UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM
Facultad de Ciencias de la Vida
Carrera de Ingeniería en Ecosistemas

BIOACCUMULATION AND TOXICITY OF MICROPLASTICS AND PESTICIDES IN FIDDLER CRABS FROM A RAMSAR SITE IN THE ESTUARY OF GUAYAS RIVER, ECUADOR

Lipsi Nathalie Villegas Borja
28 de abril de 2021, ciudad de Tena, Napo, Ecuador.

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ÍNDICE GENERAL

Artículo desarrollado bajo las directrices de la revista Ecotoxicology and Environmental Safety

Bioaccumulation and toxicity of microplastics and pesticides in fiddler crabs from a Ramsar site in the estuary of Guayas River, Ecuador.

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Abstract

Pesticides and Microplastics (MPs) pose a major threat to ecosystem functionality and to biodiversity in estuarine ecosystem. In this study, we assessed two objectives. 1) The concentration of organophosphate pesticides (OPs) and MPs in water and sediments from the burrows, and tissues of the fiddler crabs *Leptuca festae* and *Minuca ecuadoriensis*. Both species from Isla Santay, a Ramsar site in the estuary of the Guayas River, Ecuador. 2) The effects of the exposure experiment to microplastic (MP) and its interaction with malathion (MLT) in the survival of *M. ecuadoriensis* and MPs bioaccumulation. MPs concentrations in the burrows were up 660 ± 174.36 items kg^{-1} wet weight and 26 items L^{-1} in collected sediments and water, respectively. Regarding OPs, water and sediment concentrations were up to 26 times above the United States Environmental Protection Agency (US EPA) thresholds for chronic exposure. The highest abundance of MPs in the tissues was found in the gills. OPs concentrations in tissues were below the detection limits. The results of the exposure experiment showed a higher decrease in the survival in MLT+MP treatment (80%) compared to MLT (20%), MP (0%), and control (8%). The bioaccumulation of MPs was reported in the gills, digestive tract, and hepatopancreas. However, the higher concentration of MPs was reported in the MLT+MP treatment. Because MPs can increase the toxicity of malathion in fiddler crabs, which are chronically exposed to MPs and OPs, they represent suitable bioindicators to monitor Isla Santay and to comprehend the effects of human activities on the coastal environment of Ecuador.

Key words: *Leptuca festae*, *Minuca ecuadoriensis*, monitoring, microplastic, malathion

1. Introduction

Protected marine and estuarine areas allow the survival of fish stocks and the reduction of anthropogenic impacts (IUCN 2013). The establishment of a protected area implies restrictions on land use changes and polluting activities (Kelleher, 2005). However, the protection of estuarine environments has often been ineffective in meeting its conservation objectives due to water pollution (Jameson et al., 2002). In Ecuador, estuaries and mangroves have been severely affected by agricultural and urban expansion (Calle et al., 2018). Isla Santay located across of the city of Guayaquil, the second most populated city in Ecuador. Isla Santay is a Ramsar wetland considered a biodiversity hotspot. Despite being a protected area, due to the proximity to the urban area and because it is located at the estuary of the Guayas River, it is possible that Isla Santay could be exposing to contamination.

The Gulf of Guayaquil has the highest densities of plastic debris on the Ecuadorian coast (Gaibor et al., 2020). The lack of adequate treatment of solid waste, the population's consumption habits, and high urbanization, contributes to high amounts of solid waste that reach Isla Santay daily. Additionally, the pesticide concentrations in the rivers are a chronic problem due to the weak legislation, the use of hazardous pesticides and the lack of adequate information, like the knowledge gap regarding the correct use and management of pesticides by small farmers (Cambien et al., 2020; Mollocana Lara and Gonzales-Zubiate, 2020). The Guayas province is the most productive agricultural region in Ecuador, contributing to 70% of the national crop production, which makes this watershed indispensable for the country's economy (Frappart et al., 2017). The most common crops in Ecuador (sugar cane, banana, palm oil, cacao, rice, and corn) are

cultivated in the Guayas River basin. A study carried out in the Guayas River basin showed that contamination of the freshwater environment by pesticides was widely present in 60% of the sampled locations, with a total of 26 pesticide substances present in the rivers and estuaries (Deknock et al., 2019).

Among the main contaminants in estuarine environments, pesticides and plastic litter pose a major threat to ecosystem functionality and biodiversity (Syberg et al., 2015). When plastic litter reached the marine environment, it breaks down into small fragments by photo- and thermo-oxidative processes as well as through mechanical impacts such as wave action and mechanical abrasion (Andrady, 2011). The resulting particles smaller than 5 mm are then defined as microplastics (MPs) (GESAMP, 2016). If the buoyancy of these particles is negative, they sink to the seafloor where they becomes available for benthic invertebrates. Besides, particles with positive buoyancy can change to negative as consequence of the colonization by other marine organisms (e.g., marine algae) forming a plastic-organic aggregates that sink in the seafloor (Kvale et al., 2020). MPs ingested by organism (feeding and/or ventilation processes) can have mechanical and chemical effects. The mechanical effects are related with the attachment of MPs to external surface. Among these effects are the reduced of the mobility and the clogging of the digestive tract. While chemical effects can be inflammation, neurotoxicity, genotoxicity, hepatic stress, oxidative stress, disturbance of energy metabolism (de Sá et al., 2018; Setälä et al., 2016). MPs also provide surface areas that can adsorb, absorb, concentrate, and transport organic pollutants, influencing the mobility and bioavailability of pollutants for organisms (Lambert and Wagner, 2018). In addition, Wang et al., (2020) concluded that MPs particles can adsorb and act as vectors of some organophosphate pesticides, including malathion.

Malathion (MLT) is a broad-spectrum organophosphate pesticide (OPs) and one of the most widely used pesticide in the world for agricultural and non-agricultural purposes (Atwood and Paisley-Jones, 2017). The main mechanism of action of this pesticide is the inhibition of the enzyme acetylcholinesterase (AChE) which is responsible for nerve synapsis (Yu, 2011). As a consequence of the AChE inhibition, the hydrolysis of the neurotransmitter acetylcholine does not occur causing uncontrollable movements, convulsion, slow reflexes, paralysis and death (Correia and Smee, 2018; Schroeder-Spain et al., 2018; Wendel and Smee, 2009). Besides, MLT can also causes others physiological, histological, biochemical and genotoxicological damages (Ullah et al., 2018). MLT it is still allowed to be used with risk mitigation, and it is considered as moderately hazardous by the Sustainable Agriculture Network (SAN) and Ecuadorian regulation (INEN, 2008; Sustainable Agriculture Network, 2017). Therefore, in areas with high concentrations of pesticides, as in the case of the estuary of the Guayas River, the presence of MPs can enhance the toxic effects of these substances. For example, MPs can increase the toxicity of the OPs chlorpyrifos in *Acartia tonsa*, increasing the mortality of this marine copepod (Bellas and Gil, 2020). It has been demonstrated that MPs increase the toxicity of chlorpyrifos in the fish *Oncorhynchus mykiss*, producing histopathological lesions, necrosis, infiltration of inflammatory cells, and shed of villi tips, especially in the gills (Karbalaei et al., 2021). Thus, it is important to evaluate the ecotoxicological effects of the uptake of MPs and its interactions with other contaminants like MLT to marine and estuarine organisms.

Fiddler crabs are estuarine semi-terrestrial crabs characterized by their marked sexual dimorphism. Males have asymmetric bodies and a large cheliped representing from one-third to one-half of their body mass. (Rosenberg, 2014). They play essential ecological roles in biogeochemical cycles, nutrient recycling, and helping to stimulate the microbial activity in mangroves (Gribsholt et al., 2003; Zeil and Hemmi, 2006). Several studies indicate that fiddler crabs can be considered bioindicators of ecosystem health (Azpeitia et al., 2013; Capparelli et al., 2019, 2017, 2016; Lavezzo et al., 2020; Yáñez-Rivera et al., 2019). Fiddler crabs build burrows and live in direct contact with the contaminated sediment, from which they feed on organic matter adhering to the sediment particles, absorbing contaminants via the diet. Therefore, they constitute a suitable model to investigate the bioaccumulation mechanisms *in situ*. Being territorial and lacking extensive mobility, fiddler crabs are chronically exposed to local environmental contamination. Thus, the species of fiddler crabs from Isla Santay, *Leptuca festae* and *Minuca ecuadoriensis*, could be used as bioindicators of ecosystem health. *L. festae* only inhabits the riverbank of the Guayas River, where it is most exposed to the debris carried by the tides, due to the proximity to the intertidal zone. *M. ecuadoriensis* inhabits in the supratidal zone, where the tidal water arrives only during greater amplitude tides.

The proximity to multiple sources of contamination may be affecting the biodiversity of protected areas in estuarine environments. In this study, our objectives were two. The first objective was to make a preliminary assessment of the concentration of organophosphate pesticides (OPs) and microplastics (MPs) in situ at Isla Santay. We quantify MPs and OPs in the water and the sediment of the burrows of two species of fiddler crabs, *L. festae* and *M. ecuadoriensis*. We also assessment the bioaccumulation of MPs and OPs in the tissues of the two species. The second objective was to evaluate the effects of the bioaccumulation of MPs and its interaction with MLT in the survival of *M. ecuadoriensis* in a laboratory.

2. Methodology

2.1.Study location

Isla Santay (2°13'04°S, 79°52'40°W) is an island located in the Guayas River estuary. The island (Figure 1) is included in the National System of Protected Areas of Ecuador (SNAP) and declared a Ramsar site by the International Convention on Wetlands in 2000. The Guayas River is the largest Ecuadorian river that flows into the Pacific Ocean, with a flow rate higher than 1600 $m^3.s^{-1}$. It has a drainage area of approximately 34 000 km^2, where a population of 4.8 million inhabitants live (Cambien et al., 2020; Montaño and Sanfeliu, 2008). Isla Santay is located only 800 m away from Guayaquil and near to Duran, and receives direct contamination (e.g., plastic litter and pesticides) from multiple sources like agricultural industry, domestic sewage and urban activities (Deknock et al., 2019). Our sampling campaign was performed in November 2019.

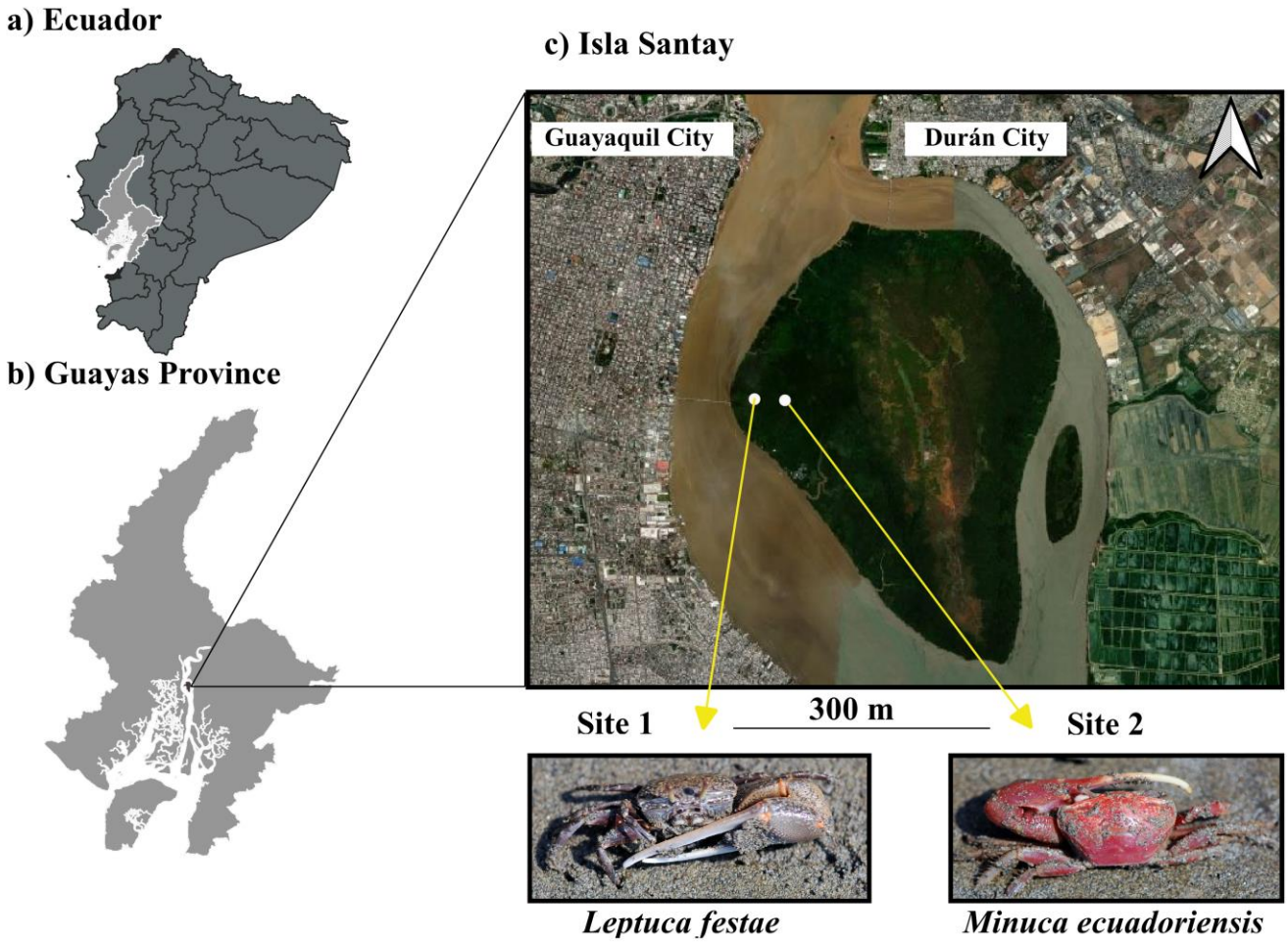

Figure 1. Study area. a) Location of the Guayas province and b) the location of Isla Santay, in the estuary of the Guayas River. c) Collection sites of the mudflat fiddler crabs (site 1, *Leptuca festae*; site 2, *Minuca ecuadoriensis*). The background image shows the urbanization around Isla Santay.

2.2. *Assessment of contamination by pesticides and MPs bioaccumulation in situ*

2.2.1. Water and sediment sampling collection

Surface water and sediment were collected from crab burrows for MPs and OPs analysis (Fig. 1). At each site, one water sample was collected for MPs analysis (1 L in transparent glass bottles) and three water samples for pesticide analysis (1L in glass amber bottles). From the crabs' burrows three sediment samples (500 g of sediment for each sample) were collected for MPs analysis in pre-clean plastic bags, and three sediment samples (500 g for each sample) were taken for OPs analysis in pre-clean plastic bags covered with aluminum foil to protect the samples from sunlight). Samples refrigerated at 4 °C were transported to the Laboratorio Nacional de Referencia del Agua at Universidad Regional Amazónica Ikiam. Samples were kept in the dark at −20 °C until analysis.

2.2.2. Crab collection and transport

30 adult crabs each species were manually collected at each sampling site. Only non-ovigerous, intermolt crabs of carapace width greater than 10 mm from M. ecuadoriensis and 5 mm for *L. festae* species were used in a bioaccumulation analyses. Crabs were transported to the Laboratorio Nacional de Referencia del Agua at the Universidad Regional Amazónica Ikiam in pre-cleaned plastic boxes of approximately 1500 cm^3 (15 cm × 15 cm × 6 cm), containing small sponge cubes moistened with water from the sampling sites. Approximately 20 crabs were placed in each box for transport. Immediately upon arrival at the laboratory, the crabs were cryo-anesthetized in crushed ice for 10 min. The gills pairs, the hepatopancreas and the digestive tract (esophagus, stomach, and intestines) were dissected for MPs quantification. The muscle tissues were dissected for OPs analysis.

2.2.3. MPs extraction and quantification

2.2.3.1. Water and sediment samples

The methodology for MPs extraction was adapted from the National Ocean and Atmospheric Administration (NOAA) (Masura et al., 2015). Water samples were filtered using two-level stainless-steel sieves of 5000 µm and 63 µm. The 5000 µm sieve was used to screen out larger particles not considered as MPs; its content was discarded. Microparticles from 63 µm sieves are transferred, aided by a minimal amount of deionized H_2O_2, to 100 ml glass collection jars Then, the samples were dried at 60 °C for 24 h and digested with a solution of Hydrogen peroxide (H_2O_2) 30% in an oscillation incubator (60 °C at 100 rpm for 2 h). Finally, the samples were filtered with a membrane filter (0.45 µm pore size) in a vacuum filtration system. For sediment samples, the MPs were extracted by density separation with the NaCl solution (1.20 kg L^{-1} density). The supernatant passed through a sieve of 63 µm and then, the same methodology applied for water samples was followed. The filters with MPs were stored in capped glass Petri dishes for further visual identification.

2.2.3.2. MPs abundance in tissues

MPs abundance was measured in 3 replicates (with 10 crabs each) for each species. Chemical digestion of the tissues was performed using H_2O_2 (30%) (200 ml for every 5 g of tissue) in an oscillation incubator at 60°C at 100 rpm for 48 h -72 h. Then, the solution was 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.

2.2.3.3. Contamination mitigation and blank samples

Precautions were taken to avoid background plastic contamination during sample treatment and analytical steps. All laboratory materials were rinsed first with Milli-Q water and then with ethanol before usage. Clean filter papers were placed in Petri dishes and exposed to the air in the laboratory during the processing time to account for atmospheric contamination. In parallel to the MPs analysis in water and sediment, blank samples were prepared with 1 liter of Milli-Q water following the same methodology used in the field samples. Filtered water was used as procedural controls for sediment samples, as described by Frias et al., (2018). For tissue samples, the blanks were prepared using H_2O_2 and followed by the protocol described above. The filters were then analyzed visually, and the MPs found were subtracted from the total samples.

2.2.3.4. MPs quantification

Filters were divided into four sections to facilitate manual counting of the MPs. MPs were counted using a stereomicroscope Amscope with a magnification of 20X, equipped with 10 MP digital camera and

software AmScope. MPs identification was performed based on the descriptions provided by Mohamed Nor and Obbard, (2014) and Masura et al., (2015). Two observers used stainless steel tweezers to actively search for MPs and to separate them from other sediment fragments. The plastic fragments were categorized by color and shape. Under the stereomicroscope, the fragments were manipulated or dragged around with the aid of tweezers to confirm the makeup of the plastic particles. If the materials crumbled or were easily crushed, they were not considered plastic compounds. If the particles kept their shape, they were included in the counting (Mohamed Nor and Obbard, 2014).

2.2.4. OPs extraction and quantification

2.2.4.1 Water and sediment samples

The water samples were transferred into separating funnels and 50 g of NaCl was added to each funnels. Samples were shaken until the NaCl was thoroughly diluted. Pesticides were extracted by shaking the samples for 1 min using 15 ml of dichloromethane, three times. Then, all the extract was filtered with 3g sodium sulfate anhydrous and rota-evaporated to 1 ml. The water samples were spiked for recovery calculations with a solution of 1,3-dimethyl-2-nitrobenzene. Sediments were oven-dried at 60 °C and passed through a sieve of 250 μm. Five-gram aliquots were spiked with the surrogate mixture (1,3-dimethyl-2-nitrobenzene) and extracted three times by sonication using 15 mL of dichloromethane for 15 min. After centrifuging (4000 rpm for 5 min), the organic extracts were concentrated and analyzed as the water samples (Montuori et al., 2015; Triassi et al., 2019).

2.2.4.2. OPs Tissue content

Muscle tissue was removed, weighed, and freeze-dried. The dried tissues were grounded into powder. Next, 1 g aliquots were extracted three times by sonication using 15 mL of dichloromethane for 30 min. After that, the extract was centrifuged at 4000 rpm for 30 min. The extract was rota-evaporated, and the residual was mixed with 1 ml of dichloromethane (Cheng et al., 2019).

2.2.4.3. Analytical determination of OPs

Extracts were analyzed by a gas chromatography with 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 standards. A reference standard mixtures (96–99% certified purity) of 7 OPs (Dimethoate, EPN, malathion, monocrotophos, ethyl parathion, sulfotep and tetraethyl pyrophosphate) were obtained from the Restek Corporation (USA). A 1000 μg L^{-1} stock solution of the standard mixture containing each pesticide was prepared in hexane and kept in the dark at −20 °C. The GC/NPD responses were linear in the concentration range of 5–1000 ng mL^{-1} with a regression coefficients between 0.996-0.999. The LODs for organophosphate pesticides ranged between 0.08 and 0.015 μg L^{-1} in water samples, 0.25-0.5 ug Kg^{-1} in dry sediments, and 1 and 2 ug Kg^{-1} in tissues. The percent recovery of each pesticide was 55 to 95% in water, 50 to 90 % in sediment. These OPs were chosen based on its frequent use in Western Ecuador (Deknock et al., 2019).

2.2.4.4. Quality control and quality assurance

Quality control measures were assessed through analysis of solvent blanks, procedure blanks. All reagents used were analytical grade. Samples were analyzed in triplicate concurrently with quality control analysis (blanks and recovery determinations). Before analysis, glassware was washed and rinsed with pesticide-free Milli-Q water and dichloromethane and then heated to 100 °C for 2 h. Contamination from the analytical procedure was neglected because pesticides were not detected in the blanks.

2.3. Exposure experiment in laboratory

2.3.1. Crab collection

Adults specimens of *M. ecuadoriensis* of both sexes were randomly collected from Isla Santay (2°13'04°S, 79°52'40°W), Guayas estuary, in the Coast of Ecuador. The crabs were transported in pre-cleaned plastic boxes (15 cm × 15 cm × 6 cm) of approximately 1500 cm^3, containing sponge cubes moistened with water and a thin layer of sediment from their habitat to the "Laboratorio Nacional de Referencia del Agua" at Universidad Regional Amazónica Ikiam. Only non-ovigerous, intermolt crabs of carapace width greater than 10 mm were used. Before the experiment, all the crabs were acclimatized to laboratory conditions exposing them to 12 hours of light and 12 hours of darkness at room temperature for 3 days.

2.3.2. MP and MLT exposure

MPs solution was prepared by addition of 200 mg of High-Density Polyethylene (HDPE) particles smaller than 250 µm in 1 L of previously filtered Milli-Q water. Then, the solution was shaken vigorously until a homogeneous solution was obtained.

A stock MLT solution of 50 mg L^{-1} was prepared using the commercial malathion 25% Proficol®. For the mixed solution of MP plus MLT, 200 mg of HDPE MP was adhered in 1L of the stock MLT solution (50 mg L-1).

In addition, the capacity of HDPE MPs particles to adsorb MLT was measured after 120 hours. To determine the partition of MLT between MP and water, we adapted the methodology by (Garrido et al., 2019), MPs were separated from the water by filtration using a microfiber filter of 0.45 µm pore diameter. Next, each filter was cut into 4-6 pieces, which were placed into a tube with 15 ml of dichloromethane, using as a solvent for the extraction. Then, the MLT was extracted by sonication using an ultrasonic bath for 30 min. The sonication was repeated twice, changing the dichloromethane.

The extracts of MLT in MPs and stock solution were analyzed by gas chromatography with 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 (99% purity). The concentrations were calculated from the calibration curves for MLT (RESKET, CT 32278).

The partitioning coefficient for sorption of MLT to MP (Kd) was calculated as *Kd= MLTMP/MLTW*, where *MLTMP* was the concentration of MLT adhered to MP expressed as mg kg^{-1}, and *MLTW* was the concentration of MLT in water as mg L^{-1}. After 120 hours, the concentration of MLT adsorbs on the MP particles were 2.7 mg g^{-1}. Around 5% of the MLT was sorbed onto MP surfaces resulting in a partitioning coefficient, Kd, of 54 L kg^{-1}.

2.3.3. Experimental procedures

A total of 100 crabs (carapace width ranges 1.4 cm – 2.5 cm) were used and divided in 25 individuals for each treatment. Each crab was put into a glass container (12 cm high and 73.2 mm in diameter), semi submerged in 10 ml of distilled water with 5 ‰ of salinity, media were prepared using distilled water and Instant Ocean® seawater salts. The exposure experiment consisting in four treatments (Table 1). Water changes and feeding was performed every 48 h. And the mortally was recorded every 24 h. After the 5 days of exposure, the crabs were cryo-anesthetized in crushed ice for 10 min after which all gill pairs, the hepatopancreas and the digestive tract were dissected from each crab for MPs quantification.

Table 1. Experimental group.

Treatment	*Replicas*	*Salinity*	*MPs dosage*	*MLT concentration*
Control	25	5 mgL^{-1}	0 mgL^{-1}	0 mgL^{-1}
MLT	25	5 mgL^{-1}	0 mgL^{-1}	50 mg L^{-1}
MP	25	5 mgL^{-1}	200 mgL^{-1}	0 mgL^{-1}
MLT+MP	25	5 mgL^{-1}	200 mgL^{-1}	50 mg L^{-1}

2.3.4. Chemical digestion and filtration of Tissue

The tissues were pooled (3-5 crabs per replicate). The chemical digestions of the tissues were performed using H_2O_2 (30%) (200 ml for every 5 g of tissue) in an oscillation incubator at 60°C at 100 rpm for 48 h -72 h. Then, the solution was maintained at 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.

2.3.5. MPs quantification

Filters were analyzed using a stereo microscope Amscope with a magnification of 20X, equipped with 10 MPs

digital camera and software AmScope.

Contamination mitigation and blank samples were performed the same as for the bioaccumulation of MPs *in situ*.

2.4 Numerical and statistical analyses

2.4.1. Bioaccumulation *in situ*

Results were expressed as mean ± standard error of the mean (SEM). To assess the bioaccumulation of MPs in tissues, a Two-way analysis of variance (ANOVA) was performed. The model was set as *MPs items g tissue^{-1}~ Specie (L. festae and M. ecuadoriensis)* Type of tissue (Gills and DT+H (digestive tract and hepatopancreas))*. Differences between means within the same factor were determined by the Student–Newman–Keuls multiple comparisons procedure. A significance level of $p < 0.05$ was employed. The OPs concentration in water was compared to water quality guidelines established by the United States Environmental Protection Agency (US EPA , 2010); the OPs concentration in sediment was compared with the Freshwater Sediment Screening Benchmarks (US EPA , 2006).

2.4.2. Exposure experiment in laboratory

Results were expressed as mean ± SEM. To assess the bioaccumulation of MPs in tissues between treatments and type of tissues a Generalized Linear Model (GLM) was performed. GLM is an extension of the Linear Model that is used when the data have a non-normal distribution and heteroscedasticity, being an alternative to ANOVA. The model was set as *MPs items g tissue^{-1}~Tratamiento*Type of tissue (Gills and DT+H (digestive tract and hepatopancreas))* using a gamma distribution. The toxicity effects of MPs, MLT and the combined exposure to MPs and MLT were evaluated through a survival analysis comparing treatment survival curves against controls using a Log-rank test. A minimum significance level of $p< 0.05$ was employed for all procedures.

3. Results and Discussion

3.1.Assessment of contamination by pesticides, MPs and bioaccumulation in situ

3.1.1. MPs in sediment samples

The average concentration of MPs in the sediments was 600 ± 174.36 and 373.33 ± 61.10 items kg-1 w. w., from sites 1 and 2 respectively (Fig.2B). Site 1, being closer to the intertidal zone, accumulates more MPs in sediment than site 2, in the supralittoral zone. The same pattern was observed in highly impacted estuaries in China (Li et al., 2018; Yao et al., 2019). In the sediments from site 1, fragments were the most abundant MPs shape (31.11%) followed by films (28.88%), fibers and beads (20% each one). In site 2, the most frequent shape were films (57.14%), followed by fragments (21.43%), fibers (17.86%), and beads (3.57%). The diversity of shape and colors of MPs is related to the source (wastewater treatment plant, domestic sewage and urban activities) and the environmental processes of the plastic debris fragmentation, such as photochemical, abrasive, biodegradation (Deng et al., 2021; Khatmullina and Isachenko, 2017). In both sites, irregular shapes (fragments and films) were the most abundant. Fragments are the result of the degradation of larger plastic waste, such as bottles, packaging, containers (Cole et al., 2011; Zhang et al., 2015). Films derived from the fragmentation of plastic packaging, bags, and agricultural films (Mohamed Nor and Obbard, 2014; Wang et al., 2019). Regarding color, white particles were the most abundant in both locations (46.66% at site 1 and 46.43% at site 2.) The same pattern was observed in the Qinzhou Bay estuary, China (Li et al., 2018). The main source of this color of MPs may be the breakdown of single-use plastic products (Napper and Thompson, 2016).

3.1.2. MPs in water samples

The concentration of MPs in the water samples was higher at site 2, supralittoral zone (26 ± 1 items L^{-1}) than at site 1 (16 ± 1 items L^{-1}), infralittoral zone (Fig. 2A). Due to occasional flooding during tidal amplitude events, supralittoral zones can accumulate significantly more plastic debris than intertidal zones, where there may be less deposition of MPs due to the constant water dynamics (Ivar do Sul et al., 2014). Regarding the shape of the particles, the fibers were more abundant in the water samples from site 1 (43.75%), while fragments were more abundant at site 2 (41.38%). Fibers are often related to laundries wastewater, to the inefficiency of wastewater treatment plants in retain MPs (Browne et al., 2011; Henry et al., 2019) and to water from fishing activities (Cole et al., 2011). Regarding color, the white MPs were the most abundant in site 1 (43.75%), while brown MPs were the most abundant in site 2 (47.29%).

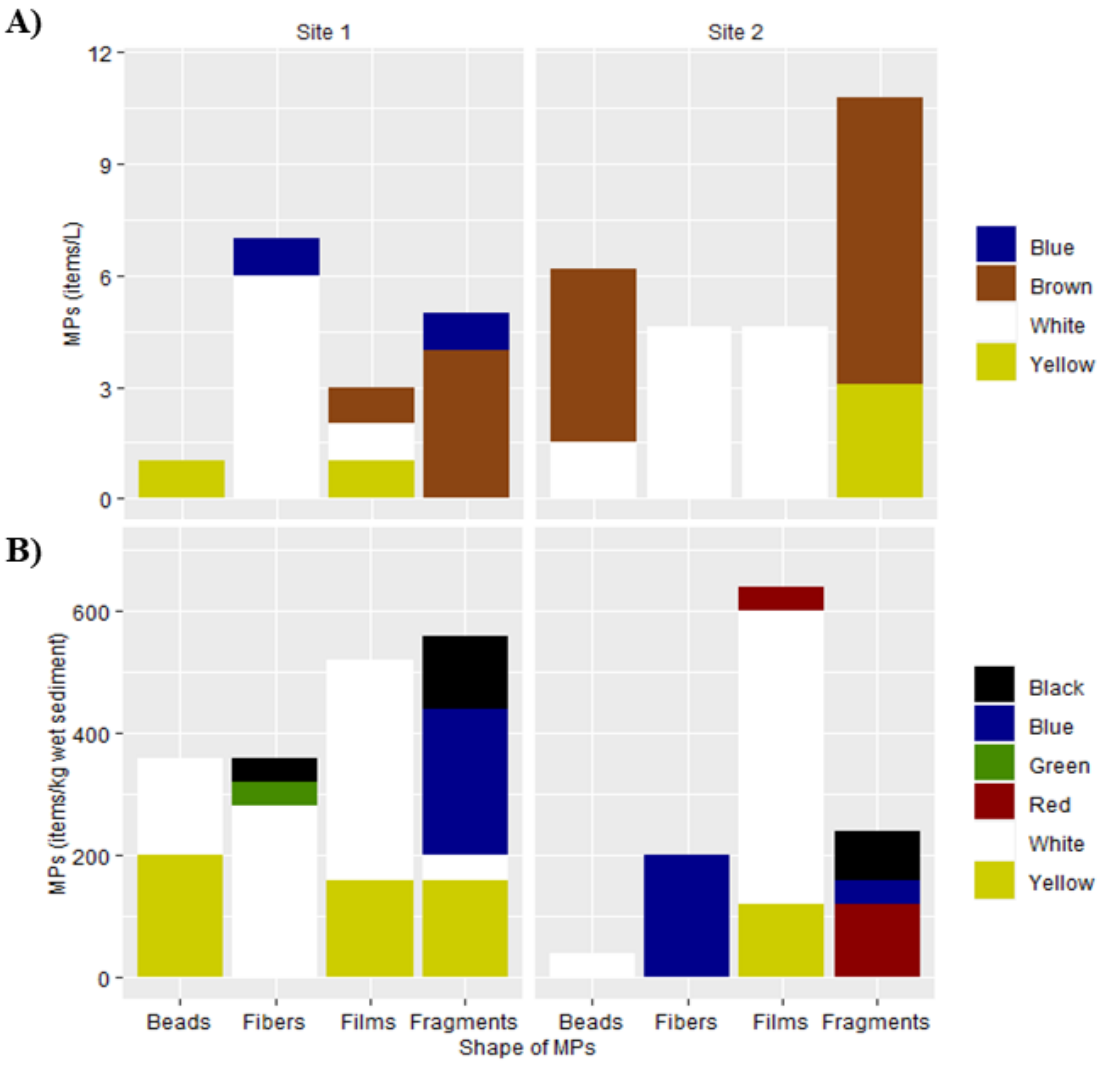

Figure 2. Abundance of MPs classified by color and shape as registered in water (A) and sediments (B) from the burrows of the two species of fiddler crabs. Site 1: infralittoral zone inhabited by Leptuca *festae*. Site 2: supralittoral zone inhabited by *Minuca ecuadoriensis* (see Fig.1 for the location of species in the study area). MPs values are given as the number of items per liter and the number of items per kilogram of wet sediment.

3.1.3. MPs in tissues

No significant difference was observed in the MPs concentration found in the tissues of both fiddler crab species. However, higher MPs accumulation was observed in the gills than in the hepatopancreas of the two species ($P<0.05$) (Fig. 3). In *L. festae*, the average concentration of MPs in the gills and in the DT+H (digestive tract and hepatopancreas) was 29.81 ± 18.13 and 7.58 ± 3.96 items g tissue^{-1}, respectively. In *M. ecuadoriensis* the average concentration was of 22.93 ± 10.77 items g tissue^{-1} in the gills and 0.50 ± 0.87 items g tissue^{-1} for in the DT+H. Variable MPs shapes and color, the greatest diversity was reported in the gills and DT+H of both species (Fig. 4).

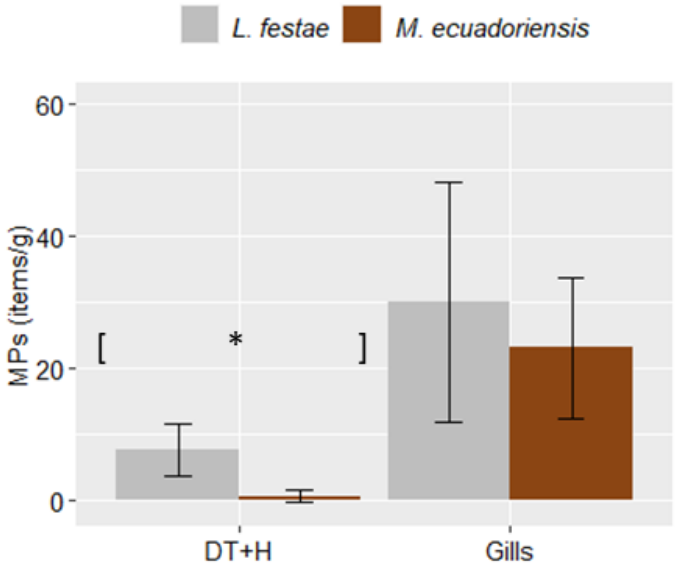

Figure 3. Abundance of MPs in the tissues of *L. festae* (grey) and *M. ecuadoriensis* (brown) from Isla Santay. Mean number ± standard deviation of MPs per gram of tissue in the gills and DT+H (digestive tract and hepatopancreas) in both species of fiddler crabs. The asterisk (*) above the square bracket indicates differences in the MPs concentration between the types of tissues, gills and DT + H ($p < 0.05$).

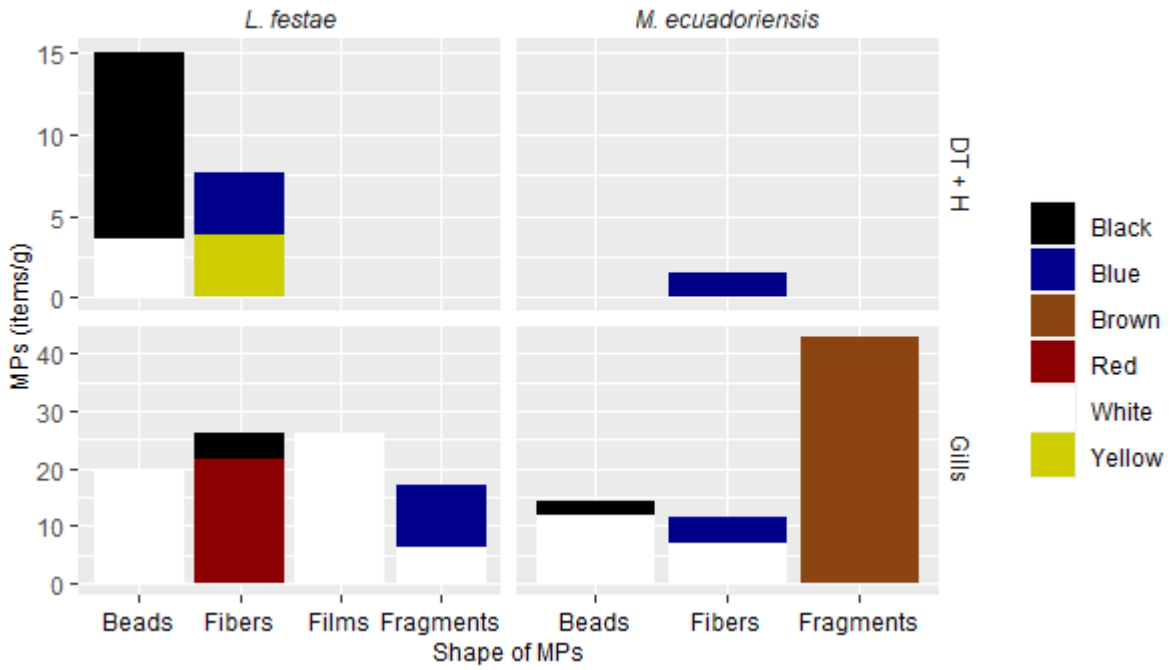

Figure 4. Abundance of Microplastics (MPs) in the tissues of *L.festae* and *M. ecuadoriensis* from Isla Santay per gram of tissue classified by color and shape.

A previous study on another specie of fiddler crab, *Minuca rapax*, reported that MPs were present more often in the gills than in the stomach, suggesting that MPs are more likely to get accumulated in the fine structures of the gills. However, MPs may pass through the digestive tract without bioaccumulating (Brennecke, 2015). During low tide the fiddler crabs come out of their burrows to feed. Fiddler crabs filter

sediment pellets using water stored in the gills chamber (Dye and Lasiak, 1987). They use the small chela to feed; small portions of sediment are placed in the buccal cavity, which is flooded with water from the branchial chamber. In the buccal cavity the sediment is washed away from the food. The food is ingested, and the sediment is expelled from the mouth as a small pellets 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), and during this process some water evaporated. The water trickles down to the opening above the legs and returns to the gill chamber and will eventually be recycled. As this active filtering process is repeated, it is likely that MPs become more concentrated in the gill chamber. The accumulation of MPs on gills may reduce the respiratory and osmoregulatory capability of the crab, however, these mechanisms need to be better studied to be confirmed.

3.1.4. OPs in water and sediment samples

The concentration of total OPs in water samples was 2.73 ± 1.64 µg L^{-1} (site 1) and 0.74 ± 0.01 µg L^{-1} (site 2); the concentration in sediment samples was 5.05 ± 0.99 µg kg^{-1} (site 1) and 5.07 ± 1.12 µg kg^{-1} (site 2) (Table 2). Parathion and EPN are in the list of prohibited pesticides by the Sustainable Agriculture Network (SAN) because of the acute toxicity and chronic effects for humans and the environment, even at low-level exposure. These pesticides are also considered extremely hazardous by the World Health Organization (World Health Organization, 2010). MLT is in the Sustainable Agriculture Network list (SAN) for use with risk mitigation and is considered as moderately hazardous by the SAN and by Ecuadorian regulations (INEN, 2008; Sustainable Agriculture Network, 2017).

Table 2. Concentrations of OPs in water and sediment samples from each species habitat. Site 1, infralittoral habitat for *L. festae* and Site 2, supralittoral habitat for *M. ecuadoriensis*. Values in bold highlight concentrations above the limits established by US EPA guidelines (EPN is not included in the guideline). *ND= No detected

Sample	*Site*	*Malathion*	*Ethyl-parathion*	*Sulfotep*	*Dimethoate*	*EPN*	*Total*
Water (µg L^{-1})	*Site 1*	**2.63 ± 1.63**	**0.10 ± 0.01**	ND	ND	ND	2.73±1.64
	Site 2	**0.66 ± 0.01**	**0.08 ± 0.001**	ND	ND	ND	0.74± 0.01
Sediment (µg kg^{-1})	*Site 1*	ND	**2.27 ± 0.29**	ND	ND	2.78 ± 0.70	5.05± 0.99
	Site 2	**2.20 ± 0.16**	**2.87 ± 0.96**	ND	ND	ND	5.07 ± 1.12

The concentration of MLT and Ethyl-parathion in water for site 1 and site 2 was ~ 26 times higher than the recommended for chronic exposure by US EPA (0.1 µg L^{-1} and 0.013 µg L^{-1}, MLT and Ethyl-Parathion respectively). Although Isla Santay is a protected area, the concentrations of OPs reported in our study for water samples were higher to those reported by Deknock et al., (2019) in the Guayas River basin in 2016. Besides, the cultivated area of sugarcane, bananas, maize and cacao increased from 2016 to 2019 (SIPA, 2021). The used of MLT is related to these crops (Deknock et al., 2019).

The concentrations of MLT and Ethyl-parathion in sediment were approximately up to 11 and 4 times higher than the Freshwater Sediment Screening Benchmarks by US-EPA (0.203µg kg^{-1} and 0.757 µg kg^{-1}, for MLT and Ethyl- Parathion respectively) suggesting a significant risk for the biota. In sediment samples, MLT was only detected at site 2. The concentration was 3 times higher in the sediments than in the water. Ethyl-parathion was ~ 23 to 35 times higher in sediment than in water, indicating that estuary sediment could be a significant sink for some of these contaminants. EPN was only found in sediments at site 1, which is of great concern since this pesticide is forbidden to use in Ecuador due to its high toxicity. Bound residues of OPs in

the soil can decline because of volatilization, interaction with microorganisms, soil type and other abiotic factor (e.g., pH, moisture) (Gervais et al., 2009). The concentration ranges of OPs found in our study were greater (5 to 15 times higher) than those reported in rivers considered highly contaminated in Europe (Montuori et al., 2015; Triassi et al., 2019).

3.1.5 OPs tissue content

Although high concentrations of some OPs were detected in water and sediment from the burrows where the fiddler crabs were collected, the concentrations in the tissues of both species were below our detection limit (1-2 μg^{-1}). The OPs found in our study do not have high rates of bioaccumulation due to faster metabolization and detoxification by aquatic organisms (Deka and Mahanta, 2016). However, small rates of bioaccumulation of MLT and Ethyl-parathion have been reported in the embryos of the crabs *Hemigrapsus oregonensis* and *Pachygrapsus crassipes* (Smalling et al., 2010), and in tissues of *Ambystoma tigrinum* (Henson-Ramsey et al., 2008) and *Heteropneustes fossilis* (Maurya and Malik, 2016). Despite the low bioaccumulation rates, some studies indicated toxicity at the environmental concentrations detected in our study. For instance, when *Daphnia magna* is exposed to MLT in concentrations lower than those found in Isla Santay (0.23 $\mu g\ L^{-1}$ and 0.47 $\mu g\ L^{-1}$), it presents DNA damage (Knapik and Ramsdorf, 2020). Acute and chronic exposure to OPs is associated with toxicological effects, including metabolic, enzymatic (AChE inactivation), protein, physiological, histological, biochemical and genotoxicological disorders (Sidhu et al., 2019), as have been reported in crustaceans (Duarte-Restrepo et al., 2020; Liu et al., 2012) and fish (Cook et al., 2005; Fulton and Key, 2001).

3.2.Exposure experiment in laboratory to MP, MLT and MLT+MP in M. ecuadoriensis.

3.2.1. Effects on the MPs and MLT on survival of *M. ecuadoriensis.*

A marked decrease in survival was observed in individuals exposed to MLT+MP treatment (80%) compared to MLT (28%), Control (8%), and MP (0%). Besides, two peaks of mortality were observed after 24h (32%) and 72h (36%) of exposure in the MLT+MP treatment.

The survival analysis (Fig. 5) shows significant differences in the survival probabilities between control and MLT+MP ($p < 0.001$), MLT and MP ($p < 0.01$), and MLT and MLT+MP ($p < 0.001$) treatments. The highest risk of mortality was found in the MLT+MP treatment with a survival probability of 20% at the 120 h of the experiment compared to MLT (76%), MP (100%), and Control (92%).

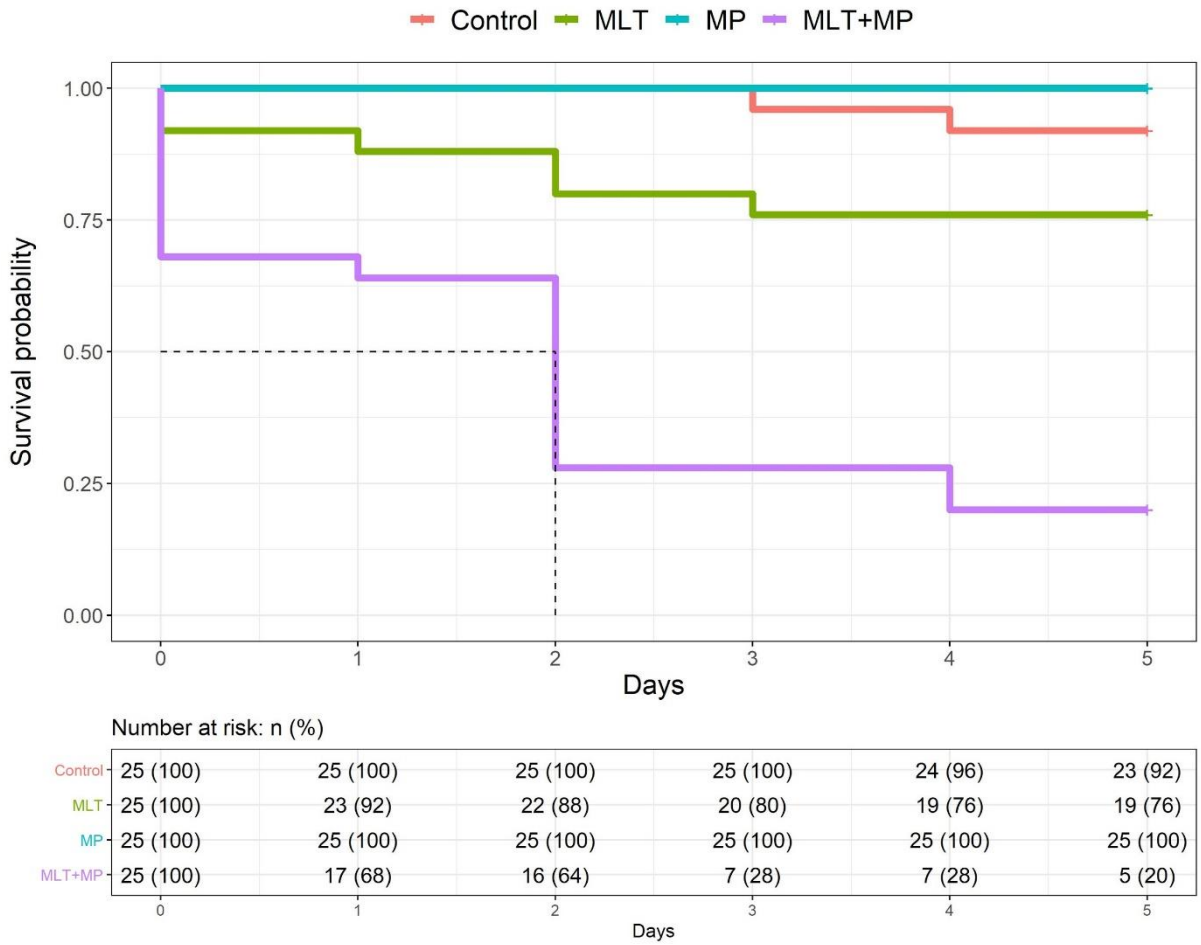

Figure 5. Survival curve probability for the Control (pink), MLT (green), MP (blue) and MLT+MP (purple) treatments. The table below gives information of the number of individual for treatment and the percentage of survival, predicted from the beginning (0 days) until the end of the experiment (5 days).

MPs did not cause a decrease in the survival of *M. ecuadoriensis* after 5 days of exposure. Our results are similar to the those reported by Brennecke, (2015) in *M. rapax* that after been exposed for 2 months to high level of MPs contamination did not show negative effects on the survival. Although MPs did not increase the mortality in *M. ecuadoriensis*, the exposure may cause other damages like an increase of oxidative stress (Wang et al., 2021), and irregularities in body weight (Torn, 2020).

Minuca ecuadoriensis exposed to MLT showed a mortality of 28% after 5 days. In the blue crab, *Callinectes sapidus*, the exposure of MLT caused a decrease in the survival of 33% at a lower concentration that used in *M. ecuadoriencsis* (0.1 mg L^{-1}) after 56 h of exposure (Schroeder-Spain et al., 2018). The exposed to MLT (3 mg L^{-1}) in the zebrafish, *Dario rerio*, caused a reduction in the survival of 50% after 120 h. MLT produced negative effects on hatching and body morphology developing (Cook et al., 2005). A LC_{50} of 15.77 mg L^{-1} after 96 h of exposure of ML was reported in the fish *Colossoma macropomum*. MLT caused histopathological damages in the gills, and increase the expression of proteins related to cancer development (Silva de Souza et al., 2020).

The synergy of MLT and MPs increase the mortality of *M. ecuadoriensis* to 80% after 5 days of exposure and was significantly more toxic than the isolated MLT exposure ($p < 0.001$). The mixture of these contaminants in the environment may have negatives effects in the fitness of *M. ecuadoriensis*, affecting the ecological roles in which this specie participates like the biogeochemical cycles, the oxygenation of the sediment and the nutrient recycling (Gribsholt et al., 2003; Zeil and Hemmi, 2006).

The adsorption of MLT on the MP surface reported in our study was 6 times higher than the reported for the adsorption of MLT on MP polyethylene films (Kd= 9.218 L kg^{-1}) (T. Wang et al., 2020b). However,

the microplastics adsorption process depends on its polymer types molecule composition, structure and the characteristics of the chemicals (F. Wang et al., 2020).

However, our results suggest that MP can act as a vector of MLT, contributing to the leached of MLT inside the tissues of *M. ecuadoriensis* causing the higher decrease in the survival reported in this treatment. In addition, our results are similar to the reported by Felten et al., (2020) in *Daphnia magna* that show a decrease in the survival as a consequence of the combined exposure to MPs and deltamethrin. Besides, in the marine copepod *Acartia tonsa*, the effects of the exposure to MPs and chlorpyrifos was 4-25 times more toxic that the isolated chlorpyrifos exposure (Bellas and Gil, 2020).

3.2.2. MPs content in experiment exposure in *M. ecuadoriensis.*

The bioaccumulation of MPs (Table 3) was reported only in the MP and MLT+MP treatments in the gills and the DT+H (digestive tract and hepatopancreas). Although the higher bioaccumulation of MPs was observed in the combined treatment, MLT+MP, the GLM model results did not show significant differences in the bioaccumulation of MPs between treatments and type of tissues. The size of the MPs particles bioaccumulated were smaller than 250 µm. Our results are similar to those reported by Brennecke, (2015) for Minuca *rapax*, which can bioaccumulate MPs particles in the size range of 180–250 µm in the gills, stomach and hepatopancreas.

Table 3. Bioaccumulation of MPs per gram of tissues in the gills and DT+H (digestive tract and hepatopancreas). The number of crabs used in each treatment was 13-20. The MPs count was performed in the individuals who survive until the end of the experiment and the ones who died during the exposure. The tissues for the individual that were not used for the bioaccumulation of MPs assessment were stored for future enzymatic analyzes not included in this research.*-- = No measured.

Time (h)	***Replica***	***Bioaccumulation of MPs (items g tissue-1)***							
		Control		***MLT***		***MP***		***MLT+MP***	
		Gill	***DT+H***	***Gills***	***DT+H***	***Gills***	***DT+H***	***Gills***	***DT+H***
24	***2***	--	--	--	--	--	--	53.23 ± 7.98	342.95 ±330.36
48		--	--	--	--	--	--	--	--
72	***2***	--	--	--	--	--	--	72.15 ± 15.27	77.98 ± 12.55
96	***1***	--	--	--	--	--	--	16.99	9.44
120	***3***	0	0	0	0	41.15 ± 20.05	29.17 ± 3.87		
Total		0	0	0	0	**41.15 ± 20.05**	**29.17 ± 3.87**	**142.37 ± 23.25**	**430.37 ± 342.91**

The bioaccumulation pattern found in the MP treatment was similar to the reported for the bioaccumulation in situ of MPs in the tissues of *M. ecuadoriensis*, being the concentration of MPs higher in the gills (58.52%) compared to the DT+H (41.48%). Also, a higher bioaccumulation of MPs in the gills has been reported in the crabs *Minuca rapax* (Brennecke, 2015) and *Neohelice granulate*, (Villagran et al., 2020) compared to other organs like the digestive tract and hepatopancreas.

On the other hand, the opposite pattern was observed in the MLT+MP treatment where the concentration of MPs was higher in the DT+H (76.07%) compared to the gills (23.93%). In addition,

acetylcholinesterase inhibitor like malathion may increase the metabolic rates producing a higher consumption of energy that could lead to an increase in the foraging activities in the crabs in response to energy demand (Holmberg et al., 1972; Roex et al., 2003; Sastry and Sharma, 1981). A study carried out in the blue crab, *Callinectes sapidus*, showed that the crabs exposed to MLT searched food more frequently than crabs from the control group (Correia and Smee, 2018).

The high bioaccumulation of MPs in the DT+H tissues could be responsible for the high mortality reported in crabs from the combined treatment. In addition, some studies demonstrated that MPs can increase the bioaccumulation of organic pollutants (T. Wang et al., 2020a; Wardrop et al., 2016; Zhao et al., 2020). And also Sun et al., (2021) demonstrated that MPs can increase the bioaccumulation of the pesticide dufulin in the earthworm, *Eisenia fetida*. Thus, it is likely that a high amount of MLT would be bioaccumulating in the hepatopancreas as consequence of bioaccumulation of MPs. The damage in the hepatopancreas could be affect several vital functions like the absorption and storage of nutrients, the synthesis of digestive enzymes (Vogt, 1994; Wang et al., 2014), and the response of the antioxidant defense system (Vogt, 2002; Wang et al., 2014). However, these mechanisms need to be evaluated for *M. ecuadoriensis*.

The cumulative proportion curve of MPs (Fig. 6) shows that during the first 72 h of exposure the percentage of accumulation of MPs was higher in the DT+H tissues compared to the gills. However, after that time the percentage of accumulation of MPs in the gills and DT+H were be similar. The low accumulation of MPs in the DT+H after 72 h could be associated with the capacity to the individual to egest the ingested MPs. For example, in *Rhithropanopeus harrisii*, 36 h after feeding, no fragments were detected in the digestive tract due to excretion of the MPs through the fecal pellets (Torn, 2020). In the shore crab, *Carcinus maenas*, was reported a significant decrease in the number of MPs in the foregut after the first 24 h (Watts et al., 2014).

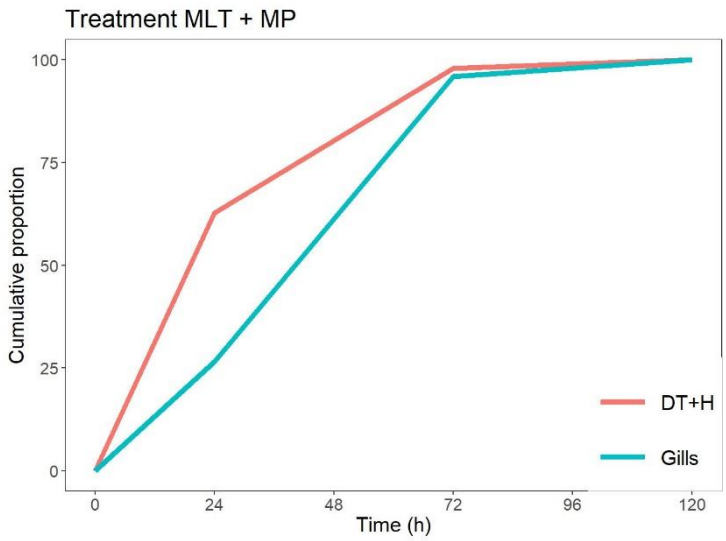

Figure 6. Cumulative proportion of MPs (%) by tissue, Gills (blue) and DT+H (red), in MLT+ MP treatments across the 120 h of the experiment.

4. Conclusion

Although Isla Santay is a protected area, it is highly contaminated by MPs and OPs. These contaminants were found in water and sediment samples from the habitat of the both species of fiddler crabs, *L. festae* and *M. ecuadoriensis*. Regarding the bioaccumulation of MPs, our results suggest that MPs are more likely to get accumulated in the gills than in the DT+H (digestive tract and hepatopancreas). The bioaccumulation of pesticides has not been observed *in situ*; however, these compounds were found at toxic levels, above those allowed by the US EPA, for water and sediment samples. Being OPs a potential risk at the individual and ecosystem level. Therefore, we suggest that current environmental protection measures must be evaluated for an effective safeguard of Isla Santay biodiversity.

Regarding exposure experiments, MPs showed an important role in the toxicity of MLT in *M.*

ecuadoriensis, with a prominent increase in mortality in the MLT + MP treatment, and the increase in the bioaccumulation of MPs especially in the hepatopancreas and digestive tract. However, further studies with the incorporation of multiple biomarkers are needed to better understand the effects of MPs and its interaction with other contaminants in fiddler crabs.

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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