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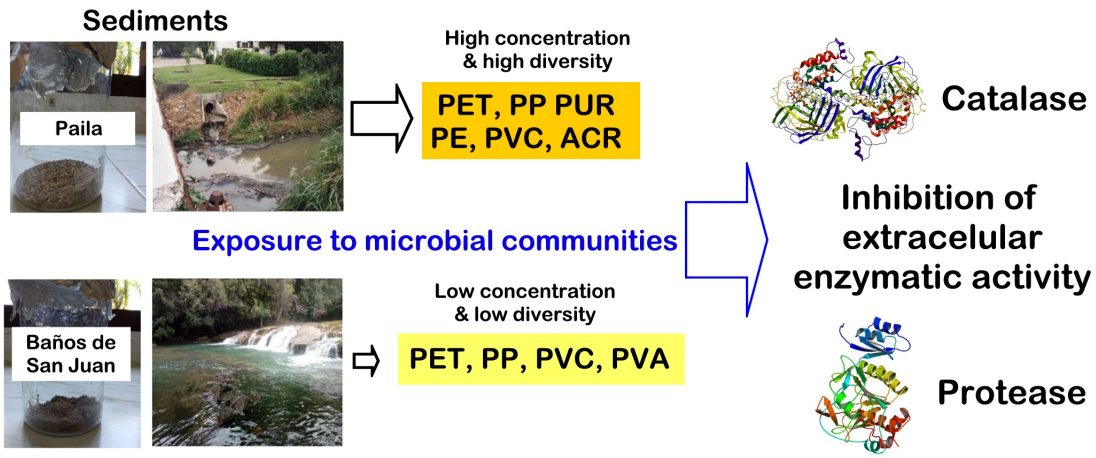

Microplastics in Cuban freshwaters: diversity, temporal changes, and effects on extracellular enzymatic activity

Jeny Adina Larrea-Murrell[1,*], Virginia Gálvez-Blanca[2], Alice L. Petre[2], Beatriz Romeu-Álvarez[1], Daysi Lugo-Moya[1], Marcia María Rojas-Badía[1], Jose Antonio Perdigón-Melón[2], Karina Boltes[2], Roberto Rosal[2]

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Abstract

Plastics, as synthetic polymers, are emerging contaminants that can harm organisms and ecosystems. This study investigates the presence of microplastics in sediments of two rivers in western Cuba, assessing their temporal variability, diversity, and characterizing the types of microplastics in these ecosystems. Additionally, the study examines the relationship between microplastic concentrations, the extracellular enzymatic activity of benthic microbial communities, and nutrient levels in sediments. Sediments from two stations, the Paila (urban river) and Baños del San Juan (rural river), were analyzed using micro-FTIR for chemical identification, and nutrients and extracellular enzyme activities were determined by colorimetric methods. The results showed higher microplastic concentrations at the Paila station compared to the Baños del San Juan station. The identified microplastics included polyethylene terephthalate (41.9 %), polypropylene (25.8 %), acrylic (6.5 %), polyvinyl chloride (6.5 %), polyethylene (3.2 %), polyurethane (3.2 %), and polyvinyl alcohol (3.2 %), with polyethylene terephthalate being the most abundant in both sampling stations. The highest microplastic diversity was observed at the Paila station in April, with June showing the highest concentrations of microparticles. Redundancy analysis showed that nitrite, polypropylene, ammonium, and precipitation were the variables influencing extracellular enzyme activities at both sampling stations. Higher levels of polypropylene were associated with increased levels of nitrite and ammonium. Additionally, it is suggested that polypropylene inhibits proteolytic and catalase activity in the sediments of the studied stations. This investigation is the first report in Cuba of the presence of microplastics in freshwater ecosystems and one of the few studies in the Latin American and Caribbean region.

1. Introduction

Plastic contamination is a global issue, posing a threat to the health of organisms and ecosystems (Stubbins et al., 2021). This material is extensively utilized in various sectors such as cosmetics, packaging, electronics, agriculture, and automotive manufacturing, due to its durability, lightweight nature, corrosion resistance, versatility, and cost-effectiveness (Yang et al., 2021). Global plastic production reached 400.3 million metric tons in 2022, an increase of 9.6 million tons from 2021 (Plastics Europe, 2022, 2023). Plastics can be categorized according to their size into macroplastics (> 25 mm), mesoplastics (5-25 mm) and microplastics (1 µm- 5 mm) (GESAMP, 2019). The GESAMP's definition of microplastics includes flakes and fibers, whose longest dimension is < 5 mm.

Microplastics (MPs) can be found in different environments, including the sea, surface waters, soil and sediments, plants and animals (Karim et al., 2024). They can be created in a size of 1 µm- 5 mm for use in cosmetics or decorative products (primary MPs) or they can originate from the fragmentation of larger plastic materials (secondary MPs) (Nkosi et al., 2023). In addition, MPs exhibit different shapes, colors and sizes (Kumar et al., 2023; Negrete Velasco et al., 2020). Rivers receive plastic pollutants from human activities conducted along their basins, including industrial processes, agriculture, and domestic activities like washing (Gonzalez-Saldias et al., 2024; Soltani et al., 2022). Once delivered, rivers contribute to the translocation of microplastics to other environments, such as soils and seas (Gonzalez-Saldias et al., 2024). Sediments act as a long-term sink for MPs, as plastics with a density greater than 1 g cm-3 settle and accumulate in sediments (Rodrigues et al., 2018), while those with lower density float on the surface or remain suspended in the water column (Yang et al., 2021). Polyethylene (PE), polypropylene

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(PP) and polystyrene (PS) are frequently detected in aquatic sediments (Fan et al., 2019; Yuan et al., 2021) and can induce changes on bacterial communities, affect enzymatic activity, nutrient cycles and sediment chemistry (He et al., 2020; Guo et al., 2024; Song et al., 2024). Various studies suggest that biofilm formation and adsorption and accumulation of pollutants onto microplastics, contribute to the increase in plastic debris density, which is the primary reason for the presence of light microplastics in sediments (Xu et al., 2020; Yang et al., 2021). Assessing the adverse effects of microplastics on freshwater ecosystems, especially on sediments, is challenging due to their diverse physical characteristics and chemical compounds that influence their fate, transport, and bioaccumulation in ecosystems (Miller et al., 2021; Gonzalez-Saldias et al., 2024).

Globally, freshwater sediments have received less attention compared to marine sediments (Thushari & Senevirathna, 2020; Nkosi et al., 2023), with freshwater sediments in the Global South being particularly understudied (Nkosi et al., 2023). The study of MPs in freshwater systems, especially rivers, is crucial due to the environmental risks posed by these pollutants (Gonzalez-Saldias et al., 2024). In the Caribbean region, there is limited research on the quantification of microplastics (Bosker et al., 2018; Garcés-Ordóñez et al., 2020). Studies in Latin America and the Caribbean account for only 4.8 % of global scientific production (Orona-Návar et al., 2022). Microplastics research in Cuba is still in its early stages, with only two published studies conducted in the province of Cienfuegos. One study focused on sediments in Cienfuegos Bay (García-Chamero et al., 2020), while the other examined atmospheric depositions in the province (Pescoso-Torres et al., 2023). However, there is a lack of data on the extent of plastic pollution in Cuban ecosystems, and no studies have been conducted on freshwater ecosystems. Previous research on the Almendares River in Havana and the San Juan River in Artemisa has primarily focused on microbiological and chemical analyses, including the detection of fecal contamination indicators, analysis of bacterial communities, and assessment of nutrients and heavy metals (Larrea-Murrell et al., 2020; Larrea-Murrell et al., 2021; Salgado Bernal et al., 2024). Recent studies have also identified pharmaceutical contaminants of emerging concern in these rivers (Larrea-Murrell et al., 2024). However, the presence of microplastics and their potential impact on extracellular enzymatic activities as indicators of microbial community activity have not been investigated in these ecosystems.

This study aims, for the first time, to determine the presence of microplastics in the sediments of two rivers in western Cuba, assess their temporal variability and diversity, and characterize the types of microplastics in these ecosystems. Additionally, it aims to investigate the relationship between MPs concentrations, the extracellular enzymatic activity of benthic microbial communities, and nutrient concentrations in the sediments.

2. Materials and methods

2.1. Study area and sampling

Sediments from two sampling stations were analyzed, one in the Almendares River (Havana, Cuba) and another in the San Juan River (Artemisa, Cuba) during the months of February, April and June 2024. The station analyzed in the Almendares River was the Paila station (23° 03′ 23.94″ N, 82° 24′ 09.75″ E) and in the San Juan River, the Baños del San Juan station (22° 49′ 24.02″ N, 82° 55′ 35.08″ E). These stations have been previously characterized according to their chemical and microbiological quality and the presence of pharmaceutical contaminants of emerging concern (Larrea-Murrell et al., 2022; Larrea-Murrell et al., 2024). The Paila station is located in an urbanized area and receives contaminants from industries (galvanizing, electronics, and pharmaceutical laboratories), domestic waste, and landfills (Larrea-Murrell et al., 2024). Meanwhile, the Baños del San Juan station is located in a rural area, where its main source of contamination is the oxidation pond system that treats domestic waste from the Las Terrazas community in Artemisa and human activities related to tourism (Larrea-Murrell et al., 2021).

The sediment samples were collected at a depth of 5 cm with a metal spoon previously disinfected with methanol and rinsed with Milli-Q water and then placed in glass jars. Three replicates were collected at a distance of approximately 1 meter from each other and then pooled into a composite sample weighing approximately 40 g. The samples were transported to the laboratory in a cooler at 4ºC, and subsequently frozen until analyzed for the detection of microplastics. The sediments from the Paila station were characterized as muddy, while those from Baños del San Juan were clayey (Figure S1, Supplementary Materials, SM).

2.2. Determination of physicochemical indicators and extracellular enzymatic activity of microbial communities in sediments

During sampling, the temperature and pH of the sediments were determined in situ using a Hanna

HI98129 multimeter. Once the samples were transferred to the laboratory, the concentration of nutrients (PO_4^{3-}-P, NH_4^+-N, NO_2^--N, NO_3^--N) was determined using the methodologies described in the manual of procedures for the handling and chemical analysis of sediment and water samples (Plumb, 1981). In brief, one gram of wet sediment was weighed and transferred to a 250 mL flask. 100 mL of bidistilled water was added, shaken vigorously, and allowed to settle overnight. The contents were then transferred to a centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant was decanted into a 250 mL flask. 50 mL of bidistilled water was added to the pellet, mixed, and centrifuged again. The supernatant was combined with the previous extract and diluted to volume with bidistilled water. The solution was filtered using GF/F filters, and the concentration of PO_4^{3-}-P (molybdenum blue method), NH_4^+-N (Nessler method), NO_2^--N (spectrophotometric method) and NO_3^--N (nitration of salicylic acid) were analyzed. The nutrient concentration (NC) in the sediment was calculated using the following formula:

$$NC(mg/kg[wet\,weight]) = \frac{(x)(y)(100)}{g} \quad (1)$$

where:
x = concentration of nutrient in the extract, mg L^{-1}
y = volume of the extract, in L (0.25 L)
g = wet weight of sediment in grams

Nutrient concentration in the extract was determined by calculating from calibration curves using KH_2PO_4 for PO_4^{3-}-, NH_4Cl for NH_4^+-N, $NaNO_2$ for NO_2^--N, and $NaNO_3$ for NO_3^--N.

For the determination of extracellular enzymatic activities such as proteases, acid and alkaline phosphatases, lipases and catalase, the methodology described in Larrea-Murrell et al. (2024) was used. Briefly, for the determination of each enzymatic activity, one gram of sediment was weighed and dissolved in 5 mL of buffer according to the enzyme to be determined in each case. This constituted the sludge mixture. Protease and catalase activities were determined using 0.05 M pH 7 phosphate buffer. Acid phosphatase activity was determined using 0.05 M pH 4.8 citrate buffer. Alkaline phosphatase activity was determined using 0.05 M pH 9 glycine buffer, and lipase activity was determined using 0.05 M pH 8 phosphate buffer. Proteolytic enzymatic activity was determined following the method described by Izquierdo et al. (2020). In this assay, casein was used as a substrate to measure proteolytic activity. The assay involved using a sample and a control to confirm that casein hydrolysis was not due to heating. Specifically, 500 µL of 1 % (w/v) casein and 500 µL of sludge were combined in the sample tube and incubated at 30 °C for 10 min. The reaction was then stopped by adding 0.4 M trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 10 min. In the control tube, 500 µL of casein was incubated at 30 °C for 10 min, followed by the addition of 1 mL of TCA and 500 µL of the sludge mixture. The control tube was also centrifuged at 5000 rpm for 10 min. In both the sample and control tubes, 500 µL of the supernatant was transferred to a test tube along with 2.5 mL of 0.4 N sodium carbonate and 500 µL of Folin's reagent (1:5). The mixture was allowed to react at 30 °C for 30 min, and the absorbance was measured at 660 nm. The absorbance values were compared to a tyrosine standard curve, and the neutral protease units were expressed as µmol of tyrosine produced per gram of sediment per minute.

Acid phosphatase activity was performed following the method of Larrea-Murrell et al. (2022). Briefly, 1.5 mL of 0.05 M pH 4.8 citrate buffer, 1.5 mL of 1 mM p-nitrophenyl phosphate (final concentration 0.3 mM), and 1.5 mL of sludge mixture were added to a test tube. The reaction was incubated at 30 °C for 10 min. To stop the reaction, 0.5 mL of the mixture was added to 4.5 mL of sodium hydroxide (0.02 M), and the absorbance was measured at 410 nm after centrifugation. Alkaline phosphatase activity was carried out following the protocol used for acid phosphatase but using a 0.05 M pH 9 glycine buffer. In all cases, prior to reading, the samples were centrifuged at 5000 rpm for 10 min to prevent sediment interference in the reading. The absorbance values were compared to a p-nitrophenol standard curve, and the acid and alkaline phosphatases units were expressed as µmol of p-nitrophenol produced per gram of sediment per minute.

Total lipase determination was performed according to the methodology proposed by Kumar et al. (2012) with modifications using p-nitrophenyl palmitate as a substrate. Briefly, 1.5 mL of 0.05 M pH 8 phosphate buffer, 1.5 mL of 1 mM p-nitrophenyl palmitate (final concentration 0.3 mM), and 1.5 mL of sludge mixture were added to a test tube. The reaction was incubated at 30 °C for 10 min. To stop the reaction, 0.5 mL of the mixture was added to 4.5 mL of absolute ethanol, and the absorbance was measured at 410 nm after centrifugation. The absorbance values were compared to a p-nitrophenol standard curve. Lipase activity units were expressed as µmol of p-nitrophenol produced per gram of sediment per minute. Total catalase activity was determined using the Sinha method (1972). In a test tube, 4 mL of 0.2 M hydrogen peroxide (final concentration 0.08 M), 5 mL of 0.05 M pH 7 phosphate buffer, and 1 mL of sludge mixture were added. The reaction was

incubated at 30 °C for 10 min. Subsequently, 1 mL of the reaction mixture was added to a tube with 2 mL of dichromate/acetic acid. The addition of the reagent instantly produced a color change to unstable blue chromic acid. After heating for 10 min in a boiling water bath, the solution color changed to stable green. After cooling to room temperature, it was centrifuged, and the absorbance was read at 570 nm. The absorbance values were compared to a hydrogen peroxide standard curve. Catalase units are expressed as hydrogen peroxide consumed per minute per gram of sediment. All assays were performed in triplicate, and a negative control was used to assess the absence of color development. For the negative control, sterile bi-distilled water was used along with buffer and substrate for each enzymatic determination, except for the catalase enzyme determination where sterile bi-distilled water and buffer were used. In this study, high substrate concentrations were utilized to ensure the measurement of the highest possible extracellular enzymatic activity. This approach was necessary because in natural conditions, the low concentration of extracellular enzymes poses challenges for their accurate determination. By using high substrate concentrations, enzymatic activity is not limited by substrate availability, and the reaction rate is solely dependent on enzyme concentration. This rate represents the maximum achievable rate for that enzyme concentration at the specific temperature and pH (Wallenstein and Weintraub, 2008). Therefore, the enzymatic activities determined in this study are potential values and do not reflect the actual proportion of enzyme-catalyzed reactions under natural conditions (Li et al., 2019).

2.3. Processing of sediment samples and recovery of microplastics

The sediment samples were vacuum-dried at 65 °C for 48 h and were then divided into three subsamples of 5 g each for microplastic analysis. The sediment subsamples were suspended in 100 mL of zinc chloride ($ZnCl_2$) solution with a concentration of 700 g L^{-1} (density 1.70 ± 0.05 g cm^{-3}) and magnetically stirred for 10 min. This high concentration enables the separation of MPs in the samples based on their density, provided that they have a density lower than 1.70 g cm^{-3} (Rodrigues et al., 2020). The samples were allowed to settle overnight, and the supernatant was subsequently filtered using 25 µm stainless steel filters and 1 µm glass fiber filters (Edo et al., 2022). The 1 µm filters were placed in a muffled Petri dish and dried in the incubator at 60 °C. The 25 µm filters were treated with 20 mL of 30 % hydrogen peroxide at 60 °C overnight to remove any remaining organic matter. After digestion, the supernatant was filtered through new 25 µm and 1 µm filters. The processed filters were washed with ultrapure water, dried at 60 °C, and placed on a clean Petri dish for subsequent visualization and analysis. Petri dishes were cleaned by heating to 400 °C to remove any rests of fibres and plastic materials.

2.4. Microplastics analysis, classification and calculation of oxidation

All particles suspected of being plastics visualized under a stereoscope were collected with needles and placed in a previously muffled Petri dish. The particles were photographed and identified using the Leica Ivesta 3 stereomicroscope equipped with Image Focus software. Potential plastics were classified according to their typology into: fibers, fragments, films and filaments according to Edo et al. (2022). Quantitative results were expressed as the number of microplastics per kg of sediment (dry weight). The chemical identification of potential microplastics and other artificial contaminants was performed using Micro-Fourier transformed infrared spectroscopy (micro-FTIR). Particles < 1 mm were placed on KBr discs and analyzed using a Nicolet iN5 FTIR microscope coupled to a Nicolet iS20 FTIR spectrometer (Thermo Fisher Scientific). The micro-FTIR equipment operated in transmission mode in the range of 550–4000 cm^{-1} with a spectral resolution 8 cm^{-1}. The spectral resolution was selected after checking that it provided a balance between polymer identification with the desired matching and signal-to-noise ratio. This procedure allowed the acquisition of high-quality spectra for most particles. The spectra were compared against a custom polymer library that integrated multiple databases, including those from Omnic 9 software by Thermo Scientific, as well as contributions from our research group and collaborating research partners. Pearson correlation was used with a minimum of 60 % coincidence for positive identification according to Edo et al. (2020). The amount of microplastics per kilogram of dry weight was calculated by multiplying the particle count with the percentage of microparticles identified as microplastics by micro-FTIR. To assess the surface oxidation level of MPs, the carbonyl index (CI) was determined. The CI is a widely used indicator for measuring the extent of surface oxidation in plastics (Rodrigues et al., 2018; Bayo et al., 2022). The formula used for CI calculation is as follows:

$$Carbonyl\ index\ (CI) = \frac{A(1)}{A(2)} \quad (2)$$

Where A(1) represents the absorbance of the carbonyl group at 1715-1735 cm^{-1} (Rodrígues et al.,

2018) and A(2) represents the absorbance of the reference peaks of each polymer detected.

2.5. Prevention of contamination and QA/QC assessment

Throughout the procedure, precautions were implemented to prevent cross-contamination. Only glassware was used in the laboratory and all plastic materials were avoided throughout all processing steps. Additionally, all materials were washed with ultrapure water and heated at 400 °C for 4 h prior to use. The laboratory coats worn by the personnel were made of brightly colored cotton.

Procedural controls were deployed during sampling and laboratory procedures that consisted of beakers exposed to the same experimental conditions as the samples and Petri dishes containing clean 25 μm opening size stainless steel filters. Transparent cellulose fibers were detected in only two of the controls (4 transparent fibers in one control and 3 transparent fibers in the other). Consequently, transparent cellulose fibres were not taken into account in the corresponding samples.

2.6. Prevention of contamination and QA/QC assessment

The normality and homogeneity of variance of the data were assessed using the Kolmogorov-Smirnov and Cochran-Bartlett tests, respectively. The comparison between the total amounts of microparticles (refers to potential plastics including MPs and artificial cellulosic particles (ACPs) that represent emerging contaminants consisting of primarily cellulose fibers with evidence of industrial processing), microplastics, artificial cellulosic particles, and unidentified particles obtained in the samples from the Paila and Baños del San Juan stations was conducted using the t-Student test of independent samples with a significance level of $p < 0.05$. Meanwhile, the comparison of the concentration of microplastics and the Shannon diversity index in each sample collected in the months of February, April, and June at the Paila and Baños del San Juan stations was performed using the Tukey HSD post hoc test ($p < 0.05$). For all these tests, the statistical software Statistica 8.0 (StatSoft, 2007) was utilized. The calculation of the Shannon diversity index of the microplastics found in the samples in the different sampling months was conducted using the statistical software Past 4.0 (Hammer et al., 2001).

To investigate the relationship between MPs concentration, physicochemical variables, and extracellular enzyme activities from February to June 2024, redundancy analysis (RDA) was conducted using the CANOCO statistical package, version 4.5 for Windows (Ter Braak and Šmilauer, 2002). The choice of redundancy analysis was based on the linear response of extracellular enzyme activities to gradients of physicochemical variables and MPs concentration as determined by detrended correspondence analysis (DCA). Physicochemical indicators and MPs that were statistically significant ($p < 0.05$) were selected a priori using the Monte Carlo permutation test (999 unrestricted permutations) under a reduced model. Correlated variables were eliminated before conducting the redundancy analysis. Triplot graphs representing the relationships between sampling stations, physicochemical variables, MPs, and extracellular enzymatic activities were generated using the CANODRAW program within the CANOCO 4.5 statistical package.

3. Results

3.1. Characterization of MPs from sediments of Paila and Baños del San Juan stations

A total of 465 microparticles (219 and 246 microparticles in Paila and Baños del San Juan stations respectively) were collected from the three samples taken in February, April, and June 2024. Among these, transparent fibers were the most abundant, accounting for 76.3 % and 62.6 % of the microparticles at the Paila and Baños del San Juan stations, respectively. A random subsample of 93 microparticles was selected for micro-FTIR characterization, with 40 from the Paila station and 53 from Baños del San Juan. The subsample size was selected to reach an accuracy < 10 % as indicated elsewhere (Anger et al., 2018; Kedzierski et al., 2019). Of these, 29 were identified as microplastics, 40 as artificial cellulosic particles (37 cellulose and 3 cellophane), and 24 could not be unambiguously identified. The Paila station had the highest number of MPs, while the Baños del San Juan station had the highest number of ACPs (Figure 1).

The concentration of MPs and ACPs in samples collected during the study period, along with their typology and color, are depicted in Figure 2. Transparent, blue, and red fibers were the most prevalent MPs, while transparent fibers dominated among ACPs. The sample from the Paila station in June (P-Jun) and the sample from the Baños del San Juan station (S-Jun) collected in the same period exhibited the highest number of MPs and ACPs compared to other months analyzed. Sample S-Feb had the lowest MPs values, and sample P-Apr showed the lowest concentration of ACPs. Among the 29 MPs, polyethylene terephthalate (PET) (48.3 %), polypropy-

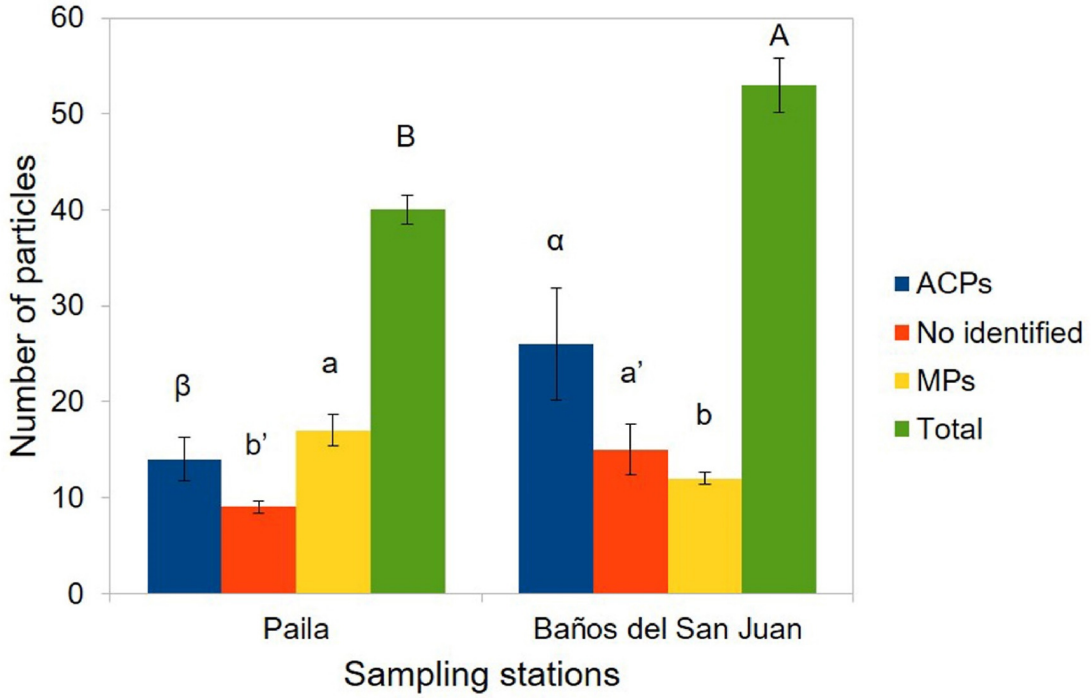

Figure 1: *Number of total particles, microplastics (MPs), artificial cellulosic particles (ACPs) and unidentified particles at the Paila (Almendares River) and Baños del San Juan (San Juan River) sampling stations in the period February-June 2024. Different letters indicate significant differences between the number of MPs (a and b), ACPs (α and β), No identified (a' and b') and Total microparticles (A and B) found at the Paila station with respect to the Baños del San Juan station, according to ANOVA-Tukey HSD ($p < 0.05$). Error bars correspond to standard deviation.*

lene (PP) (27.6 %), acrylic (ACR) (6.9 %), polyvinyl chloride (PVC) (6.9 %), polyethylene (PE) (3.4 %), polyurethane (PUR) (3.4 %), and polyvinyl alcohol (PVA) (3.4 %) were identified, with PET being the most prevalent (Figure 3 and Figure S2 in SM).

Micro-FTIR spectra were utilized to assess the extent of surface oxidation of PP, PE, PVC, PVA, PUR, and ACR by measuring the carbonyl index (CI). The CI calculation was performed for six out of the seven microplastics identified in samples from the Paila and Baños del San Juan stations, namely PP, PE, PVC, PVA, PUR, and ACR. PET microplastic was excluded from the analysis due to a consistent peak at 1714 cm^{-1} attributed to the C=O double bond stretching (Miranda et al., 2021). The CI values for the mentioned MPs were 1.09, 0.84, 0.72, 0.80, 1.01, and 1.63, respectively, indicating oxidation of these plastics in sediments. The Shannon diversity index shows that the highest diversity of microplastics was detected at the Paila station in the April sample, followed by the Baños del San Juan sample from the same month. In both instances, there was a decrease in the relative abundance of PET and an increase in the relative abundance of PP and other polymers in April. The lowest diversity of microplastics was observed in the samples collected in February and June at the Baños del San Juan station (Figure 4).

3.2. Relationship between microplastics, nutrient concentration and extracellular enzymatic activities

To analyze the relationship between extracellular enzymatic activities, nutrient concentrations, and the concentration of different microplastics detected at the Paila and Baños del San Juan stations, redundancy analysis was conducted (Figure 5). The concentrations of nitrite, ammonium, polypropylene, and precipitation were identified as the key factors influencing the variability of extracellular enzymatic activities at the Paila and Baños del San Juan stations during the study period ($p < 0.05$). The Monte Carlo test for the first canonical axis was not significant ($p = 0.2320$; $p > 0.05$), but it was significant for all canonical axes ($p = 0.008$; $p < 0.01$), indicating that the selected variables (nitrite, ammonium, polypropylene concentrations, and precipitation) can explain the variability of extracellular enzymatic activities at the stations studied.

Based on the redundancy analysis, the species-environmental variables relationship explained 84.0 % of the accumulated variance, with the first and second canonical axes accounting for this variance (Figure 5, Table S2 (SM)). The species-environment correlations for the first and second axes were 0.991 and 0.994, respectively. Extracellular enzymatic ac-

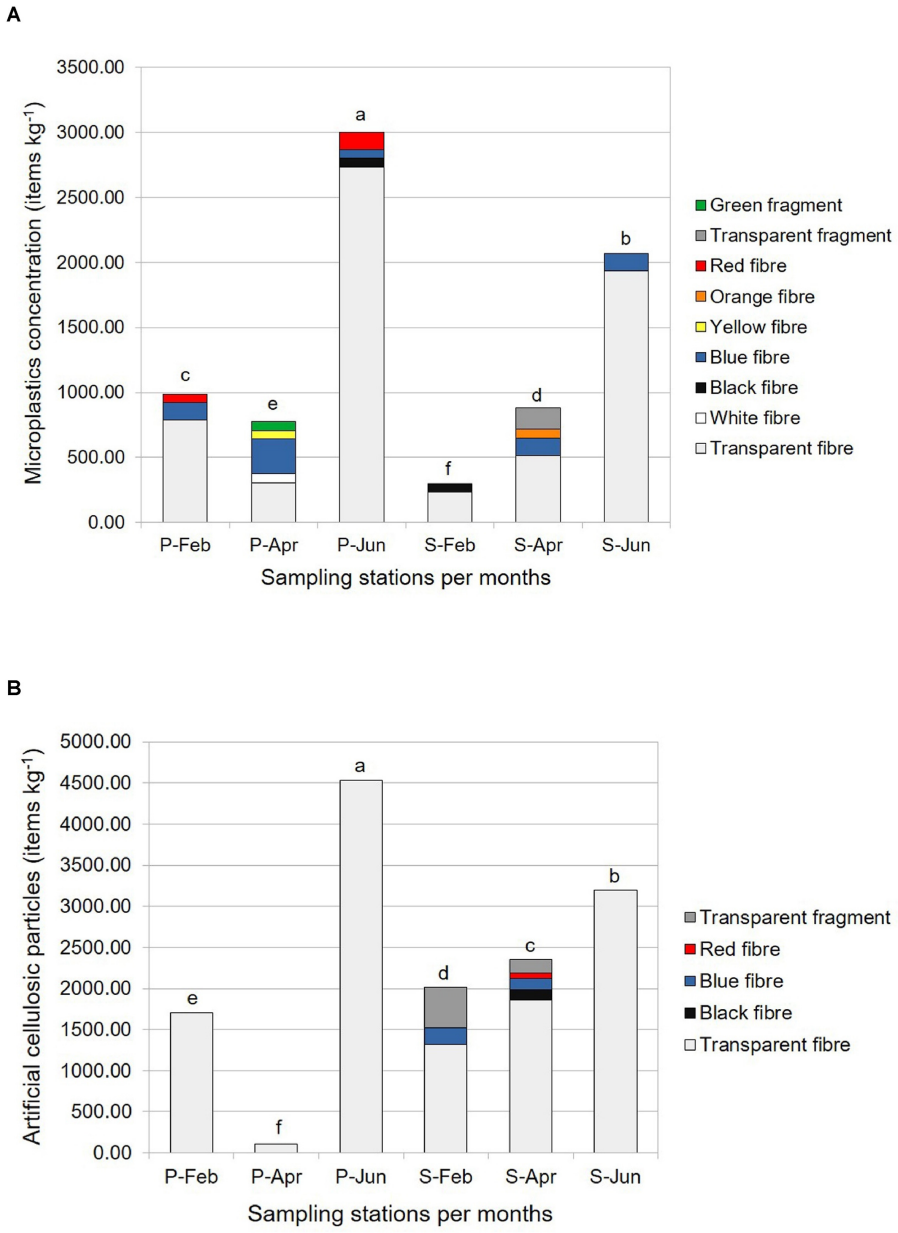

Figure 2: *Concentration of microplastics (A) and artificial cellulosic particles (B) per kg of dry weight of sediment, typology and color of the microparticles characterized in the samples from the Paila (P) (Almendares River) and Baños del San Juan (S) (San Juan River) stations in the months of February (Feb), April (Apr) and June (Jun) 2024. Different letters indicate significant differences between the total concentrations of microplastics (A) and artificial cellulosic particles (B) obtained in each sample according to ANOVA-Tukey HSD ($p < 0.05$).*

tivities were categorized into five groups based on the characteristics of each sampling station in February, April, and June 2024. The first group formed at the Paila station in February showed high activity of acid and alkaline phosphatases and lipases, low activity of proteases and catalases, high concentrations of polypropylene, ammonium, and nitrites, and relatively abundant precipitations. The second group at the Paila station in April and June exhibited low enzymatic activity, high concentrations of nitrites, polypropylene, and ammonium, and abundant precipitations. The third group at the Baños del San Juan station in February had high activity of proteases and catalases, low activity of lipases, acid and alkaline phosphatases, low concentrations of nitrites, polypropylene, and ammonium, and abundant precipitations. The fourth group at the Baños del San Juan station in June showed high activity of proteases, catalases, acid and alkaline phosphatases, and low activity of lipases, with low concentrations of polypropylene, nitrites, and ammonium, and scarce precipitations. The fifth group had low activity of proteases and catalases, high activity of lipases, acid and alkaline phosphatases, high concentrations of

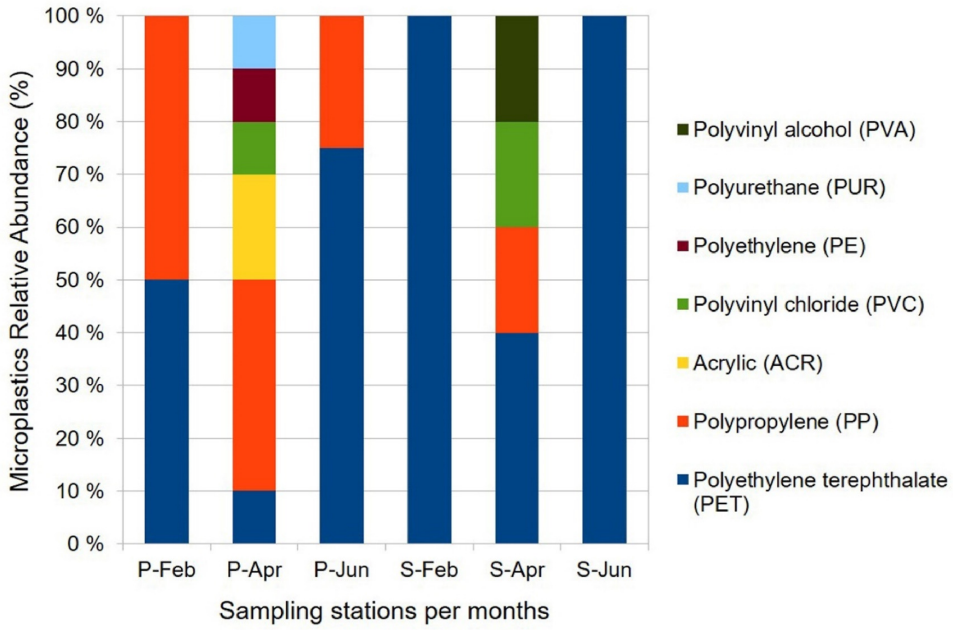

Figure 3: *Relative abundance of microplastics identified at the Paila (P) (Almendares River) and Baños del San Juan (S) (San Juan River) stations in the months of February (Feb), April (Apr) and June (Jun) 2024.*

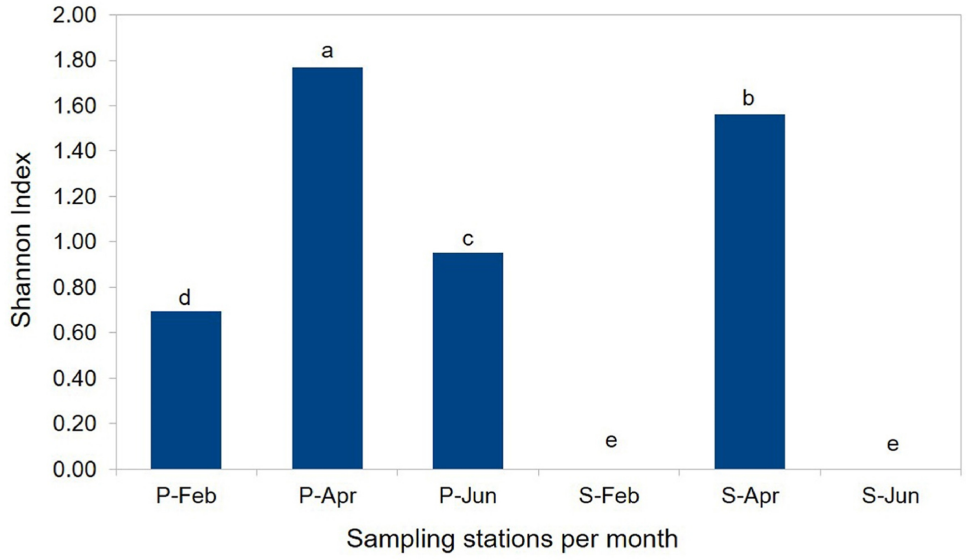

Figure 4: *Shannon diversity index of microplastics at the Paila (P) (Almendares River) and Baños del San Juan (S) (San Juan River) sampling stations in February (Feb), April (Apr), and June (Jun) 2024. Different letters indicate significant differences between Shannon index in each sample according to ANOVA-Tukey HSD ($p < 0.05$).*

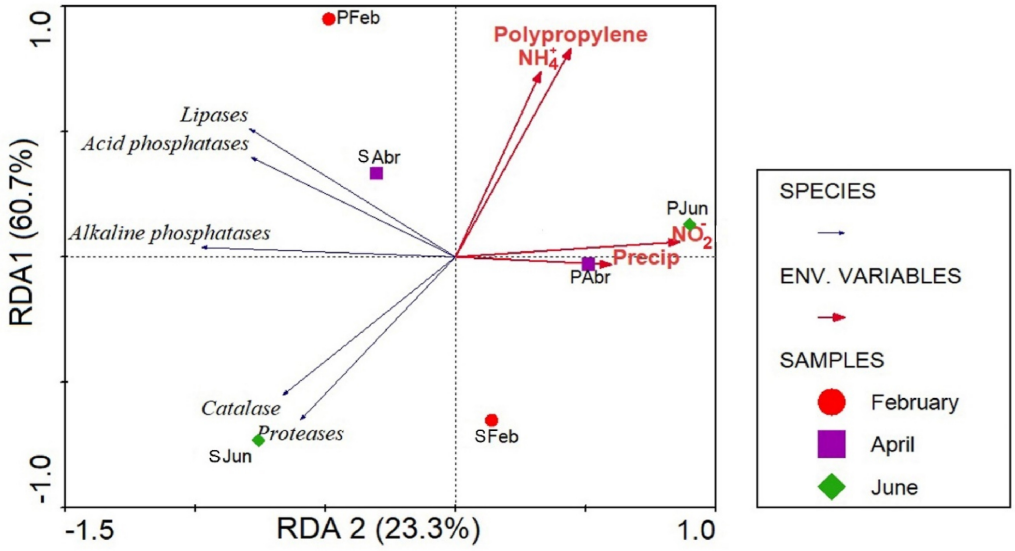

Figure 5: *Triplot of the redundancy analysis (RDA) of the extracellular enzymatic activity in sediments at the sampling stations Paila (Almendares River) and Baños del San Juan (San Juan River) in the period February-June 2024 using physicochemical indicators. NH_4^+: ammonium; NO_2^-: nitrite; Polypropylene (PP); Precip: Precipitations. Sampling stations: P: Paila station; S: Baños del San Juan station.*

polypropylene, low concentrations of nitrites, high concentrations of ammonium, and scarce precipitations.

4. Discussion

4.1. Abundance, temporal changes and diversity of MPs in Paila and Baños del San Juan stations

The study of microplastic presence in rivers is crucial as these environments transport microplastics to seas and oceans (Yang et al., 2021). Sediments tend to accumulate the highest concentrations of microplastics due to their density compared to water, making them a focal point of global research (Edo et al., 2020; Negrete Velasco et al., 2020; Soltani et al., 2022; Álvarez-Troncoso et al., 2024). However, freshwater sediments have been less studied compared to marine sediments (Yang et al., 2021; Orona-Návar et al., 2022).

In this study, microplastics and artificial particles were found in the sediments of the Paila (Almendares River, urban river) and Baños del San Juan (San Juan River, rural river) stations, indicating the presence of these pollutants in environments with varying characteristics, consistent with global reports (Edo et al., 2020; Negrete Velasco et al., 2020; Mutshekwa et al., 2023; Nkosi et al., 2023; Gonzalez-Saldias et al., 2024). The Paila station had a higher concentration of microplastics compared to the Baños del San Juan station, likely due to diverse pollution sources such as pharmaceutical laboratories, the galvanizing industry, cement production, untreated domestic wastewater, and landfill waste (Larrea-Murrell et al., 2024; Salgado Bernal et al., 2024). Similar findings were reported by Mutshekwa et al. (2023) in South Africa's recreational reserves with varying anthropogenic influences. Sediment characteristics also play a role in microplastic abundance, with muddy sediments like those in Paila having higher microplastic levels compared to sandy sediments (Van Daele et al., 2024) as occur in Baños del San Juan.

The Baños del San Juan station showed a higher presence of artificial cellulosic particles, likely influenced by recreational bathing in natural pools at this location (Larrea-Murrell et al., 2021), potentially introducing these particles from bathers' clothing. Runoff from oxidation lagoons upstream of the Baños del San Juan station, treating wastewater from the Las Terrazas Community could also contribute to the presence of artificial cellulosic particles (Larrea-Murrell et al., 2021). These particles, which are industrially processed materials, may contain various additives like colorants, softeners, flame retardants, biocides, and antistatic agents, which can be released into the environment from these particles (Darbra et al., 2011; da Costa et al., 2023).

Most of the microplastics detected at both sampling stations were fiber-type, indicating their secondary origin from the fragmentation of larger plastic materials textiles. Fibers have been frequently found in various aquatic environments worldwide (Yang et al., 2021; Orona-Návar et al., 2022). For instance, Alam et al. (2019) reported that 91 % of the

quantified microplastics in the Ciwalengke River in Indonesia were fibers. Similarly, Martínez-Silva and Nanny (2020) found that fibers accounted for 85 % of the total microplastics in the Magdalena River in Colombia. It has been suggested that fibers are released into the environment through washing water disposed of via domestic wastewater (Peng et al., 2017; Yang et al., 2021). In terms of color, transparent fibers were the most abundant in the samples from both stations, among both microplastics and artificial cellulosic particles (Fig. 2). Transparent microparticles typically originate from plastic waste which have a short lifespan (Yang et al., 2021). In addition, these fibers can reach aquatic ecosystems from the air environment (Stanton et al., 2019).

Samples collected in June showed the highest abundance of microparticles, especially at the Paila station where there was significant rainfall (205 mm) ten days prior to collection (Tables S1 and SM). This rainfall likely contributed to the transport of microparticles from terrestrial to aquatic environments due to soil drag and drainage (Gonzalez-Saldias et al., 2024). While it is generally believed that sediment resuspension decreases microplastic abundance during the rainy season (Tang et al., 2020), this phenomenon is less common in muddy sediments, leading to higher retention of microplastics and increased abundance (Xia et al., 2021; Gonzalez-Saldias et al., 2024). During rainy events, untreated wastewater from sewers intensifies (Niu et al., 2024), potentially contributing to the accumulation of microplastics and other pollutants at downstream stations like Paila. Suburban sewers have been shown to be a significant source of microplastic pollution, with an average concentration of > 500 items L^{-1} in the Beijing River in China (Niu et al., 2024). Additionally, urbanized areas near rivers can experience increased microplastic concentrations in rainwater (Niu et al., 2024), as observed at the Paila station. At the Baños del San Juan station, low rainfall in June (6.8 mm) contributes to the accumulation of microplastics in sandy sediments, exacerbated by continuous wastewater discharge from oxidation lagoons treating domestic waste from the Las Terrazas Community (Larrea-Murrell et al., 2021). Furthermore, increased public activity at Baños del San Juan during the summer months may introduce textile microparticles from bathers' clothing into the water, potentially contributing to higher microplastic concentrations (Larrea-Murrell et al., 2021). Browne et al. (2011) estimated that washed clothing releases around 1900 fibers per piece, while De Falco et al. (2020) suggested that a single person could release over 900 million polyester fibers into the air through clothing use, which could eventually deposit in river waters and contribute to microparticle sedimentation.

The contrasting results between Paila and Baños del San Juan stations in June and February suggest that sediment characteristics could play a role in microplastic concentrations fate. The sandy sediment at Baños del San Juan may favor resuspension of microparticles, leading to lower sediment concentrations despite higher rainfall (87.7 mm) in February (Tables S1 and SM). In April, Paila station exhibited the lowest concentration of artificial cellulosic particles, coinciding with lower precipitation values (22.2 mm) (Tables S1 and SM), highlighting the significant contribution of rainfall to microparticle pollution during the rainy season (Niu et al., 2024). The temporal variability in microplastic and artificial cellulosic particle concentrations may be influenced by river hydrodynamics, pollution sources, and sediment characteristics, as suggested in the literature (Niu et al., 2024; Ololade et al., 2024; Van Daele et al., 2024).

The microplastics identified in this study (PET, PP, ACR, PVC, PE, PUR, and PVA) are consistent with the chemical nature of microplastics found globally (Xia et al., 2021; Queiroz et al., 2022; Nkosi et al., 2023; Niu et al., 2024; Ololade et al., 2024; Van Daele et al., 2024). PET was the most prevalent type at both sampling sites, which aligns with findings in various freshwater environments such as the Great Lakes (Fuschi et al., 2022), Nigerian river sediments (Ololade et al., 2024), Ox-Bow Lake (Yenagoa, Nigeria) (Oni et al., 2020), and Arvand River sediments in Iran (Soltani et al., 2022). PET particles have a high density (1.38-1.41 $g.cm^{-3}$), leading to their accumulation in the lower water column and sediment surface (Fuschi et al., 2022; Soltani et al., 2022). Common sources of PET in rivers include plastic bottles used for household cleaning products, textile industry, cable coatings, plastic beverage containers, and packaging (Soltani et al., 2022; Ololade et al., 2024). The presence of plastic containers, serving as a source of these microplastics, was evident in both ecosystems studied.

The diversity of microplastics in samples from Baños del San Juan and Paila was determined using the Shannon index (H'), revealing that samples with higher diversity (Paila April and Baños del San Juan April) had lower microplastic concentrations, consistent with findings by Niu et al. (2024) in freshwater ecosystems in Beijing. In April, both sampling stations experienced minimal precipitation, resulting in low water flow that facilitated the sedimentation of microplastics, particularly those with lower density like PP (0.900-0.910 g cm^{-3}). Additionally, the continuous discharge of domestic, industrial, and agricultural wastewater introduced various types of microplastics (Huang et al., 2023), predominantly

at the Paila station (Larrea-Murrell et al., 2024; Salgado Bernal et al., 2024), which exhibited greater microplastic diversity compared to the Baños del San Juan station. In the current study, the oxidation level of microplastics was assessed using the carbonyl index. The carbonyl index is a measure of the absorption band of carbonyl species generated during photo- or thermo-oxidation processes in the 1850-1650 cm^{-1} range, calculated by comparing the relative carbonyl peak to a reference peak (Almond et al., 2020). The reference peaks of the polymers detected PP, PE, PVC, PVA, PUR, and ACR were 1460/1471/2910/1462/1654/2856 cm^{-1}, respectively. The obtained carbonyl index (CI) values ranged from 0.72 to 1.63 for various types of microplastics, except for PET, indicating oxidation of these polymers. Determining the CI for PET was challenging due to a common peak at 1714 cm^{-1} from C=O stretching (Miranda et al., 2021). The oxidation of microplastics is influenced by factors such as exposure time, microplastic characteristics, and the presence of chemical additives like flame retardants or UV stabilizers (Rodrigues et al., 2018). Photo-oxidation is a complex process that alters the physicochemical properties of microplastics, leading to fragmentation and release of organic compounds such as polymer molecules and additives (Miranda et al., 2021; Xu et al., 2024). Photo-oxidation can affect the adsorption capacity of contaminants, change their mobility (Kim et al., 2022), increase toxicity to organisms, and impact microorganisms in the surrounding environment, including biofilm formation on microplastics, growth of planktonic microorganisms, and the microbial community in sediments (Chen et al., 2022; Xu et al., 2024).

4.2. Relationship between microplastics, nutrient concentrations and extracellular enzymatic activities

The high concentrations of nitrites, ammonium, and polypropylene, along with the abundant rainfall at the Paila station compared to the Baños del San Juan station, were the primary factors influencing the response of extracellular enzymatic activities in the sediments and, consequently, the microbial communities at these sampling stations. The redundancy analysis (RDA) revealed that it is not a single variable but rather a combination of factors that determine the enzymatic activities' response. The Paila station in the Almendares River exhibits high contamination levels, creating an anoxic ecosystem with low oxygen concentrations in both the water column and sediments, as reported by Larrea-Murrell et al. (2024). In contrast, the Baños del San Juan station represents a well-oxygenated ecosystem with lower contamination levels (Larrea-Murrell et al., 2021). The low dissolved oxygen concentrations, coupled with continuous discharges of domestic and industrial wastewater at the Paila station, result in elevated levels of reduced nitrogen forms (nitrite and ammonium) in both the water column (Larrea-Murrell et al., 2022, 2024) and sediments (Tables S1 and SM). These reduced nitrogen forms positively correlate with the high polypropylene concentrations, particularly at the Paila station compared to the Baños del San Juan station. This relationship suggests a common source of pollutants, likely originating from untreated or poorly treated domestic and industrial wastewater constantly entering the Almendares River (Larrea-Murrell et al., 2024; Salgado Bernal et al., 2024). Additionally, the decomposition of polypropylene can release additives, including nitrogenous compounds like ammonium polyphosphate, a flame retardant (Seidi et al., 2020; Bellayer et al., 2024), potentially contributing to increased ammonium concentrations in the vicinity of microplastics.

In this study, it was observed that high concentrations of ammonium and polypropylene negatively affected proteolytic and catalase activities. Ammonium, which can come from wastewater discharges and polypropylene decomposition, is known to be harmful to aquatic organisms (Liu et al., 2022; Edwards et al., 2024), leading to the inhibition of proteolytic enzymatic activity (Jiang et al., 2019). This inhibition may be due to end-product inhibition, disrupting the synthesis pathway of these enzymes. Furthermore, increased levels of ammonium can impact the microorganisms responsible for enzyme production, reducing their abundance and diversity (Shen et al., 2019; Edwards et al., 2024), ultimately resulting in lower protease concentrations. Wang et al. (2023) studied the effects of aged microplastics (PE, PET, PVC, and PLA) and their leachates on activated sludge, finding significant reductions in protease activity caused by PE, PET, and PVC microplastics, indicating their potential to hinder protein hydrolysis processes. Additionally, Cau et al. (2024) examined the impact of microplastics on marine sediments using a microcosm model and observed a 35 % decrease in aminopeptidase activity compared to control treatments.

All these effects would be reflected in the biogeochemical cycle of nitrogen, which occurs under low oxygen conditions at the Paila station (Figs. S3 and SM). The inhibition of proteolytic activity could impact the mineralization of organic matter, particularly the decomposition of nitrogen compounds (proteins, peptides, etc.), leading to their accumulation in sediments. This accumulation affects water quality by contributing to the depletion of dissolved oxygen, re-

sulting in ecosystem anaerobiosis (Liang et al., 2018). Miao et al. (2019) demonstrated that microplastics can significantly reduce the activity of the enzyme N-leucine aminopeptidase, a key protease in the nitrogen cycle, disrupting the cycle in freshwater environments. Additionally, elevated concentrations of nitrite, ammonium, and polypropylene, along with anoxic conditions, can stimulate the growth of denitrifying bacteria and anaerobic ammonium-oxidizing bacteria (ANAMOX) while causing the death of nitrifying bacteria (aerobic), impacting the nitrification process. Li et al. (2020) found that PP microplastics create an anaerobic environment on their internal surface, promoting the growth and activity of denitrifying bacteria, facilitating the conversion of reactive nitrogen into final gaseous products (N_2O and N_2) (Chen et al., 2024). Moreover, in redox cycles, accessible nitrogen is converted to ammonium, removing reactive nitrogen from the environment, whereas the presence of MPs increases ammonium concentration in the water column, leading to poor nitrification, reduced ammonium flux fluctuations, and increased eutrophication (Cluzard et al., 2015; Salam et al., 2024). These conditions are observed in the water column of the Paila station (Izquierdo et al., 2020; Larrea-Murrell et al., 2022).

On the other hand, the high concentrations of nitrites, ammonium, and polypropylene were also associated with the low catalase activity detected in Paila compared to the Baños del San Juan station. Li et al. (2022) demonstrated that the addition of PVC and PE microplastics led to a decrease in catalase activity. Similarly, Starosyla (2020) and Liu et al. (2022) observed the inhibition of the catalase enzyme in the sediment depths where anthropogenic contamination is most pronounced. Catalase is an enzyme responsible for breaking down hydrogen peroxide produced by aerobic microorganisms (Milgrom, 2016), which are impacted under anoxic conditions. Therefore, the reduction in catalase activity in the sediments could be linked to the absence of aerobic microorganisms that produce it. In general, microplastics can disrupt biogeochemical cycles, particularly the nitrogen cycle, by directly affecting enzymatic activities, microbial community structure, additive release, and reactive oxygen species generation (Shen et al., 2022). However, the specific mechanisms through which different types of microplastics influence the nitrogen cycle in freshwater ecosystems have not yet been fully elucidated.

In this study, multiple correlations between microplastics and physicochemical factors, as well as enzymatic activities of sediments from two stations with different characteristics (urban river and rural river), confirmed the effect of microplastics on freshwater ecosystems. The relationship between microplastics (MPs) and nutrient concentration suggests that they may originate from the same source of contamination and could help explain the impact of MPs on nutrient biogeochemical cycles. This research is the first study on microplastics in sediments of freshwater ecosystems in Cuba and one of the few in the Latin American and Caribbean region, contributing to filling the information gap on this topic.

5. Conclusions

Microplastics and artificial cellulosic particles were detected for the first time in Cuba in the sediments of two stations, one from an urban river and the other from a rural river. The concentrations of MPs were higher at the urban river station, while the concentrations of ACPs were higher at the rural river station, indicating different sources of contamination at each sampling site. Temporal variability was observed in both ecosystems, with the highest concentrations of microparticles detected in June at both stations. This trend was associated with increased precipitation at the Paila station (urban river) and recreational bathing at the Baños del San Juan station (rural river).

Transparent fibers were more frequently found compared to fragments and films, suggesting a secondary origin of the microplastics in both ecosystems. The highest diversity, according to the Shannon index, was observed in April at the Paila station, where six out of the seven microplastics identified in the samples from both stations were present. The microplastics PET, PP, ACR, PVC, PE, PUR, and PVA were identified, with PET being the most concentrated in both ecosystems. According to carbonyl index the MPs showed surface oxidation. Redundancy analysis showed that nitrite, polypropylene, ammonium and precipitations were the variables influencing extracellular enzyme activities at Paila and Baños del San Juan stations. Higher levels of polypropylene were associated with increased levels of nitrite and ammonium. Additionally, it is suggested that polypropylene inhibit proteolytic and catalase activity in the sediments of Paila and Baños del San Juan stations.

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Supplementary Materials

Microplastics in Cuban freshwaters: diversity, temporal changes, and effects on extracellular enzymatic activity

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Contents:

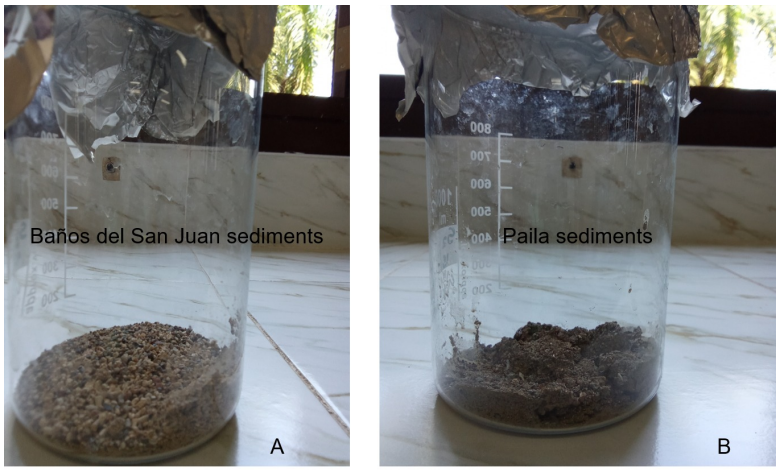

Figure S1: *Dry sediments from Baños del San Juan station (San Juan River) (A) and Paila station (Almendares River) (B).*

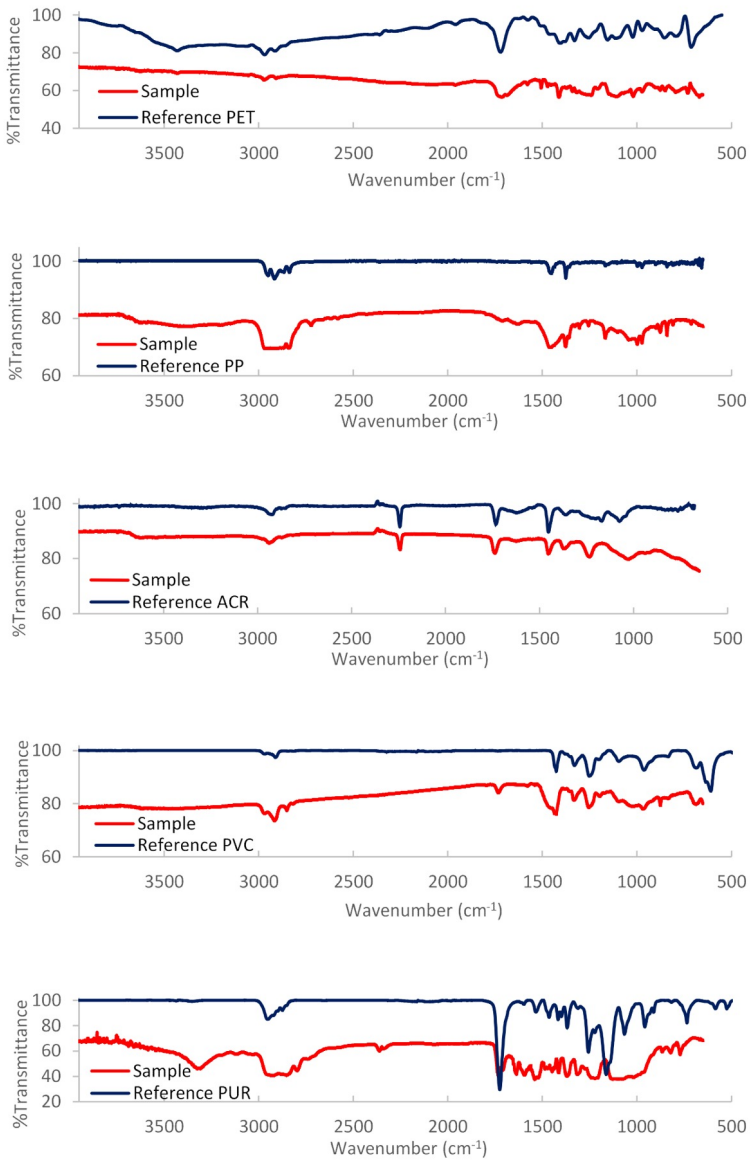

Figure S2: *Representation of the FTIR spectrum of the presence of Polyethylene terephthalate (PET), Polypropylene (PP), Acrylic (ACR), Polyvinyl chloride (PVC) and polyurethane (PUR).*

Table S1. *Mean values of the physicochemical indicators and extracellular enzyme activities determined in the sediments of the sampling stations Paila and Baños del San Juan in the period February-June 2024.*

Physicochemical indicators	Baños del San Juan			Paila		
	Feb. 2024	April 2024	June 2024	Feb. 2024	April 2024	June 2024
Temperature (ºC)	20.7	22	27.3	23.4	27.9	27.7
pH	7.8	7.5	8.6	8.1	8.2	8.1
NO_2^--N (mg kg^{-1})[a]	ND	ND	ND	10.5	ND	9.8
NO_3^--N (mg kg^{-1})	185.2	ND	22.2	ND	37.0	728
NH_4^+-N (mg kg^{-1})	ND	288	ND	18.8	ND	69.3
PO_4^{3-}-P (mg kg^{-1})	16.1	18.7	17.9	35.6	6.6	7.5
Proteases (µmol g^{-1} min^{-1})	0.33	ND	1.17	0.20	0.18	0.02
Acid Phosphatase (µmol g^{-1} min^{-1})	0.22	0.30	0.57	0.87	0.14	0.18
Alk. Phosphatase (µmol g^{-1} min^{-1})	0.22	0.69	0.90	0.62	0.13	0.02
Lipases (µmol $g_{dry\ mass}{}^{-1}$ min^{-1})	0.01	0.20	0.12	0.18	0.01	0.02
Catalase (µmol g^{-1} min^{-1})	1279	1196	1460	221	164	113
Precipitations (mm)[b]	87.7	0.00	6.8	45.4	22.2	205

ND: Not detected.
[a] Nutrient concentration data are expressed in wet weight.
[b] In the case of Paila station (Almendares River) the data were obtained from the meteorological station of Casablanca, Institute of Meteorology, Havana. For Baños del San Juan (San Juan River), the data were obtained from the Ecological Station Sierra del Rosario, Artemisa. It was taking into account the precipitations of 10 days before the sampling date.

Table S2. *Statistical summary of the redundancy analysis (RDA) for extracellular enzyme activities, physicochemical indicators and microplastics concentration in sediments of Paila (Almendares River) and Baños del San Juan (San Juan River) stations.*

Axes	RDA 1	RDA 2	RDA 3	RDA 4	Total variance
Eigenvalues	0.598	0.230	0.138	0.020	1.000
Species-environment correlations	0.991	0.994	1.000	0.986	
Cumulative percentage variance (%):					
— of species data	59.8	82.8	96.5	98.5	
— of species-environment relation	60.7	84.0	98.0	100.0	
Sum of all eigenvalues					1.000
Sum of all canonical eigenvalues					0.985
Axes correlations:					
— Nitrite concentration (NO_2-N)	0.86	0.06	-0.26	-0.43	
— Ammoniun concentration (NH_4-N)	0.33	0.74	-0.58	0.07	
— Precipitations	0.60	-0.03	-0.59	0.54	
— Polypropylene concentration (PP)	0.44	0.83	-0.02	-0.33	

Note: In bold the variables considered significant on each axis.

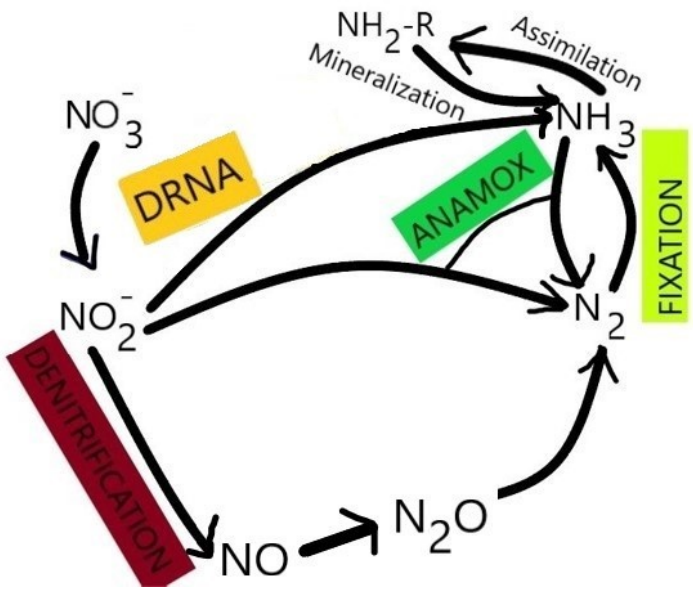

Figure S3: *Nitrogen cycle under anoxic conditions. DRNA: dissimilatory reduction of nitrate to ammonia ($NO_3^- \rightarrow NO_2^- \rightarrow NH_3$); ANAMOX: anaerobic ammonia oxidation ($NO_2^- + NH_3 = N_2$); Denitrification: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$.*