtures and MP (Li et al., 2021; Trevisan et al., 2020; Zong et al., 2021; Choi et al., 2020). On the other hand, we demonstrated herein that the combined action of MLT and MP increased *M. ecuadoriensis* mortality to 80% and was more lethal than the isolated MLT exposure. Thus, we suggest a synergistic effect between MLT and MP. Most likely, MP acts as a MLT vector. This is corroborated by the detected MLT concentration adsorbed to the MP particles (2.7 mg g^{-1}, Kd = 54 L kg^{-1}). OPs can adsorb to MP by various mechanisms, such as partition effects or hydrophobic, electrostatic, and non-covalent interactions (Fu et al., 2021). Wang et al. (2020c) indicated a significantly positive correlation between the Log Kow and the adsorption capacity of OPs on MP, proposing that hydrophobic pesticides exhibit high affinity to MP. MLT is considered as moderately soluble in water (Log K_{ow} = 2.36, National Center for Biotechnology Information, 2021). Consequently, hydrophobic interactions can explain part of the MLT adsorbed on MP surfaces.

In crabs exposed to MP alone, 87.5 MP items per g $tissue^{-1}$ (mean = 21.9, sd = 14.9) were quantified in DT + H and 123.5 MP items per g

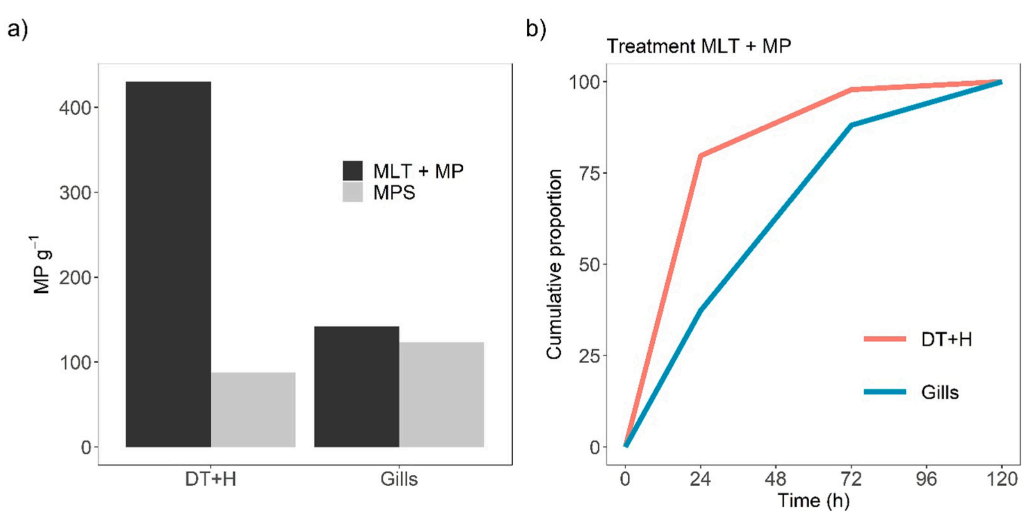


Fig. 2. a) Amount of microplastic bioaccumulated by fiddler crabs in the MLT + MP and MP only treatments after 5 days. Bioaccumulation was determined for all the five crabs that survived the MLT-MP treatment. Bioaccumulation was determined for a sample of 5 crabs subject to the MP only treatment to enable data comparisons. b) Cumulative proportion of MPs (%) in the MLT + MP treatments during the 5 days of experiment. DT + H = digestive tract and hepatopancreas.

tissue^{-1} were found in gills (mean = 30.9, sd = 26.3) after 5 days (Fig. 2a). Previous studies concerning the fiddler crab *Minuca rapax* also reported more MP in the gills than in the digestive tract, suggesting that MP are more likely to become trapped and accumulate in the fine structures of gills, and may pass through the digestive tract without bioaccumulating (Brennecke, 2015). Fiddler crabs filter sediment pellets using water stored in the gill chamber (Dye and Lasiak, 1987). Thus, this active filtering could contribute to MP gill retention. The feeding behavior of fiddler crabs has been associated with particle selection ability (Robertson and Newell, 1982), as these animals use small chela to feed, where small portions of sediment are placed in the buccal cavity and the substrate is washed to float away food which is ingested. The remaining substrate is then discharged to the surface. On the other hand, the water in the branchial chamber is forced up and out onto the face of the carapace for gas exchange (Miller, 1961). The water trickles down to the openings above the legs and returns to the gill chamber, where it will be eventually recycled and evaporated again. As this process is repeated, MP may become more concentrated in the gill chamber.

In the combined MLT + MP exposure, 430 MP items per g tissue^{-1} were found to bioaccumulate in the DT + H (mean = 107.6, sd = 160.7) and 142 MP items per g tissue^{-1} were detected in gills (mean = 35.5, sd = 32.9) (Fig. 2a). It has been previously reported that acetylcholinesterase inhibitors, like MLT, may increase metabolic rates, which could lead to increased foraging activities in crabs in response to energy demands (Holmberg et al., 1972; Roex et al., 2003; Sastry and Sharma, 1981), a pattern that has been also detected in the blue crab *Callinectes sapidus* (Correia and Smee, 2018). Thus, MLT exposure in *M. ecuadoriensis* may be associated to increased MP intake. However, the mechanism underlying this pattern requires further investigations for confirmation.

Since high mortality was detected in the MLT + MP treatment, the cumulative proportion of ingested MP every 24 h could be calculated (Fig. 2b). After 24 h, MP content was higher in DT + H tissues compared to the gills. Thus, MP gill accumulation and in DT + H level out. After 72 h, the low MP accumulation in DT + H may be associated with crab capacity in excreting ingested MP (Torn, 2020; Watts et al., 2014). Mortality in the MP + MLT was also higher during this period (Fig. 1). This suggests that the high MP bioaccumulation in the DT + H tissues could have contributed to the high mortality observed in the combined treatment.

We conclude that the presence of MP increases MLT toxicity. Furthermore, the MLT + MP mixture increases MP tissue bioaccumulation. Due to the significant decrease in the survival of crabs exposed to MP and MLT, this association is synergistic and affects MLT bioavailability, causing acute toxicity. Our results raise concerns, as MP contamination in aquatic environments is ubiquitous and may result in synergistic effects in mixture with other environmental contaminants. Further investigations incorporating specific biomarkers are required to better understand the mechanisms that lead to the toxicity of MP mixture with other contaminants.

CRediT authorship contribution statement

Lipsi Villegas: Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft. **Marcela Cabrera:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Gabriel M. Moulatlet:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Mariana Capparelli:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the present investigation.

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