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Assessment of Microplastic and Organophosphate Pesticides Contamination in Fiddler Crabs from a Ramsar Site in the Estuary of Guayas River, Ecuador

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Abstract
We assessed the concentration of organophosphate pesticides (OPs) and microplastics (MPs) in water and sediments from the burrows, and tissues of the fiddler crabs *Leptuca festae* and *Minuca ecuadoriensis*, from Isla Santay, a Ramsar site in the estuary of the Guayas River, Ecuador. MPs concentrations in the burrows were 660 ± 174.36 items kg^{-1} (w.w.) and 26 ± 1 items L^{-1} in collected sediments and water, respectively. Regarding OPs, water and sediment concentrations were up to 26 times above the USEPA thresholds for chronic exposure, indicating environmental risk. MPs were found in tissues collected from both species. The highest abundance was in the gills followed by the digestive tract and hepatopancreas. OPs concentrations in tissues were below the detection limits. Because fiddler crabs are chronically exposed to environmental contamination, they are suitable bioindicators to monitor Isla Santay and to comprehend human impacts in coastal environments of Ecuador.

Keywords Bioaccumulation · Isla santay · *Leptuca festae* · *Minuca ecuadoriensis* · Monitoring

Marine and estuarine areas have been protected for different purposes, such as the protection of fish stocks and the reduction of anthropogenic impacts (IUCN 2013). The designation of a given location as a protected area implies in restrictions on land use changes and polluting activities (Kelleher 2005). However, the protection of marine and estuarine environments has often been ineffective in meeting 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 in front of the city of Guayaquil, the most populated city in Ecuador, is a Ramsar wetland and a biodiversity hotspot. Due to the proximity to the urban area and because it is located at the estuary of the Guayas River, the status of protected area may not be sufficient in protecting this site and needs to be evaluated.

The Gulf of Guayaquil has the highest density of plastic debris in the Ecuadorian coast (Gaibor et al. 2020). The lack of adequate treatment of solid wastes, added to the population's consumption habits and high urbanization, contribute to the high amounts of solid waste that reach Isla Santay daily. Additionally, the pesticides pollution of the Guayas River is a chronic problem. 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 on the numerous arable lands of the Guayas River basin. A study carried out in the Guayas River basin showed that contamination of the freshwater by pesticides was present in 60% of the sampled locations, with a total of 26 different pesticide substances detected (Deknock et al. 2019).

Pesticides and plastic litter pose major threats to estuarine 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

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2011). The resulting particles smaller than 5 mm are then defined as microplastics (Gesamp 2016). If the buoyancy of these particles is or becomes negative after colonization processes, they sink to the seafloor where they become available for benthic invertebrates. MPs ingested by organisms (by feeding and/or ventilation processes) can induce negative mechanical effects due to their attachment to external surfaces (e.g., reducing mobility and clogging the digestive tract) as well as chemical effects (e.g., inflammation, neurotoxicity, genotoxicity, hepatic stress, oxidative stress, disturbance of energy metabolism and accumulation in the tissues (Setälä et al. 2016; De Sá et al. 2018). MPs also provide surface areas that can absorb, concentrate, and transport organic pollutants, influencing the mobility and bioavailability of pollutants for organisms (Lambert and Wagner 2018). In areas with high concentrations of pesticides, as the estuary of the Guayas River, the presence of MPs can enhance the toxic effects of these substances.

Several studies indicate that fiddler crabs can be considered bioindicators of ecosystem health (Azpeitia et al. 2013; Capparelli et al. 2019, 2016, 2017; Lavezzo et al. 2020; Yáñez-Rivera et al. 2019). Fiddler crabs play an important role in biogeochemical cycles and nutrient recycling in mangroves (Gribsholt et al. 2003; Zeil and Hemmi 2006). They constitute an attractive model to investigate the bioaccumulation mechanisms *in situ*, particularly because 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. Being territorial and lacking extensive mobility, fiddler crabs are chronically exposed to local environmental contamination. Thus, the species of fiddler crabs from Isla Santay, could be used as bioindicators of ecosystem health.

The proximity to multiple sources of contamination may be affecting the biodiversity of protected areas in estuarine environments. In this study, our objective is 1) to make a preliminary assessment of the concentration of organophosphate pesticides (OPs) and microplastics (MPs) in the water and in the sediments of the burrows inhabited by two species of Fiddler crabs, *Leptuca festae* and *Minuca ecuadoriensis* inhabit, at the Isla Santay, and 2) to evaluate the concentration of OPs and MPs in the tissues of these two species. The accumulation capacity of contaminants is a key factor to consider when using model species as sentinels to monitor impacted regions and to assess the efficiency of protected areas in avoiding direct anthropogenic impacts. Based on our assessment, we propose the continuous monitoring of Isla Santay using fiddler crab species as bioindicator species.

Isla Santay (2°13'04°S, 79°52'40°W) is an island located in the Guayas River estuary. The island is included (Fig. 1) 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 $m^3 s^{-1}$ and a drainage area of approximately 34,000 km^2, where a population of 4.8 million inhabitants live (Montaño and Sanfeliu 2008; INEC 2020). Isla Santay is located only 800 m away from the cities Guayaquil and Duran and receives direct contamination from multiple sources, including plastics litter (Nikita Gaibora et al. 2020) and agrochemicals (Deknock et al. 2019). Our sampling campaign was performed in November 2019.

Surface water and sediment were collected from crab burrows (Fig. 1, site 1, *Leptuca festae*; site 2, *Minuca ecuadoriensis)* for MPs and OPs analysis. At each site, one water sample was collected for MPs (1 L in transparent glass bottles) and three water samples for pesticides analysis (1 L in each glass amber bottle). From the crabs' burrows three sediment samples were collected for MPs analysis (500 g in pre-clean plastic bags) and three sediment samples for OPs analysis (500 g in pre-clean plastic bags covered with aluminum foil to protect the samples from sunlight). Samples were transported refrigerated (4°C) to the Laboratorio Nacional de Referencia del Agua at the Universidad Regional Amazónica Ikiam. Samples were kept in the dark at − 20°C until analysis.

Leptuca 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* lives in the supratidal zone, where the tidal water arrives only during greater amplitude tides. 30 adult crabs of 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 the 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, after which all gill pairs, the hepatopancreas and the digestive tract (esophagus, stomach, and intestines) were dissected for MPs quantification and muscle tissues were dissected for OPs analyisis.

The methodology for MP 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 and minimize the clogging of the smaller sieve; its content was discarded. Microparticles from the 63 µm sieves were transferred, aided by a minimal amount of deionized H_2O, to 100 mL glass collection jars Then, the samples were dried at 60°C for 24 h and digested with a solution of Hydrogen

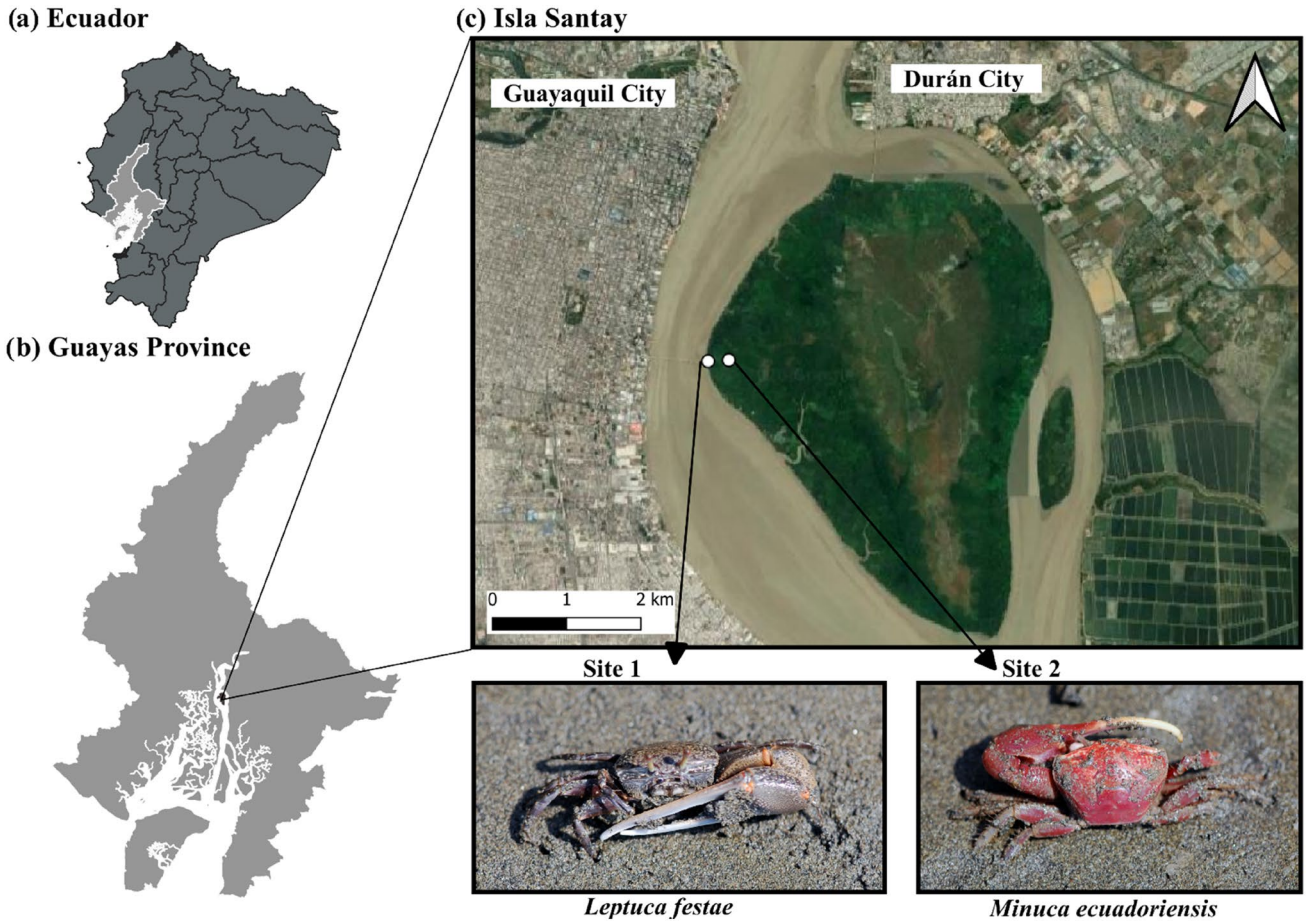

Fig. 1 **a** Location of the Guayas province and **b** the location of Isla Santay, in the estuary of the Guayas River. **c** Collection sites of the mudflat fiddler crabs (site 1, *Leptuca festae*; site 2, *Minuca ecuadoriensis*). The background image shows the urbanization around Isla Santay.

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. In sediment samples, MPs were extracted by density separation with 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.

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/5 g of tissue) in an oscillation incubator at 60°C at 100 rpm for 48–72 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.

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 MP 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. Drinking and filtered water were 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 the protocol described above. The filters were then analyzed visually, and the MPs found were subtracted from the total samples.

Filters were divided into four sections to facilitate an accurate 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. The patterns used to identify types of MPs were based on the descriptions provided by Mohamed Nor and Obbard (2014) and Masura et al. (2015), as well as on visual inspection. Two observers used stainless steel tweezers to actively search for MPs and to separate them from sediment

fragments. MPs 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 as plastic compounds. If the particles kept their shape, they were included in the counting (Mohamed Nor and Obbard 2014).

The water samples were transferred to separating funnel where 50 g of NaCl was added until completely diluted. Pesticides were extracted by shaking the samples for 1 minute using 15 mL of dichloromethane, three times. Then, the extract was filtered with 3 g 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).

Muscle tissue was removed, weighed, and freeze dried. Then, 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. Then, the extract was rota-evaporated, and the residual was mixed with 1 mL of dichloromethane (Cheng et al. 2019).

Extracts were analyzed by a 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 standards. A reference standard mixture (96%–99% certified purity) of 7 OPs (Dimethoate, EPN, malathion, monocrotophos, ethyl parathion, sulfotepp and tetraethylpyrophosphate) 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. GC/NPD responses were linear in the concentration range of 5–1000 ng mL^{-1}, with regression coefficients between 0.996 and 0.999. The detection limits (LODs) for organophosphate pesticides ranged between 0.08 and 0.015 µg L^{-1} in water samples, 0.25–0.5 µg kg^{-1} in dry sediments and 1–2 ug kg^{-1} in tissues. The percent recovery of each pesticide was 55%–95% in water and 50%–90% in sediment samples. The OPs were chosen based on their frequency of use in Western Ecuador (Deknock et al. 2019).

Quality control was assured through solvent blanks analysis and procedure blanks. All reagents used were analytical grade. Samples were analyzed in triplicate concurrently with quality control analysis (blanks and recovery determinations). Before analysis, the 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 can be neglected because pesticides were not detected in the blanks.

Results were expressed as mean ± SEM. To assess the bioaccumulation of MPs in tissues, a two-way analysis of variance (ANOVA) was performed, being tissue (gills and the digestive tract and hepatopancreas (DT + H) and species the independent factors. Differences between means within the same factor were determined by the Student–Newman–Keuls multiple comparisons procedure. The minimum significance level of $p < 0.05$ was employed throughout. The OPs concentration in water was compared to the water quality guidelines established by the United States Environmental Protection Agency (USEPA 2010); the OPs concentration in sediment was compared to the Freshwater Sediment Screening Benchmarks (USEPA 2006).

Results and Discussion

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, c). Site 1, being closer to the intertidal zone accumulated 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 MPs shape were films (57.14%), followed by fragments (21.43%), fibers (17.86%), and beads (3.57%). Films derive from the fragmentation of plastic packaging and bags (Wang et al. 2019; Mohamed Nor and Obbard 2014). 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 white MPs may be the breakdown of single-use plastic products (Napper and Thompson 2016).

The concentration of MPs in water samples were higher in site 2, supralittoral zone (26 items L^{-1}) than in Site 1 (16 items L^{-1}), infralittoral zone (Fig. 2a, b). 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 MP due to the constant water dynamics (do Sul et al. 2014). Regarding the shape of the particles, fibers were more abundant MPs in site 1 (43.75%), while fragments were more abundant MPs in in site 2 (41.38%). Fibers are often related to laundries wastewaters, to the inefficiency of wastewater treatment plants in retain MPs (Browne et al. 2011; Henry et al.

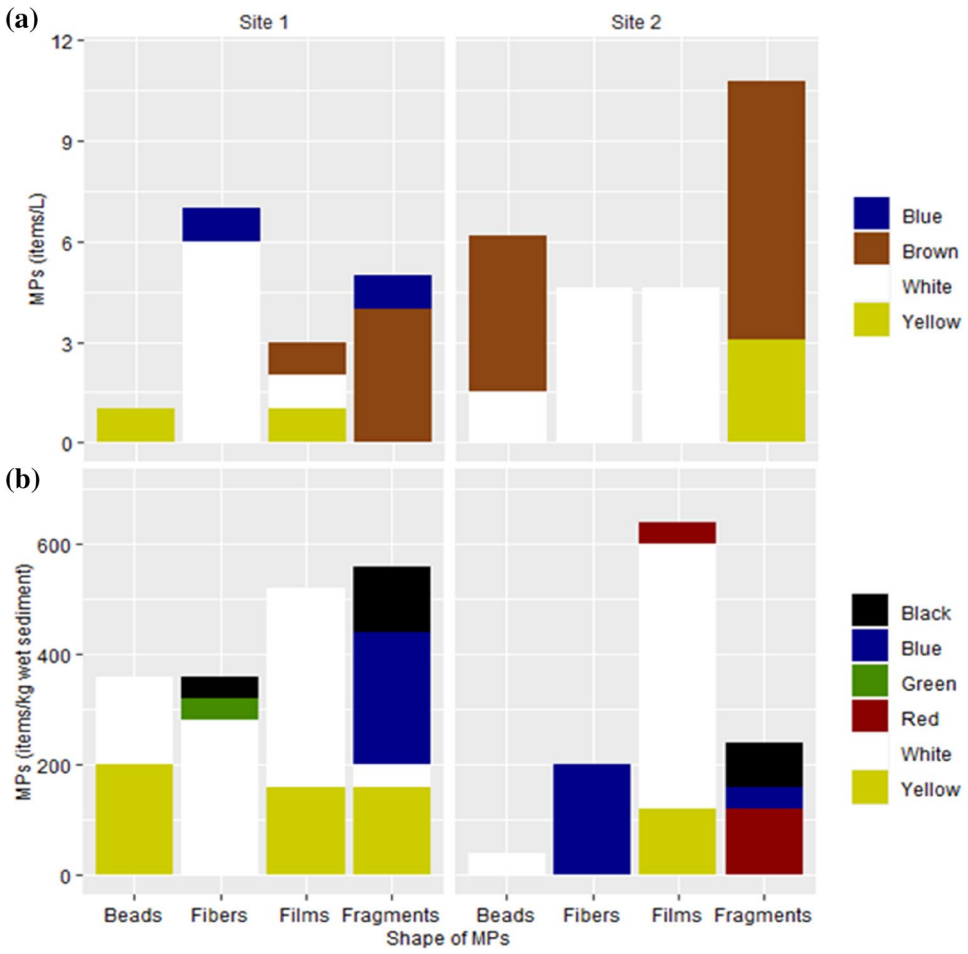

Fig. 2 Abundance of microplastic (MPs) classified by color and shape as registered in water (**a**) and in sediments (**b**) from the burrows of the two species of fiddler crabs. Site 1: infralittoral zone inhabited by *Leptuca festae*. Site 2: supralittoral zone inhabited by *Minuca ecuadoriensis* (see 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.

2019) and to waster 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%).

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 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. Regarding MPs shapes and color, the greatest diversity was reported in the gills and DT + H of both species (Fig. 4).

A previous study on another species of fiddler crab, *Minuca rapax*, reported that MPs were more often found in the gills then in the stomach, suggesting that MPs are more likely to get trapped and accumulated in the fine structures of the gills, and may pass through the digestive tract without bioaccumulating (Brennecke 2015). Fiddler crabs filter sediment pellets using water stored in the gills chamber (Dye and Lasiak 1987). Thus, this active filtering could contribute

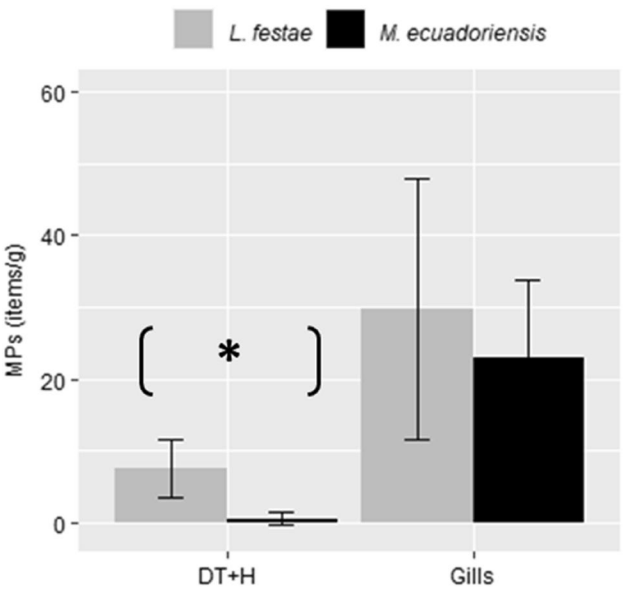

Fig. 3 Abundance of microplastics (MPs) in the tissues of *L. festae* and *M. ecuadoriensis* from Isla Santay. Mean number ± standard deviation of MPs per gram of tissue in the gills and DT+H in both species of fiddler crabs. * indicates significant differences ($p < 0.05$) in the MPs concentration between gills and DT + H of each species

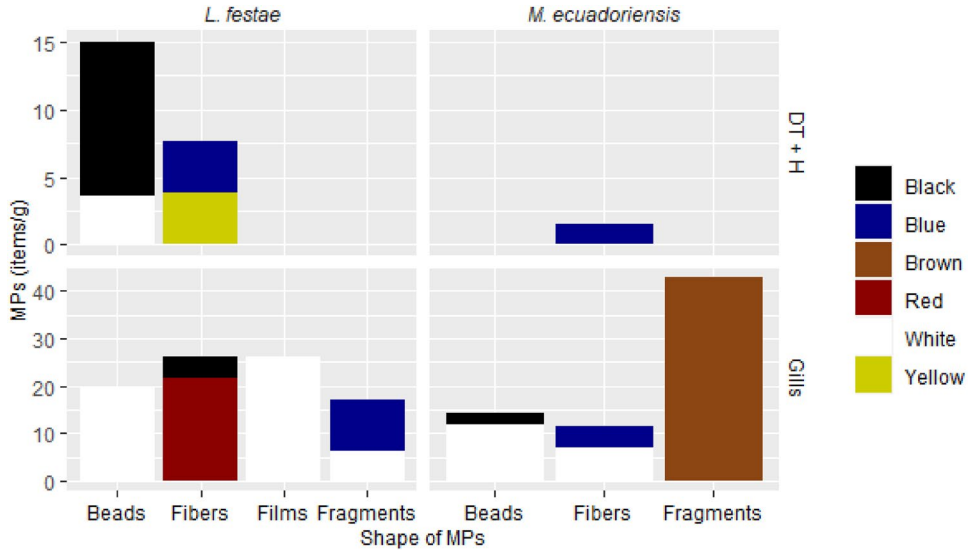

Fig. 4 Abundance of microplastics (MPs) in the tissues of *L. festae* and *M. ecuadoriensis* from Isla Santay per gram of tissue classified by color and shape.

to the retention of MPs in the gills. The feeding behavior of fiddler craps has been associated with particle selection ability (Robertson and Newell 1982). They use the small chela to feed; 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 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 opens above the legs and returns to the gill chamber and will eventually be recycled and evaporated again. As this 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.

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 1). 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 as extremely hazardous by the World Health Organization (World Health Organization 2010). Malathion is in the Sustainable Agriculture Network (SAN9), which is the list of pesticides for use with risk mitigation. It is considered as moderately hazardous by the SAN and by Ecuadorian regulations (Sustainable Agriculture Network 2017; INEN 2008).

The concentration of Malathion and Ethyl-parathion in water for site 1 and site 2 were approximately 26 times higher than the recommended for chronic exposure by the USEPA (0.1 µg L^{-1} and 0.013 µg L^{-1}, Malathion and Ethyl-Parathion respectively). Our results were 10 times higher than the reported by Deknock et al. (2019) for the Guayas River basin.

The concentrations of Malathion and Ethyl-parathion in sediment were approximately 11 and 4 times higher than the Freshwater Sediment Screening Benchmarks by the USEPA (0.203 µg kg^{-1} and 0.757 µg kg^{-1} for Malathion and Ethyl-parathion, respectively) suggesting a significant risk for the biota. In sediment samples, Malathion was only detected at site 2, in concentration 3 times higher in the sediments than in the water. Ethyl-parathion was 20 to 35 times higher in

Table 1 Concentrations of OPs in water (µg L^{-1}) and sediment samples (µg kg^{-1}) from each species habitat. Site 1, infralittoral habitat for *L. festae* and Site 2, supralittoral habitat for *M. ecuadoriensis*

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 ± 0.00**	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

Values in bold highlight concentrations above the limits established by USEPA guidelines (EPN is not included in the guideline)

ND Not detected

sediments than in water, indicating that estuary sediment could be a significant sink for this contaminant. 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. This OP has a half-life ranging from 1 to 20 days (Gibson and Burns 1997; Getenga et al. 2000). Besides, bound residues of OPs in the soil decline faster because of volatilization, interaction with microorganisms andsoil type (Gervais et al. 2009). The concentration ranges of OPs found in our study were 5 to 15 times higher than those reported in rivers considered highly contaminated in Europe (Triassi et al. 2019, Montuori et al. 2015).

Although high concentrations of OPs were detected in water and sediment from the burrows where the fiddler crabs were collected, the concentrations in the tissues of both species was below the detection limit (1–2 μg kg^{-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 Malathion 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 Malathion in concentrations lower than those found in Isla Santay (0.23 μg L^{-1} and 0.47 μg L^{-1}), it presents DNA damage (Knapik and Ramsdorf 2020). Acute and chronic exposure to OPs is associated to toxicological effects, including metabolic, enzymatic (AChE inactivation), protein, physiological, histological, biochemical and genotoxicological disorders (Sidhu et al. 2019), as have been reported in in fish (Fulton and Key 2011; Cook et al. 2005), and crustaceans (Liu et al. 2012; Duarte-Restrepo et al. 2020).

Although OPs are highly toxic for aquatic organisms, the combined exposure of these pollutants with MPs could enhance the negative effects on the ecosystem due the capacity of MPs to act as pollutant vectors (Wang et al. 2019). Because OPs and MPs have been found in sediment and water samples from Isla Santay, and that MPs can be vectors of OPs pesticides, MPs could play an important role in the bioaccumulation and toxic effects of OPs in the biota of that area. In summary, our results showed that both species of the fiddler crabs are exposed to MPs and OPs through contaminated water and sediment. Both *L. festae* and *M. ecuadoriensis* accumulate MPs in their tissues, mainly in the gills. Although bioaccumulation of pesticides has not been observed, these compounds were found at toxic levels and can cause damages at the individual and ecosystem level. Thus, although Isla Santay is a protected Ramsar site, we advertise that current environmental protection measures needs to be re-evaluated for the purpose of effectively safeguard Isla Santay biodiversity. Our work was a preliminary monitoring, and further studies on specific biomarkers are necessary to better understand the effects of these contaminants on sentinel species.

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Declarations

Conflict of 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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