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Plastic ingestion in aquatic insects: Implications of waterbirds and landfills and association with stable isotopes

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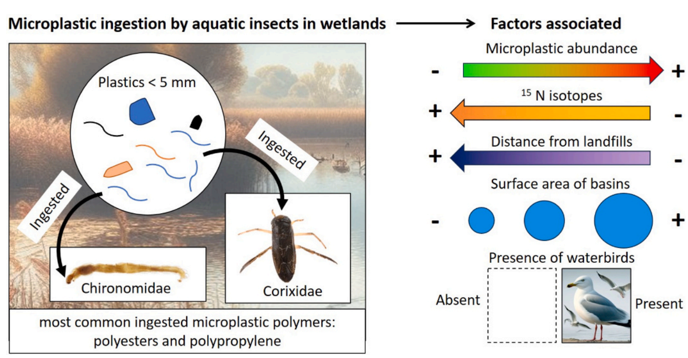
HIGHLIGHTS

- Microplastic abundance in coastal wetlands was greater in larger basins.
- Microplastic abundance increased in sites near landfills and with avian biovectors.
- Polyester and polypropylene are the most common polymers found in aquatic insects.
- Microplastic abundance in aquatic insects was correlated with ^{15}N isotopes.

GRAPHICAL ABSTRACT



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ABSTRACT

Wetlands provide numerous ecosystem services including freshwater purification. Nonetheless, their functionality is continuously impacted by many pollutants. Plastics are considered as an emerging threat for these ecosystems, but only recently have studies began to focus on plastic and microplastic (MP) contamination in wetlands, especially in biota. This study aims to investigate the abundance of MPs in two ubiquitous aquatic insect taxa (i.e. Corixidae (Hemiptera) and Chironomidae (Diptera)) collected in twelve zones within Mediterranean wetlands belonging to three basins located in Andalusia (south-west Spain). We compared MP contamination across basins and tested the proximity to landfills and presence of colonial waterbirds [i.e. white storks (*Ciconia ciconia*) and gulls (*Larus michahellis* and *L. fuscus*)] on MP abundance in aquatic insects. We also performed stable isotope analyses of nitrogen and carbon ($\delta^{15}N$ and $\delta^{13}C$) to evaluate the potential association between MP abundance and isotopic values. We detected 571 suspected MPs (mostly blue fibers) in insects of

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different developmental stages (i.e., larvae, pupae, nymphs and adults). Polyesters and polypropylene were the most frequent polymers detected. The generalized linear mixed models indicated that MP abundance decreased with increasing distance from landfills; but it also increased in sites with birds that fed on landfills and roost in wetlands. When controlling for landfill effects, sites in the smallest basin (Guadalete) had lower MP contamination than those in Odiel-Tinto and the much larger (>15×) Guadalquivir. Moreover, we found a negative association between MPs items/g (or mean MPs) and ^{15}N isotopes in adult corixids. Our findings showed that MP pollution is present in all the study areas, including strictly protected wetlands. The use of aquatic insects for biomonitoring of MP pollution can help identify priority areas for management actions to mitigate plastic pollution.

1. Introduction

Wetlands are vital ecosystems found across the globe, covering approximately 8 % of the Earth's land surface (Ballut-Dajud et al., 2022). These ecosystems provide essential services to humans such as water regulation and purification, erosion control, and climate regulation (Millennium Ecosystem Assessment, 2005). Despite the manifold advantages wetlands provide, they face significant pressures, both natural (like floods or cyclones) and human-induced, such as land use conversion and excessive water exploitation. Causes of wetland loss and degradation include agriculture, urban expansion, overexploitation for resources, and the introduction of alien species (Newton et al., 2020; Ballut-Dajud et al., 2022; Pegg et al., 2022). Nowadays, many wetlands worldwide are disappearing or continue to be degraded (Fluet-Chouinard et al., 2023). One of the most evident modern threats to aquatic ecosystems is the contamination by plastics. Since the first reports of plastic pollution in the ocean during the '70s and '80s, research activities to better understand this phenomenon have been developed as have policies to address it, underlining the detrimental effects of plastics on the ecological integrity, ecosystem functions and biodiversity of aquatic ecosystems (Fossi et al., 2020; Ouyang et al., 2022; Battisti et al., 2023; Bottari et al., 2024). Recent estimates report a peak of 390.4 million tons of global plastic production (Statista, 2022), of which only 9.8 % are recyclable or bio-based (Plastics Europe, 2022). As a result, most plastics end up in landfills, incinerators, or become mismanaged plastic waste (Plastics Europe, 2022). The latter can reach the fluvial systems that act as carriers of plastic litter towards the oceans and associated estuarine and wetland systems (González-Fernández et al., 2021; Gallitelli and Scalici, 2022). Among aquatic ecosystems, coastal wetlands can be especially prone to act as sinks for plastic accumulation as they receive waste from multiple origins, either terrestrial (e.g. rivers) or marine (e.g. deposition at high tide, and storms) (Wang et al., 2019; Ouyang et al., 2022; Plastics Europe, 2022; Ritchie and Roser, 2023; Battisti et al., 2024; Gallitelli and Scalici, 2024). Moreover, it has recently been shown that waterbirds (e.g., gulls and storks) that feed in landfills but roost in wetlands can transport hundreds of kg/year of plastic from landfills into individual wetlands via regurgitation or defecation (Cano-Povedano et al., 2023; Martín-Vélez et al., 2024). As a consequence, even wetlands in remote or protected areas can be contaminated by plastics transported by rivers, birds, wind, or sea.

Plastics can be classified based on size as macroplastics (plastics >5 mm), mesoplastics (plastics 5 mm–2 cm) and microplastics (MPs; plastics 1 μm–5 mm), with varying impacts on ecosystems and biota (Gallitelli and Scalici, 2022). Macro and mesoplastics are frequently ingested and transported by birds and are harmful in themselves, and also because they can degrade into MPs creating secondary MPs. Once in the environment, macro and mesoplastics are broken up into smaller particles by exogenous agents, e.g. solar radiation, mechanical stimuli of wind and waves (Singh and Sharma, 2008). Apart from secondary MPs, MPs are produced by e.g. textile and cosmetic industries (i.e., primary MPs). Both primary and secondary MPs may cause sublethal and lethal effects on biota – placing freshwater biota and ecosystems at risk (Franzellitti et al., 2019).

Macroinvertebrates (e.g. aquatic insects) are major components in the food web of aquatic communities and play a fundamental role in ecosystem functioning. Due to their importance, MP accumulation in macroinvertebrates has been investigated in rivers (Windsor et al., 2019; Cera and Scalici, 2021; Gallitelli et al., 2020), however research in wetland environments remains scarce (Qian et al., 2021; Bertoli et al., 2022). MP ingestion/exposure can affect invertebrate growth rates and metabolic processes (Castro et al., 2022), and alter their isotopic composition (Zhu et al., 2018). Stable isotopes of carbon and nitrogen (δ^{13}C and δ^{15}N) are commonly used to assess trophic relationships, integrating information over long time frames. Recently, they have also been utilized in MP studies, as their ingestion can induce stress that alters their energy storage, without a change in diet (Garcia et al., 2021; Huang et al., 2020).

MPs have become pervasive in all freshwater ecosystems, yet research has focused mainly on permanent rivers and lakes, while studies in other wetlands have remained scarce (Miller et al., 2017; Cera et al., 2020; Camargo et al., 2022; Cera et al., 2022). In this study, we aimed to investigate the contamination of MPs in Mediterranean wetlands in Andalusia (south-west Spain), using aquatic insects as biomonitors. We focused on wetlands within the coastal plain of three river basins varying in size more than an order of magnitude (Odiel-Tinto: 3980 km^2; Guadalquivir: 57,520 km^2 and Guadalete: 3667 km^2) but with similar land-use (Sánchez-Moyano and García-Asencio, 2011; Egüen et al., 2015; Buonocore et al., 2021; Sabater et al., 2022). Our specific objectives were: 1) to assess differences in MP accumulation between aquatic insect taxa and their developmental stages; 2) to examine differences in MP abundance and characteristics (shape, color and polymer composition) between small and large basins; 3) to evaluate the influence of landfill proximity, and waterbird presence (yellow-legged gulls *Larus michahellis*, lesser black-backed gulls *L. fuscus* and white storks *Ciconia ciconia*) on MP concentrations in aquatic insects; 4) to investigate the possible associations between MP abundance in aquatic insects and stable isotope values (δ^{13}C and δ^{15}N).

Our main hypotheses were: H1) MP accumulation would be different between insect developmental stages in a taxa-specific way, i.e. in Corixidae the larger adult stage should accumulate more MPs than the smaller nymphs despite having similar feeding habits (Coccia et al., 2016; Garcia et al., 2021), whereas Chironomidae larvae and pupa should have similar MP abundance, since pupae do not feed, but may retain the MPs ingested by larvae (Al-Jaibachi et al., 2018; Simakova et al., 2022) and H2) MP abundance would be higher in aquatic insects within larger basins, since MPs could originate from a larger area.

We also expected that H3) MP abundance in aquatic insects would be higher at sites close to landfills regardless of the site protection status, as these are the main source of MPs in the environment (Bharath et al., 2023) and that H4) MP abundance in aquatic insects would be associated with the presence of avian biovectors, known to contribute to plastic deposition in wetlands (Martín-Vélez et al., 2024). Lastly, H5) we expected that MP abundance in aquatic insects would be associated with stable isotopes of δ^{13}C and δ^{15}N. This is because exposure to stressors might alter stable isotope signatures (Karlson et al., 2018), or else trophic niche may be correlated with MP ingestion rates (e.g. sediment feeders may be more exposed to MPs).

2. Methods

2.1. Study area

This study was conducted across 12 zones in southwest Spain, with 8 situated in protected areas and 4 located in non-protected areas (Table S2). Specifically, among the protected areas we sampled:

1. Cádiz Bay Natural Park in Cadiz province. This is a coastal wetland complex including natural intertidal mudflats and solar saltworks, declared as a protected area at the Regional level since 1989 (Junta de Andalucia, 2004). Further, the park is listed as an internationally important wetland under the RAMSAR Convention and is part of the Natura 2000 Network
2. Doñana National Park in Huelva and Seville provinces. The park is designed as a RAMSAR wetland, is part of the Natura 2000 Network, and is a UNESCO World Heritage Site (Green et al., 2018).
3. The Odiel Marshes Natural Park in Huelva province. This is an estuarine complex, containing mudflats and saltpans declared as a UNESCO Biosphere reserve in 1983.
4. The Dehesa de Abajo in the province of Seville. This is a private Nature Reserve with a seasonal freshwater lake and trails allowing visitors to explore the area and its fauna (Junta de Andalucia, 2024).

Among the unprotected areas, we sampled within 1) the Cetina artificial saltpan complex (in the Cadiz Bay area), an Important Bird Area (IBA 251) one of the most important sites in Spain for salt production; 2) an unprotected area adjacent to the Cádiz Bay Natural Park; and 3) a private agricultural estate in Huelva province (Fig. 1). The studied sites belonged to three river basins: Guadalete in Cadiz province; Odiel-Tinto in Huelva province, and Guadalquivir in Seville province (Fig. 1).

The number of sampled stations varied between zones. In areas consisting of multiple water bodies (i.e. the Cadiz Bay complex), we sampled three stations belonging to different water bodies, while in areas formed by one or two water bodies, the three stations were spatially distributed within them (Table S1). In Doñana National Park we sampled four stations, and in Odiel marshes we choose two different stations that were well separated from each other but within the same marsh system. In each station, three replicates were collected. Site selection was based on the presence/absence of waterbird resting and/or breeding sites. In total, seven out of 12 sampling zones were also used as resting and breeding sites for waterbirds such as white storks (*Ciconia ciconia*), yellow-legged gulls (*Larus michahellis*) and lesser black-backed gulls (*Larus fuscus*), which are known to transport plastic from landfills to wetlands within their feces and pellets (Martín-Vélez et al., 2022; López-Calderón et al., 2023; Cano-Povedano et al., 2023). Sampled waterbodies included saltpans, temporary and permanent ponds. Distances from open-air landfills ranged from a minimum of 2176 m to a maximum of 52,411 m, Fig. 1 and Table S1.

2.1.1. Aquatic insect collection and processing

Aquatic insect sampling was conducted once within each site during June and July 2022, by sweeping a D-framed pond net (500 μm mesh; 16 × 16 cm) over 1 m for 30 s at three different points within each station. After collection, invertebrate samples were preserved in plastic

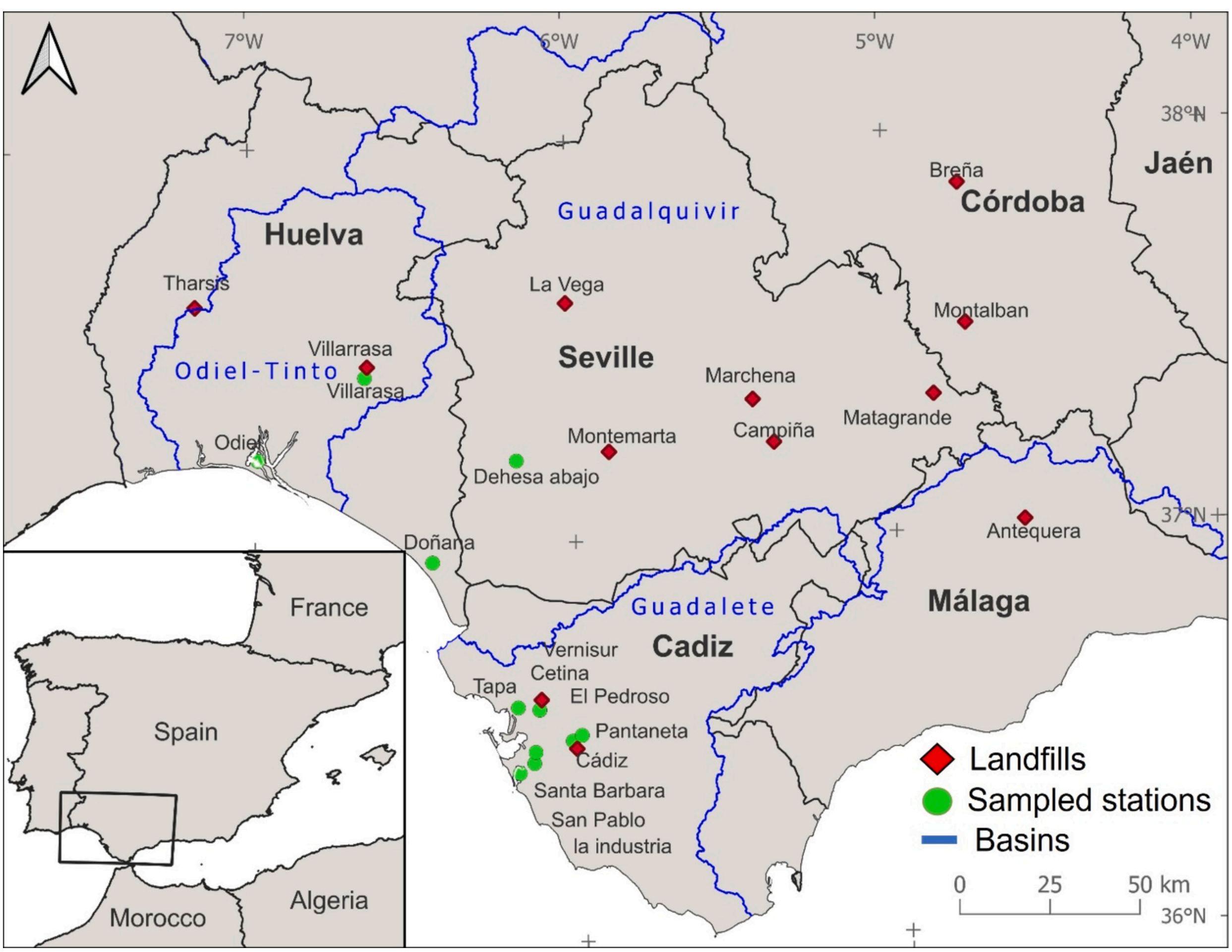

Fig. 1. Study area showing the provinces and sampled stations in Andalusia. The red dots indicate landfills, and the green circles indicate the sampled stations. The three basins are indicated on the map with a blue line.

containers filled with 70 % ethanol until further processing. When possible, 20 adult corixids of *Trichocorixa verticalis* (an alien species common in most stations, Céspedes et al., 2019) were sampled for stable isotope analyses. These were kept in plastic containers filled with water and transported to the laboratory. Upon arrival at the laboratory, individual corixids were transferred to 1.5 ml Eppendorf tubes and frozen at −20 °C. Adult corixids were dried, weighed and packed into tin capsules before analysis. *Trichocorixa verticalis* was selected based on previous research (Coccia et al., 2016) and because it was the most abundant corixid found across stations (4 zones; 7 stations from Guadalete; 1 zone; 6 stations from Odiel-Tinto and 2 zones; 4 stations from Guadalquivir).

In the laboratory, sampled insects were separated by taxonomic group and identified under a stereomicroscope. The most abundant and frequent taxa belonging to Hemiptera and Diptera were used for microplastic examination, i.e. Corixidae and Chironomidae, respectively. These were identified to the lowest practical taxonomic level using available keys, i.e. genus/species level for corixid adults and family level for Chironomidae larvae, pupae and corixid nymphs (Jansson, 1986; Tachet, 2010).

2.1.2. Microplastic processing

Aquatic insects were processed by adapting protocols from Gallitelli et al. (2020). Specifically, pooled aquatic insects were rinsed with distilled water, weighed on a precision balance (±0.01 g), put in 50 ml Falcon tubes and digested with 30 % hydrogen peroxide (H_2O_2) solution at 37 °C for 48 h. Thereafter, if fragments of aquatic insect bodies were still visible by the naked eye, the samples were kept at ca. 24 °C (room temperature) for 7 days. This was a suitable timeframe for complete degradation. The solution of digested samples was then filtered onto glass fiber filters (VWR, Grade GF/F, porosity 0.7 μm, ⌀ = 47 mm) under vacuum. The filters were stored in sterile glass petri dishes for further analyses. In practice, the filters were screened with a stereoscope (following Gallitelli et al., 2022; Taurozzi et al., 2024) for MPs detection. Suspected MPs were marked on the filter, counted and classified according to shape (i.e., beads, fibers or fragments) and colors (red, green, blue, white, transparent, or black). The color of MPs was assigned to common color categories (e.g., red, white, black, etc.) following Cowger et al. (2020) and Lusher et al. (2020).

After stereoscope identification, we selected and analyzed a subsample of 181 out of 571 suspected MPs, representing approximately 32 % of the total. First, subsamples for analyses were chosen based on color, then, to account for potential variability across provinces and sampling stations, MPs were selected in proportion to their occurrence across stations. These subsamples were chemically characterized by Raman spectroscopy. The Raman spectra were acquired by a Renishaw In Via micro-Raman spectrometer equipped with a Leica DM2700 M confocal microscope with a long work distance Olympus 20× objective, and Renishaw Wire software to set experimental conditions and for spectra acquisition. Two solid-state diode laser sources, one at 532 nm and the other at 785 nm, were used to acquire good-quality spectra. In particular, for each source, the Raman scattered light was dispersed by a diffraction grating with 1800 lines/mm or 1200 lines/mm, respectively. The scattered light is collected by a 1024 × 256 pixel CCD detector that is Peltier cooled (−70 °C). An integration time of 5 s and three accumulations (i.e., number of scans per sample) were used, being the most adequate to obtain clear spectra.

Raman spectra were processed and analyzed as described in Cowger et al. (2020). First, spectra were refined by subtracting an adaptative baseline and smoothed by Savitzky–Golay filtering using the open-source software Spectragryph (v. 1.2.16.1) (Menges, 2023). Thereafter, the processed spectra were identified by automated matching scores with reference open-source libraries available on R and online. In detail, the following libraries were loaded in the environment Rstudio (v. 2023.06.1) and the reference spectra were correlated to sample spectra using the functions "spectra.corr" and "match_spec": library "Mpdatabase" of the R package "RamanMP" v. 1.0 (Fremout and Saverwyns, 2012; Nava et al., 2021) which focuses on additives, and library "Raman" of the R package "OpenSpecy" v. 0.9.5 (Cowger et al., 2021) which includes a wide range of plastic spectra. In addition, the online version of "OpenSpecy" was also used (https://openanalysis.org/openspecy). For each sample spectrum, the output of automatic identification from the different libraries was compared and visually checked before accepting the match. For automatic matches evaluated with uncertainty because the libraries provide contrasting results, and for scores of similarities between reference and sample spectra <60 % (100 % corresponds to identical spectra for samples and reference, while 0 % represents completely different spectra), the identification was complemented by information from the scientific literature. Note that, due to the different characteristics of the libraries, a sample spectrum often matched with a pigment in RamanMP but with a plastic in OpenSpecy; under those circumstances, we report the type of plastic. If some samples could not be classified as plastics due to the presence of pigments, these samples are considered to be of anthropogenic origin (Munno et al., 2020).

2.1.3. Sample quality assurance and control (QA/QC)

To avoid external contamination of invertebrate samples, we used a quality assurance/quality control (QA/QC) approach (Gallitelli et al., 2020). During sampling activities, we used tools rinsed with distilled water and, whenever possible, plastic-free containers (e.g., glass bottles). For the Falcon tubes, they were rinsed with distilled water before being used to remove particles which might have adhered to the wall. We prevented accidental contamination of the samples in the laboratory by using nitrile gloves, cotton clothes, and clean tweezers (Gallitelli et al., 2020). In addition, we performed blanks to evaluate the potential contamination of our samples during their processing. For this, we used glass Petri dishes as controls by removing the lid during the filtration of each invertebrate sample, so that they were exposed to potential airborne contamination (following Taurozzi et al., 2024). Thereafter, control dishes were also opened each time the filters to be analyzed were exposed to air, so that external contamination of the samples could later be identified. In addition to airborne contamination, we also checked the solutions used, i.e., distilled water and hydrogen peroxide. For this, we performed three blanks for each of them separately by filtering distilled water and hydrogen peroxide by vacuum pump. Then, we observed the filters under the stereoscope to check the occurrence of potential MPs. After this, we would have subtracted all those detected in our control blanks from our results (i.e., real MPs). We found only one fiber in one liter of peroxide hydrogen. However, since we used approximately one liter of this solution to perform our analyses, this contamination was considered as negligible. We did not find any MPs in the controls of airborne contamination.

2.1.4. Stable isotope analyses

Isotopic analyses of carbon and nitrogen (C and N) contents of 143 *T. verticalis* were conducted at the UC Davis Stable Isotope Facility (University of California, Davis). Samples were analyzed using a PDZ Europa Scientific Roboprep elemental analyzer coupled with a PDZ Europa Hydra 20/20 isotope ratio mass spectrometer (Crewe, UK). Due to the small size of corixids, individuals were generally analyzed whole, which impeded MP quantification. Nonetheless, adult *T. verticalis* used for stable isotopes were from the same location as individuals used for MP quantification. All isotope results are presented in δ-notation as parts per thousand (‰) deviation and are referenced to international standards for nitrogen (Air) and carbon (Vienna Pee Dee Belemnite) using the equation: $\delta^{13}C, \delta^{15}N = [(Rsample/Rreference) - 1] \times 103$, where R $= {}^{13}C/{}^{12}C$ for carbon and ${}^{15}N/{}^{14}N$ for nitrogen. The mean standard error (based on standard reference material) for samples measured at UC Davis was ±0.07 ‰ and ±0.09 ‰ for δ13C and δ15N, respectively.

2.1.5. Statistical analyses

All analyses were conducted in the statistical programming environment R version 4.1.1 (R Development Core Team, 2009), including functions in vegan, car, lsmeans, glmmTMB, DHARMa, ggplot2 and performance.

We first used non-metric multidimensional scaling (NMDS), based on the Hellinger transformed Euclidean distances matrix of polymer abundances, to visualize differences in MPs (including polymers and additive) composition between basins. Then, we used a Permutational Multivariate Analyses of Variance with distance matrices (PERMANOVA, Adonis in R, see Oksanen et al., 2012) to test for differences in MP composition between basins. If significant differences were found, we used a Simper analysis to identify the MPs that contributed most to the differences between basins. Rare polymer/pigments that occurred only once were eliminated to avoid potential bias.

We used Generalized linear mixed models (GLMMs) to determine 1) if the distance to the nearest landfill (continuous), the level of protection (categorical), bird presence (categorical) and basin (categorical) had significant effects on MP abundance; and 2) if there were differences in MP abundance between macroinvertebrate families and developmental stages. To further explore the bird effects on MP abundance, we built an additional model excluding distance to the nearest landfill from the model. This allowed us to examine the effect of birds on MPs, independent of the potential influence of landfill proximity. Models were fitted with a gaussian or negative binomial error distribution and identity or a log link. Sampling station was used as a random factor, and the weights or log weights of the dissolved insects were used as an offset to adjust for sampling effort. Models were corrected for overdispersion and zero inflation using the functions ziformula and dispformula (Zuur et al., 2009) where necessary. Emmeans function with a Tukey adjustment was used to account for multiple comparisons. Model validations were done using DHARMa package (Hartig, 2017). We used the Variance Inflation Factor (VIF) to detect multicollinearity between variables. Due to the high collinearity (vif > 3) between the level of protection and the distance from the nearest landfill, we excluded the former from the model.

For stable isotope analyses, we used General linear models (GLMs) to determine if $\delta^{13}C$ and $\delta^{15}N$ values from individual samples were influenced by MP abundance (item/gr) from pooled samples using bird presence and sampled stations as covariates. This allowed correcting for isotope variability due to bird presence and across sampling stations. Models used the log number of individuals analyzed for stable isotope analyses as an offset and were fitted with a Gamma or Gaussian error distribution, and a log or identity link.

3. Results

3.1. Microplastic abundance in aquatic insects and MP characteristics

A total of 9139 aquatic insects were analyzed for MPs, of which 6081 belonging to Corixidae [including adults (3967) and nymphs (2114)], and 3058 to Chironomidae [including larvae (2953) and pupae (105)]. Among adult corixids, 628 were *Sigara* spp.; 1345 were *Micronecta scholtzi* and 1994 were *Trichocorixa verticalis*.

We found a total of 571 suspected MPs: 379 within Corixidae (adults 234; nymphs 145) and 192 within Chironomidae (larvae 154; pupae 38). The average abundance of suspected MPs (±SD) was 0.23 ± 0.36 (item/ind), or 45.8 ± 1489 (items/g) for Corixidae, and 0.28 ± 0.47 (item/ind), or 359 ± 1049 (items/g) for Chironomidae. However, we did not find any significant difference in MP abundance between insect families (*z-value* $= -1.52$; $P = 0.13$), nor between developmental stages within each family (chironomidae larvae vs pupae: *t-ratio* $= 0.93$; $P = 0.78$; corixidae adults vs nymphs: *t-ratio* $= -1.64$; $P = 0.35$). The majority of suspected MPs were fibers (468), followed by fragments (103), and no beads were found (Fig. 2). A total of seven MP colors were found (Table S2). Among them, blue was the dominant color in all basins for both fibers and fragments, representing on average 78.8 % of items (see Fig. 3; Fig. 4; Table S2).

Raman analysis revealed that 88.0 % of the suspected MPs were of anthropogenic origin (153 out of 174 readable spectra), of which 32.0 % were plastics, 45.0 % were additives and 11.0 % were colored cotton, cellulose or wool. A total of 12 plastic polymers were identified by Raman (Table S3). Among them, polyester (13.0 % of items) and polypropylene (10.0 %) were the most common polymers, present in 12 and

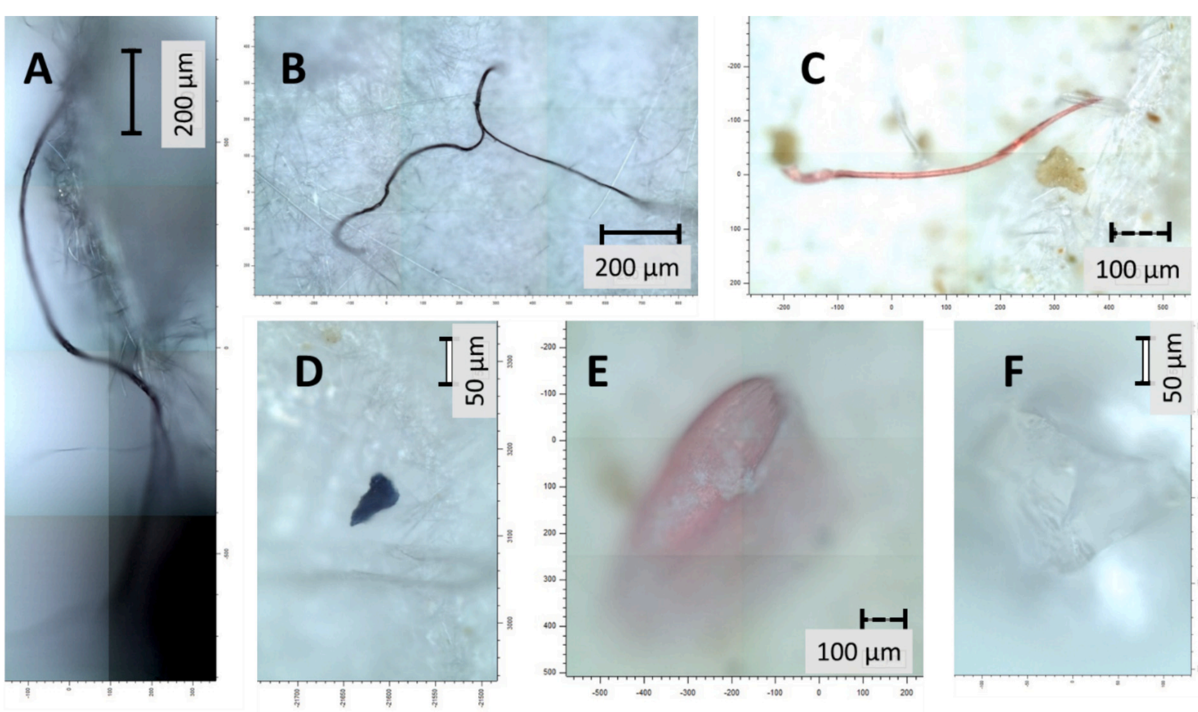

Fig. 2. Examples of the most common items observed from within aquatic insects: A) black fiber; B) blue fiber; C) red fiber; D) blue fragment; E) red fragment; F) transparent fragment.

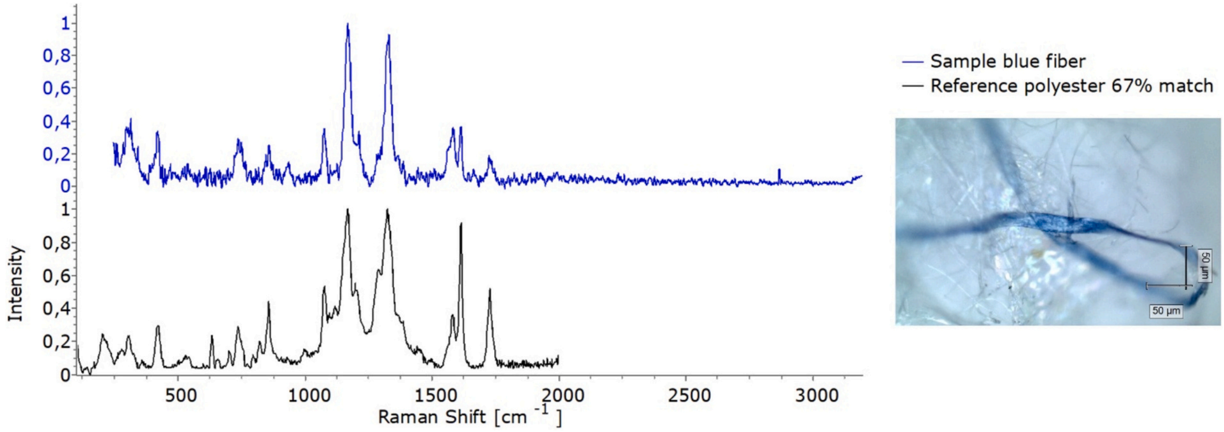

Fig. 3. Raman spectrum of polyester, the most abundant anthropogenic contaminant detected in our samples above. Above in blue, the spectrum of a blue fiber measured by 785 nm source. Below in black, the reference spectrum of blue polyester, which is available in the database SLOPP-E: “polyester3darkblue” (Munno et al., 2020). The match between sample and reference spectra displayed is 67 % using OpenSpecy (openalysis.org).

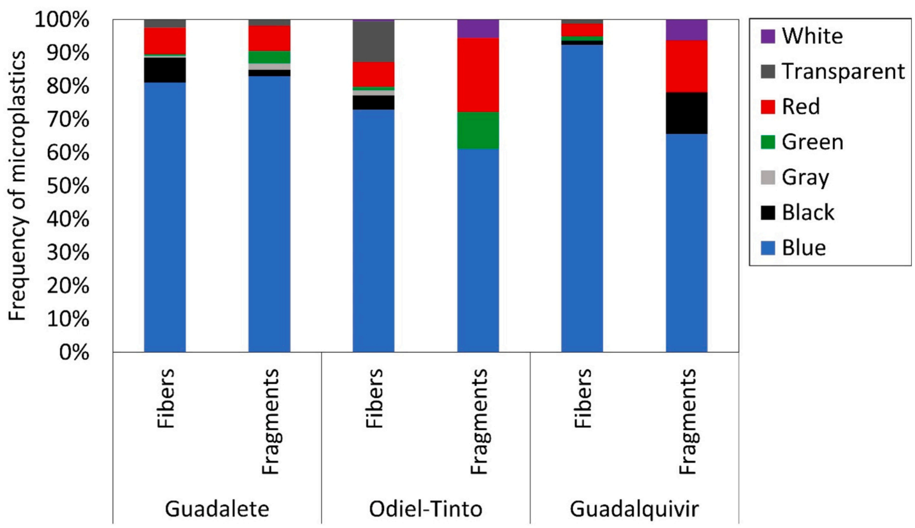

Fig. 4. Proportion of the different colors in fibers and fragments across basins.

9 sampling stations respectively, followed by polyethylene terephthalate (6.7 %) and polyacrylonitrile (4.5 %). Among additives (Table S3), blue pigments were the most common, in particular indigo was the most frequent (28.0 % of items), present in 26 out of the 36 sampled stations, followed by pigment blue 63 (PB63) (7.5 %) present in 9 sampled stations.

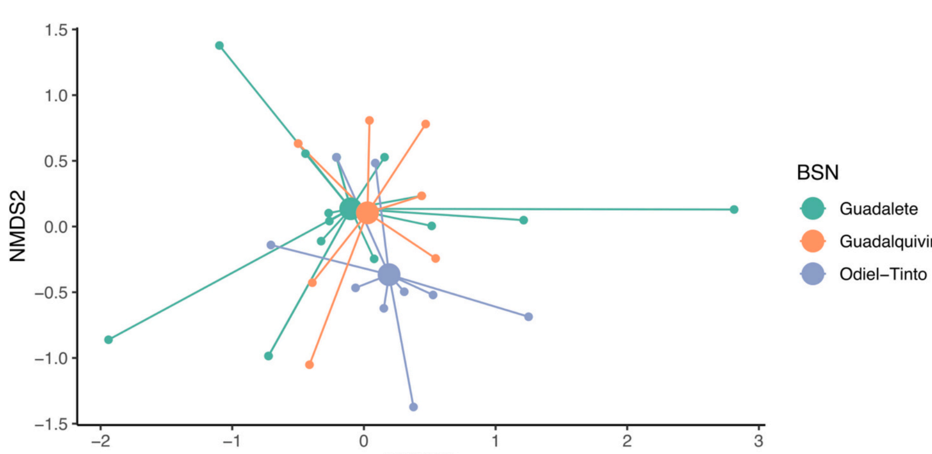

Fig. 5. Non metric multidimensional scaling (NMDS) ordination showing variations in MP (polymer+ additive) composition ingested by aquatic insects across the three basins (BSN).

3.2. Influence of basin, landfills and presence of waterbirds

The distribution of suspected MPs across locations is as follows: 254 items in the Guadalete basin, 206 in the Odiel-Tinto basin, and 111 in the Guadalquivir basin (Table S2). Maximum average values across basins were found in the Guadalete basin (Table S2).

NMDS conducted on the identified MPs showed no clear separation in MP composition between basins (Fig. 5). This was also confirmed by Adonis analyses, showing no significant differences between basins ($R^2 = 0.09$; $P = 0.07$).

Overall, the GLMMs indicated no significant differences in MP abundance across basins ($P > 0.05$). However, GLMM models showed that, after controlling for other variables, basins and the distance from the nearest landfill had significant partial effects on the total abundance of suspected MPs (Table 1). Specifically, sites within the Guadalete had significantly lower MP abundance than those within the Odiel-Tinto (emmeans Tukey; $t = -3.35$; $P = 0.006$), while differences in MPs between the Guadalete and the Guadalquivir were close to being significant (*z value* $= -2.38$; $P = 0.059$). MP abundance decreased with increasing distance from landfills (*z value* $= -2.52$; $P = 0.001$). Bird presence did not affect MP abundance (*z value* $= 1.6$; $P = 0.11$) when using the distance to the nearest landfill and basins as covariates, but it became significant after excluding landfill proximity from the model (*z value* $= 2.55$; $P = 0.01$).

3.3. Association between MP abundance and stable isotope ratios

Regarding the stable isotope analyses, *T. verticalis* was more ^{15}N enriched at the Guadalquivir stations, but less ^{13}C enriched at the Guadiana station (Table 2). After accounting for site variability and bird presence, we found a negative association between MPs item/gr (or Mean MPs) and ^{15}N isotopes (GLMM; $z = -8.49$; $P < 0.001$), but no association was detected for ^{13}C ($z = -1.19$; $P = 0.23$).

4. Discussion

This study contributed to filling knowledge gaps on the contamination of wetlands by MPs. We examined MP contamination in two families of aquatic insects (Corixidae and Chironomidae) sampled from wetlands of three river basins of Andalusia (southwest Spain). MPs are present in both families and in all sampled areas, but their abundance was significantly greater in larger basins. The accumulation of MPs was increased by both the proximity of the sampled wetlands to landfills and the presence of avian plastic biovectors (storks and gulls). We also investigated the effects of MPs ingestion on stable isotope values for aquatic insects, finding a strong negative correlation with ^{15}N isotopes.

4.1. MP accumulation in different insect taxa and developmental stages

Macroinvertebrates can accumulate MPs accidentally from water and sediment, and through the ingestion of contaminated prey (Windsor et al., 2019). For these reasons, they have also been used a proxy of ecosystem contamination (Windsor et al., 2019; Akindele et al., 2020: Lin et al., 2021). Unfortunately, we did not have MP data for sediments and water, but previous studies demonstrated that macroinvertebrate susceptibility to MP accumulation occurs mainly through direct ingestion (Alvarez Troncoso et al., 2022; Garcia et al., 2021; Scherer et al., 2017; Varg and Svanbäck, 2023; Windsor et al., 2019).

Table 1
Summary of the Generalized linear mixing model (GLMM) for the effects of different response variable on abundance of suspected MPs. The P values of the Guadalete and Odiel-Tinto refer to the comparisons with the Guadalquivir, which is aliased. Basin comparisons are reported in detail in the main text. Significant values are reported in bold.

	Estimate	Std.	z-Value	Pr (>|z|)
(Intercept)	63.846	18.744	3.406	**0.000659**
Landfill distance	−4.843	1.920	−2.522	**0.011662**
Bird presence	−2.631	1.639	−1.606	0.108332
Guadalete	−8.623	2.646	−3.258	**0.001121**
Odiel-Tinto	3.528	2.985	1.182	0.237245

Table 2
Mean stable isotopes values (±SD) of *T. verticalis* in each basin. n indicates the number of individuals analyzed for stable isotopes or the number of sampling stations.

Basin	Individuals (n)	Stations (n)	$\delta^{13}C$	$\delta^{15}N$
Guadalete	73	7	−15.2 (±1.09)	9.2 (±1.52)
Odiel-Tinto	42	6	−12.5 (±0.89)	7.2 (±0.82)
Guadalquivir	28	4	−20.4(±3.81)	10.8 (±1.25)

Corixidae and Chironomidae larvae have different feeding mechanisms (i.e., scrapers, piercers, or shredders), but we did not find any difference in MP accumulation between them, perhaps due to similarities in feeding behavior. Corixidae in our samples included the genera *Sigara*, *Micronecta* and *Trichocorixa*, all considered omnivores feeding on both plants (e.g., detritus, algae) and animals (small benthic animals) at the bottom of the water column (Coccia et al., 2016; Hadicke et al., 2017). Similarly, Chironomidae roam the bottom of water bodies in search of dead organisms and detritus (Tachet, 2010). These two taxa may thus have a similar likelihood of accidentally ingesting MPs while feeding.

Surprisingly, we did not find any differences in MP abundance, corrected for the sampled weight between insect developmental stages, contrary to our hypothesis (H1). We found that corixid adults and nymphs did not show any difference in MP concentrations, despite their different sizes (Céspedes et al., 2019). This lack of a difference may reflect their similar ecological niche and feeding mode (Tachet, 2010), leading to a similar exposure to MPs.

On the other hand, Chironomidae larvae and pupae did not show differences in MP abundance even though pupae do not feed (Marziali and Rossaro, 2006). A recent study of the closely related Culicidae (Diptera) showed that MPs can be ontogenetically transferred from larvae to pupae (Al-Jaibachi et al., 2018; Simakova et al., 2022). Therefore, as for Culicidae, MPs in Chironomidae can be transferred from larvae to pupae during development without being lost, and then into flying adults, highlighting the potential for MP movement from aquatic into terrestrial environments (including chironomid predators such as bats or terrestrial birds).

4.2. MP contamination across basins

MPs were found as fiber and in fragment forms, supporting other studies in aquatic ecosystems (Cera et al., 2022; Dalvand and Hamidian, 2023; Gallitelli et al., 2020; Qian et al., 2021; Wang et al., 2017). In particular, fibers were previously found to be the most frequent in wetlands (Almeida et al., 2023; Helcoski et al., 2020; Kabir et al., 2023). Microfibers are widely used, for example, in the textile industry and personal care products (e.g., brushes and sponges), so they are released in wastewater from clothes washing and subsequently reach the environment (rivers and wetlands) (De Falco et al., 2019; Singh et al., 2020; Kumar et al., 2021). In addition, MPs could also originate from the fragmentation of larger plastic materials due to exposure to water and atmospheric agents (Kataoka et al., 2019; Razeghi et al., 2021).

When comparing MP abundance between sites, the GLMM models showed no significant absolute differences between basins, but their partial effects were significant when controlling for the distance from landfills and the presence of biovectors. The Guadalete sites had lower MP abundance compared to the Odiel-Tinto (a highly significant difference), and the Guadalquivir (marginally significant). Since the Guadalete is the smallest basin, this result supports our initial hypothesis (H2) that coastal wetlands within larger basins are more contaminated than those within smaller ones.

On the other hand, the NMDS and Adonis analyses conducted on the MPs identified showed no clear separation in MP composition between basins, indicating a similar polymer contamination in the three basins. We found the most common plastic polymers to be polyester, and polypropylene, in agreement with other studies in Andalusia wetlands (Cano-Povedano et al., 2023; Martín-Vélez et al., 2024) and elsewhere (Qian et al., 2021). This can be related to their wide use e.g. food packaging; agriculture (i.e. mulching film). We also found polyethylene terephthalate and polyacrylonitrile, which are typically used for packaging and industrial textiles. Besides polymers, pigments, especially blue Indigo and blue 63 (PB 63- Indigo carmine), were also common, perhaps because of their use in textile industries (Jung et al., 2020; Nesterovschi et al., 2023). Additives such as pigments can include toxic materials (Namgung et al., 2019) that can be harmful when ingested by biota. Unfortunately, few studies classify the MP polymers prevalent in wetland macroinvertebrates (Qian et al., 2021), preventing us from comparing our results with other studies elsewhere. Similarly, we cannot compare our results in detail with a study of macroplastics transported by waterbirds in the same area (Cano-Povedano et al., 2023) because of the different methodology used for polymer identification (e.g. RAMAN vs. FTIR).

It is worth noting that sampling was conducted only during dry summer months, so we may have underestimated the quantity of MPs present in aquatic insects, since MP levels typically increase in water during the rainy season (Dalvand and Hamidian, 2023; Liu et al., 2022; Sarin and Klomjek, 2022).

4.3. Effect of proximity to landfills and waterbirds as biovectors on microplastic abundance

We found that sites close to landfills accumulate a greater quantity of MPs compared to more distant areas, supporting our hypothesis (H3). This is not surprising, since in general landfills can contaminate surrounding environments with plastic waste, e.g. through discharge, effluents, and open-air fires (Natesan et al., 2021). While information for a full understanding of the transport routes of MPs from landfills is missing, we found that the presence of roosts or colonies of birds that regularly feed on landfills (i.e., gulls or storks) was associated with an increased occurrence of MPs in aquatic insects, supporting our hypothesis (H4). However, this effect was masked by the proximity to landfills, suggesting that this related factor had the strongest influence on MP contamination in these wetlands. Storks and gulls tend to concentrate near landfills (López-Calderón et al., 2023; Martín-Vélez et al., 2024), so these spatial variables are non-independent. The fact that sites within Guadalete had the lowest MP concentrations, despite the presence of two landfills in close proximity and more stations with waterbirds (Fig. 1, Table S1) indicates the importance of other factors such as the size of the catchment area from which MPs may be washed or blown into aquatic systems. Other factors not controlled for in this study may also be important. For instance, the presence of vegetation along wetland shorelines can reduce the influx of plastic pollutants entering through runoff (Gallitelli et al., 2024; Gallitelli and Scalici, 2024).

Since macro and meso plastics can be biovectored to wetlands by birds (López de la Nieta et al., 2022; López-Calderón et al., 2023; Martín-Vélez et al., 2020; Martín-Vélez et al., 2024), this suggests that 1) small and light MPs are also transported by these same waterbirds, or 2) that these meso and macroplastics once in wetlands are broken down into small MPs that ultimately end up in aquatic insects. MP ingestion by other waterbirds elsewhere has been already documented (Coughlan et al., 2021; Flemming et al., 2022), as has their ability to carry MPs on their feathers (Reynolds and Ryan, 2018). In particular, the ingestion of MPs could pose a risk to these same waterbirds, as well as to other biota inhabiting coastal and inland wetlands (Coughlan et al., 2021). Therefore, MP ingestion by biota and MP occurrence in wetlands may be an important new threat to their conservation (Weitzel et al., 2021).

4.4. Association between MP abundance and stable isotopes in *Trichocorixa*

The fact that we found a negative association between MPs and *Trichocorixa* ^{15}N isotopes supports our Hypothesis 5. This result suggests that *Trichocorixa* might ingest more MPs when feeding on resources with lower N content, such as detritus and algae, perhaps because these tend to accumulate more MPs.

Additionally, the ingestion of MPs can lead to a ^{15}N depletion perhaps due to the low quality of food ingested, or a reduction in consumption rate due to changes in predatory efficiency behaviours (Haubert et al., 2005; de Sá et al., 2015; Carrasco et al., 2019). This in turn would affect macroinvertebrate nutritional status with effects that may cascade along the food chain (i.e. lower quality food for their predators). However, it is also possible that the observed $\delta^{15}N$ depletion was due to the consumption of similar resources that differed in isotopic signatures between sites. While we controlled for site variability using a mixed model, which accounts for differences between sampling stations and the presence of avian biovectors, we acknowledge that not having an isotopic baseline for correction within each site may still leave unaccounted variability. On the other hand, we cannot rule out the possibility that our results were due to a confounding variable, e.g. if the variation in MP abundance between sites was also related to the relative importance of different anthropogenic nutrient sources with distinct ^{15}N signatures (Paredes et al., 2020).

Given the key role of aquatic insects in wetland ecosystems, further studies should address this topic in detail to fully understand these relationships between MPs and stable isotopes.

4.5. Future perspectives and conclusions: on the protection of wetlands from microplastic contamination

Research on MPs contaminating biota in wetlands is an emerging field and few scientific studies are available as yet, preventing a clear picture. Nevertheless, MPs are clearly widespread in wetlands. Wetlands are now considered both potential sources and sinks of plastics (Qian et al., 2021; Battisti et al., 2024). Although plastics can accumulate in these environments, factors such as wind-storms, rains, and floods might remobilize and transport plastics (as may the emergence of flying insects such as chironomids or corixids carrying MPs). This implies that wetlands could play a key role in the dispersal of MPs to other ecosystems, both aquatic and terrestrial. Thus, effective plastic management should prevent the arrival of plastic contamination in these habitats from landfills, including reductions in the problems of biovectoring (Martín-Vélez et al., 2024).

Future research activities should address the dynamics of MP transport in water and sediment matrices, and the effects on wetland ecosystems. In addition, it will be useful to integrate data from a range of scales from the coarse resolutions (basin) to local factors (e.g. vegetation density, and spatial variation within a wetland).

We have shown that stable isotopes can be a valuable tool to detect possible consequences of MP ingestion in aquatic biota. Thus, we suggest their inclusion in MP monitoring, alongside aquatic insects, as complementary methods to further assess the effects of MPs in wetland environments.

CRediT authorship contribution statement

L. Gallitelli: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **A. Cera:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **M. Scalici:** Writing – review & editing, Visualization, Validation, Resources. **A. Sodo:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis. **M. Di Gioacchino:** Writing – review & editing, Visualization, Validation, Formal analysis. **B. Luzi:** Writing – review & editing, Visualization, Validation, Investigation. **F. Hortas:** Writing – review & editing, Visualization, Validation, Formal analysis, Conceptualization. **A.J. Green:** Writing – review & editing, Visualization, Validation, Resources, Investigation, Funding acquisition, Conceptualization. **C. Coccia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are present in the manuscript and Supplementary Materials.

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Appendix A. Supplementary data

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