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Hidden microplastic pollution in protected terrestrial ecosystems: Evidence from gray wolves and Persian leopards

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ABSTRACT

Microplastics are ubiquitous pollutants with the potential to penetrate even strictly protected terrestrial ecosystems. Here, we investigate microplastic contamination in apex carnivores, gray wolves and Persian leopards, within Golestan National Park, a national park and UNESCO biosphere reserve in northeastern Iran. A total of 34 scat samples were collected opportunistically across diverse habitats and systematically confirmed using DNA metabarcoding approach to avoid misidentification. Microplastic extraction involved oxidative digestion, density separation, and microscopic examination, followed by Raman spectroscopy for polymer identification. Microplastics were detected in 79.4 % of samples, with wolves showing a significantly higher total microplastic area than leopards, despite no significant differences in particle number or average size. Identified polymers included polystyrene, polyethylene, polyethylene terephthalate and polyamide, likely originating from tire wear, packaging, synthetic textiles, and agricultural plastics. Spatial analyses revealed negative correlations between microplastic load and distance to roads and nearby villages, highlighting anthropogenic sources as primary drivers of contamination even within fully protected zones. Wolves, as generalist and more mobile predators, exhibited elevated exposure compared to the more selective leopards, indicating dietary and behavioral influences on contaminant accumulation. These findings demonstrate that protected terrestrial habitats are not immune to diffuse human-derived pollutants and emphasize the role of infrastructure and rural settlements in shaping contamination patterns. Given the parallels observed in other protected areas globally, these results underscore the need for integrated monitoring and mitigation strategies to safeguard apex carnivores and ecosystem integrity in conservation areas worldwide facing similar anthropogenic pressures.

1. Introduction

Microplastics, defined as plastic particles smaller than 5 mm, represent a pervasive environmental contaminant that has infiltrated virtually every ecosystem on Earth, from polar ice caps to tropical rainforests [31,51]. Their widespread distribution is driven by atmospheric transport, ocean currents, and human activities, making them a global challenge that transcends borders and habitats [10,51]. Originating from the breakdown of larger plastics through physical weathering and UV degradation, as well as direct releases from products like cosmetics, textiles, and industrial abrasives, microplastics accumulate in soils, sediments, and biota, posing multifaceted risks to biodiversity [31,9]. The persistence of these particles in the environment stems from their resistance to biodegradation, allowing them to remain for centuries and interact with natural processes in unpredictable ways [67]. This ubiquity has prompted international concern, with microplastics now recognized as a key indicator of the Anthropocene, reflecting humanity's profound alteration of planetary systems [68]. As production of plastics continues to escalate, projected to quadruple by 2050, the burden on ecosystems intensifies, necessitating urgent research into their long-term ecological consequences [18].

The impacts on wildlife are profound, including physical ingestion leading to blockages, reduced feeding efficiency, and impaired reproduction, which collectively diminish individual fitness and population viability [20,52]. Chemical leaching of additives such as phthalates, bisphenols, and flame retardants further exacerbates toxicity,

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potentially disrupting endocrine systems and causing developmental abnormalities in exposed organisms [11]. Moreover, microplastics act as vectors for adsorbing and concentrating hydrophobic pollutants like persistent organic pollutants (POPs) and heavy metals, amplifying their bioavailability and facilitating transfer across trophic levels [25]. In food webs, this bioaccumulation process allows contaminants to ascend from basal organisms to higher predators, compounding existing stressors such as habitat loss, overexploitation, and climate change, which already threaten species with declines and extinctions [36]. Protected areas, intended as refuges from anthropogenic pressures, are increasingly compromised by this infiltration, with detections in remote sites like mountain catchments and national parks underscoring the failure of geographical isolation to mitigate diffuse pollution [10,4]. This vulnerability highlights a broader crisis: even the most safeguarded ecosystems are permeable to global pollutants, challenging traditional conservation paradigms and demanding integrated approaches to address transboundary threats.

Despite extensive research on marine systems, knowledge of microplastic abundance in terrestrial protected ecosystems remains limited, particularly for apex predators that serve as sentinels of environmental health [66]. While aquatic studies have documented high ingestion rates in invertebrates and fish, revealing baseline contamination and trophic amplification, terrestrial investigations lag behind, often focusing on soil biota or lower trophic levels rather than integrative indicators like carnivores [65]. This gap is particularly acute in biodiverse hotspots, where microplastics could disrupt fragile interactions and undermine ecosystem services. For instance, recent assessments indicate that microplastics alter soil microbial communities, nutrient cycling, and plant growth, with cascading effects on herbivores and their predators, yet empirical data from terrestrial mammals, especially in conserved zones, are sparse [50,66]. Apex predators, by virtue of their position at the top of food chains, accumulate contaminants over time, making fecal analysis a non-invasive tool to gauge ecosystem-wide exposure. However, some studies overlook this potential, leaving uncertainties about prevalence in fully protected terrestrial settings and hindering the development of targeted monitoring protocols [42]. Addressing this void is essential, as it could reveal hidden contamination levels and inform conservation strategies to protect vulnerable species from insidious threats.

The scarcity of data on microplastics in terrestrial apex predators extends to understanding how these particles permeate protected landscapes, where human influences are minimized but not absent. Airborne deposition, runoff from adjacent lands, and wildlife-mediated transport introduce microplastics even into core protected zones, potentially leading to bioaccumulation in species like large carnivores that roam extensive territories [3]. Moreover, microplastics ingested by top predators could cause secondary contamination of soil and water via excretion, as these animals defecate across wide areas, redistributing particles and associated toxins back into the environment [27]. Given the extensive home ranges and daily movements of large carnivores, often covering tens to hundreds of square kilometers, they can transport ingested microplastics from contaminated peripheral zones to the pristine core of protected areas through consumption of polluted prey and subsequent fecal deposition, effectively acting as vectors for intra-park pollutant dispersal and amplifying ecosystem-wide exposure. Quantifying abundance in such contexts would not only establish baselines but also highlight the inefficacy of current protection measures against global pollutants, prompting reevaluation of reserve management in an era of pervasive contamination.

Further, the role of diet in microplastic transmission to top predators is underexplored in terrestrial contexts [44]. Marine research has established correlations between prey diversity and contaminant uptake, with generalist feeders often exhibiting higher loads due to broader exposure pathways [42]. Generalists may ingest more particles through varied diets, while specialists reflect specific transmission routes, yet empirical links via dietary reconstruction are lacking, limiting insights into how microplastics reshape predator-prey dynamics. For example, if herbivores accumulate microplastics from soil or vegetation, their consumption by carnivores could amplify exposure, altering energy flows and community structures [53,59]. This knowledge deficit impedes predictions of long-term ecological impacts, such as reduced hunting efficiency or reproductive success in contaminated predators, underscoring the need for integrated dietary analyses to trace transmission pathways.

Dietary correlations also intersect with behavioral ecology, as foraging strategies influence exposure risks. In terrestrial ecosystems, opportunistic carnivores might encounter microplastics through scavenging near human-modified edges, while obligate hunters target less contaminated prey, but without comparative data, these hypotheses remain untested. Bridging this gap could reveal how dietary plasticity modulates contamination, informing models of trophic transfer and ecosystem resilience in polluted landscapes.

Spatial variations in microplastic prevalence within protected areas also warrant investigation, as proximity to human influences like roads, boundaries, and adjacent land uses may drive uneven distribution. Airborne and hydrological pathways create contamination gradients, with higher concentrations often observed near edges or infrastructure, as seen in some reserves where urban runoff elevates loads [3]. However, comprehensive comparisons across park zones are rare, leaving uncertainties about hotspots and dispersal mechanisms in fragmented habitats. Such analyses could identify vulnerable areas, guide zoning for enhanced protection, and mitigate risks in landscapes bisected by development [10].

Understanding spatial patterns is further complicated by temporal and environmental factors, which may exacerbate gradients in protected zones. Without spatially explicit data, efforts to prioritize remediation remain hampered, particularly in biodiverse areas where uneven contamination could disproportionately affect key species [65].

Specifically, we asked three research questions:

(1) What is the abundance of microplastics in fecal samples from wolves and Persian leopards in the Golestan National Park?

(2) How do species-specific foraging behaviors and dietary habits influence the total microplastic load in wolves and Persian leopards?

(3) How does microplastic abundance in scat samples vary based on collection locations across different park areas?

2. Materials and methods

2.1. Study area

This study was conducted in Golestan National Park, a UNESCO-designated biosphere reserve located in northeastern Iran [61], spanning the provinces of Golestan, Semnan, and North Khorasan (UNESCO Man and the Biosphere Programme) (Fig. 1). Established in 1957 as Iran's first national park, Golestan National Park covers approximately 91,895 ha and is renowned for its rich biodiversity. The park encompasses a wide range of ecosystems, from Caspian Hyrcanian forests clustered in the west to arid steppes and semi-desert zones in the east [2]. Golestan National Park hosts a diverse array of wildlife, including six wild ungulate species: the near-threatened wild goat, red deer *Cervus elaphus maral*, roe deer *Capreolus capreolus*, wild boar *Sus scrofa*, goitered gazelle *Gazella subgutturosa*, and the globally vulnerable urial *Ovis vignei* [19]. Large carnivores also inhabit the park, such as the endangered Persian leopard *Panthera pardus tulliana*, grey wolf *Canis lupus*, and brown bear *Ursus arctos* [30,57]. Although there are no permanent settlements within the park, 37 villages are located around the park within an 11-km radius, representing the primary sources of surrounding anthropogenic pressure.The park's varied topography, ranging from 400 to 2400 m above sea level, together with a precipitation gradient from about 700 mm in the west to 150 mm in the east [2], creates diverse ecological conditions. Combined with its proximity to human settlements, this landscape forms a unique interface where natural ecosystems

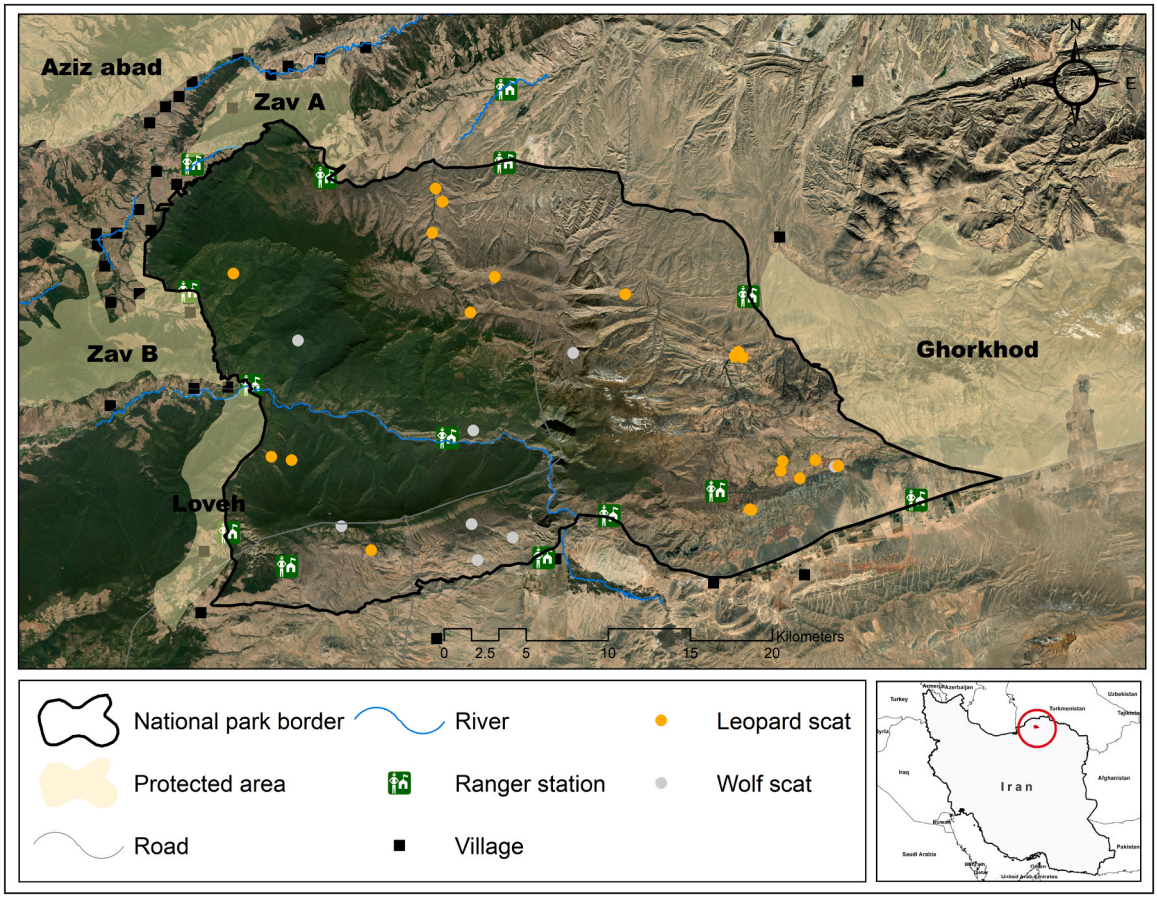


Fig. 1. Golestan National Park, located in northeastern Iran (inset map), encompasses diverse habitats including deciduous forests, shrublands, and steppes. The park harbors a wide range of wildlife, with six species of herbivores and fourteen species of Carnivora, such as the Persian leopard and the gray wolf.

meet anthropogenic influences, making Golestan National Park an ideal site for investigating environmental contaminants such as microplastics.

Gray wolves and Persian leopards, the park's two dominant apex carnivores, were selected as sentinel species due to their large body mass (wolves: 30–80 kg; leopards: 30–90 kg), high daily food intake (~2–5 kg and ~2–4 kg, respectively) [29], and extensive home ranges (100–1000 km^2 and 50–500 km^2) [16], which amplify contaminant exposure and biomagnification across trophic levels. Non-invasive fecal analysis enables ecosystem-wide monitoring without disturbing these IUCN-listed endangered populations [26].

2.2. Sample collection and preparation

Scat samples from gray wolves and Persian leopards were collected opportunistically during a concurrent camera-trapping project conducted across all regions of Golestan National Park. The park was systematically divided into 55 grids, each measuring 4.5 × 4.5 km, with one camera trap deployed per grid to monitor wildlife distribution and activity.

A total of 115 scat samples were initially collected along established walking trails, ridgelines, and riverbeds traversed during camera-trap deployment, maintenance, and retrieval. To reduce spatial autocorrelation and ensure balanced representation across gradients of anthropogenic disturbance, a stratified subset of 34 genetically confirmed carnivore scats (wolf and leopard) was selected using GIS-based distance analysis (Fig. 1).

Distances to the main highway and to the nearest of the 37 surrounding villages were calculated separately. With respect to the highway, 47.1 % (n = 16) of the selected samples were located within 4 km, 32.4 % (n = 11) between 4.1 and 8 km, and 20.6 % (n = 7) farther than 8 km from the road. Independently, 23.5 % (n = 8) of samples were within 6 km of the nearest village, 41.2 % (n = 14) were 6.1–12 km away, and 35.3 % (n = 12) were more than 12 km from any village. This stratified design effectively captured a broad gradient of human influence, driven primarily by the central highway as the main corridor of anthropogenic pressure and secondarily by the dispersed network of 36 rural settlements surrounding the park.

Samples were gathered along established walking trails and paths traversed during camera installation, maintenance, and retrieval, ensuring comprehensive spatial coverage while minimizing disturbance to the habitat. Surveys for scat collection were conducted simultaneously with camera trap deployments by field experts along footpaths most frequently used by wolves and leopards. To prevent misidentification, leopard and wolf scats were opportunistically selected and collected based on size, shape, and proximity to other signs. To distinguish leopard scat from that of wolves and other sympatric carnivores while reducing identification errors, scats with a diameter greater than 2.7 cm and accompanied by scratch marks beside them were considered leopard scat [54], whereas scats with a smaller diameter, lacking scratch marks, and placed on bushes were considered wolf scat. During autumn and winter of 2023–2024, scat samples were collected from various areas of Golestan National Park. For each sample collected, date and geographic coordinates were recorded.

For metabarcoding studies, strict sterile protocols were followed during sample collection and processing. Fresh fecal samples were collected using sterile gloves to minimize contamination. Each sample was immediately wrapped in autoclave-sterilized aluminum foil, labeled with unique identifiers, and transported under controlled conditions [55]. To preserve DNA integrity, samples were freeze-dried (lyophilization) until complete desiccation and subsequently homogenized using

a sterile porcelain mortar to ensure uniform particle size for efficient DNA extraction [14].

2.3. Microplastic characterization

Each lyophilized fecal sample (2.00 ± 0.01 g dry weight) was transferred to a pre-cleaned 100 mL glass beaker and subjected to oxidative digestion with 20 mL of 30 % H_2O_2 (analytical grade, Merck) at 50 °C for 48 h in a shaking incubator (80 rpm). Additional 10 mL aliquots of 30 % H_2O_2 were added as needed until complete dissolution of organic matter was achieved (maximum total volume 40 mL). After cooling, the digest was diluted with 100 mL pre-filtered Milli-Q water and vacuum-filtered through a 0.8 µm polycarbonate filter to remove large undigested residues.

The dried residue was subsequently transferred to a 50 mL glass centrifuge tube and mixed with 30 mL saturated NaCl solution (density 1.2 g cm^{-3}, pre-filtered through 0.4 µm). The tube was shaken vigorously by hand for 2 min and centrifuged at 4500 × g for 10 min at 20 °C (Hettich Rotina 380 R). The supernatant containing floating microplastics was carefully decanted and vacuum-filtered onto a new 0.8 µm polycarbonate filter. The density separation step was repeated twice to maximize recovery [12,39].

Filters were dried at 50 °C for 24 h and stored in covered glass Petri dishes until analysis. Particles were first examined under a stereomicroscope (Olympus SZX16) at 100 × magnification and photographed. Nile Red staining (1 µg mL^{-1} in methanol) was applied as a rapid screening method to highlight putative microplastics under blue-light excitation [15]. Polymer identification was performed using a Thermo Scientific™ Almega™ XR Dispersive Raman Spectrometer equipped with a 532 nm Nd:YLF laser (50 % power, 10 s exposure, 5 accumulations) to prevent thermal degradation. Spectra were accepted only when the hit quality index exceeded 80 % against reference libraries [6,28].

2.4. DNA metabarcoding for species identification

To ensure accurate identification of predator species fecal samples and minimize misidentification, a DNA metabarcoding approach was applied targeting ~140-bp fragment of the V5 loop of the mitochondrial 12S rRNA gene (Forward 5′-ACTGGGATTAGATACCCC-3′, Reverse 5′-TAGAACAGGCTCCTCTAG-3′) [49], designed for vertebrate metabarcoding enabling reliable differentiation between gray wolf and Persian leopard scats and their prey. Genomic DNA was extracted from 34 scat samples collected in Golestan National Park using the Qiagen DNeasy PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany). We added three DNA isolation blank to trace any cross contamination. Approximately 200 + -20 mg of lyophilized/homogenized fecal material was weighed and processed following established protocols for fecal DNA extraction [14,58]. The samples were transferred to PowerBead Pro tubes provided in the kit, and DNA extraction was performed according to the manufacturer's instructions. The procedure involved mechanical lysis, chemical digestion, removal of PCR inhibitors, and binding of DNA to silica spin columns, followed by sequential washing steps to eliminate contaminants such as bile salts and polysaccharides. This method ensured the recovery of high-quality DNA suitable for downstream PCR amplification and sequencing [5].

The PCR amplification (following protocol described in [49]) and subsequent steps [48], including library preparation (following [34]), purification of PCR products and sequencing were performed at the Department of Biogeography, Trier University, Germany. In the final step, dual-indexed and purified PCR products from all 37 samples (34 fecal samples and three negative controls (including DNA isolation blank and PCR negative controls) were sequenced using the Illumina MiSeq 300-cycle V2 chemistry platform. Raw reads were demultiplexed using the MiSeq Control Software version 2.6.2.1 (Illumina Inc., San Diego, California, USA). Low-quality reads and primer sequences were trimmed using Cutadapt [40] and VSEARCH (Rognes et al., 2016) within the APSCALE4 v2.30 pipeline (Buchner et al., 2022; https://github.com/TillMacher/apscale_gui). After quality filteration and denoising, dataset contained 107 distinct sequences and 314,753 reads. Finally, high quality sequences were clustered (threshold of 0.97) into 57 MOTUs.

The APSCALE blast tool v1.0.6 (based on blast 2.16.0 +,) was applied for taxonomic annotation by comparing MOTUs against curated GenBank reference database of MIDORI2, version GB266_ srRNA, [37]), ensuring precise attribution of microplastic data to each carnivore species [7]. Although alongside the predator DNA, the prey DNA can be sequences in this approach, but the highest relative abundance of reads principally were annotated and attributed to eighter the gray wolf or Persian leopard. Only the sequences with 100 % similarity to the reference database were retained for predator identification.This approach verified the earlier scat identification or confirmed our primary suspected ones. The R version 4.5.1 (R CoreTeam) and RStudio were applied using tidyverse 2.0.0 [45,64] for sequence reads data and OTU table management and plotting. The supplementary data containing the OTU table of identified (Table S1).

3. Result

Field surveys in Golestan National Park revealed distinct spatial patterns in carnivore scat deposition. Leopard scat, scratch marks, and tracks were predominantly documented along routes shared with park rangers, suggesting preference for established trails, whereas gray wolf scat were frequently observed on vegetation bordering secondary paths. Despite observable differences in morphology and deposition patterns, DNA barcoding was required for reliable species identification.

During field collection, a total of 34 scat samples were initially morphologically assigned to species based on physical characteristics, location, and associated signs: 19 to gray wolf and 16 to Persian leopard. However, subsequent genetic analysis via DNA metabarcoding provided definitive species identification, revealing a discrepancy with the initial field assessments. The molecular results of 33 out of 34 primary samples confirmed that 13 originated from wolves, 20 from leopards, and one sample was identified as belonging to the brown bear *Ursus arctos*. The brown bear sample was excluded from subsequent comparative analyses between wolves and leopards. All following results on microplastic load and spatial distribution are therefore based on this genetically confirmed dataset.

3.1. Microplastic abundance and characteristics in scat samples

Microplastics were detected in 27 out of 34 genetically confirmed scat samples (79 %), with a higher prevalence in wolf samples (11/13; 85 %) compared to leopard samples (16/20; 80 %). The number of microplastic particles identified per 2 g of fecal material ranged from 0 to 42 across all samples, with an overall mean abundance of 7.63 ± 8.2 particles per 2 g (mean ± SD; equivalent to 3.81 particles per g). Species-specific differences were apparent: wolf scats exhibited a mean of 10.00 ± 10.34 particles per 2 g (range: 0–42; 5.00 per g), while leopard scats showed a lower mean of 6.1 ± 5.98 particles per 2 g (range: 0–24; 2.90 per g).

The average surface area of microplastic particles per sample (where present) varied considerably, ranging from 0.002825 mm^2 to 0.720615 mm^2. For contaminated wolf samples, the mean particle size was 0.179 ± 0.214 mm^2 (n = 11), whereas for leopard samples, it was 0.082 ± 0.126 mm^2 (n = 16). Samples without microplastics were assigned a size of 0 mm^2 for summary purposes. Detailed per-sample data on microplastic abundance and average particle size are presented in Table 1.

To assess the statistical significance of differences in the number and average size of microplastics between wolf (n = 11) and leopard (n = 16) samples with microplastics (excluding 6 samples with zero microplastics), the Mann-Whitney *U* test was conducted using the data

Table 1
Number, average size (mm^2), Raman spectroscopy peaks (cm^{-1}), and types of microplastics identified in scat samples from wolves, leopards, and brown bears in Golestan National Park, including polystyrene (PS), polyethylene (PE), polyethylene terephthalate (PET), and polyamide (PA).

Sample ID	Confirm Species	Number of Microplastics	Microplastic Average Size (mm^2) ± SD	Microplastic total Area (mm^2)	Raman Pick	Polymer Type
13	Wolf	42	0.0052 ± 0.0016	0.2173	1597	PS
14	Wolf	16	0.0581 ± 0.0174	0.9288	1598, 1635	PS, PA
4	Wolf	14	0.0954 ± 0.0286	1.3359	1388, 1595, 1636	PS, PE, PA
7	Wolf	12	0.0705 ± 0.0212	0.8464	1389, 1594, 1637	PS, PE, PA
1	Wolf	8	0.0184 ± 0.0055	0.1473	1386, 1596	PS, PE, PA
8	Wolf	8	0.4598 ± 0.1379	3.6783	1385, 1598, 1638	PS, PE, PA
12	Wolf	8	0.7206 ± 0.2162	5.7649	1386, 1595, 1636, 1722	PS, PE, PA, PET
29	Wolf	8	0.4520 ± 0.1356	3.6163	1357, 1387, 1474, 1597	PS, PE
15	Wolf	6	0.0216 ± 0.0065	0.1296	1388, 1594, 1637, 1723	PS, PE, PA, PET
18	Wolf	6	0.3118 ± 0.0935	1.8707	1389, 1595, 1638	PS, PE, PA
9	Wolf	2	0.3152 ± 0.0946	0.6304	1387, 1596, 1635	PS, PE, PA
10	Wolf	0	0	0	-	None
33	Wolf	0	0	0	-	None
30	Leopard	24	0.0167 ± 0.0050	0.401	1598, 1385, 1720, 1654	PS, PE, PET, PA
24	Leopard	16	0.0028 ± 0.0008	0.0452	1596, 1721	PS, PET
25	Leopard	12	0.0646 ± 0.0194	0.7754	1389, 1598, 1635	PS, PE, PA
11	Leopard	10	0.0223 ± 0.0067	0.223	1599, 1387, 1723, 1656	PS, PE, PET, PA
16	Leopard	10	0.1320 ± 0.0396	1.3204	1596, 1385, 1721, 1655	PS, PE, PET, PA
20	Leopard	8	0.0360 ± 0.0108	0.2876	1599, 1387, 1722, 1656	PS, PE, PET, PA
21	Leopard	8	0.0080 ± 0.0024	0.0644	1596, 1384, 1723, 1653	PS, PE, PET, PA
3	Leopard	6	0.0079 ± 0.0024	0.0474	1598, 1385	PS, PE
19	Leopard	6	0.0071 ± 0.0021	0.0424	1598, 1385	PS, PE
26	Leopard	6	0.0413 ± 0.0124	0.248	1600, 1655, 1725	PS, PA, PET
5	Leopard	4	0.0606 ± 0.0182	0.2423	1597, 1384, 1722, 1654	PS, PE, PET, PA
17	Leopard	4	0.0158 ± 0.0047	0.063	1597, 1386, 1720, 1654	PS, PE, PET, PA
6	Leopard	2	0.0378 ± 0.0113	0.0756	1596, 1386, 1720, 1653	PS, PE, PET, PA
23	Leopard	2	0.1216 ± 0.0365	0.2432	1597, 1386, 1721, 1655	PS, PE, PET, PA
31	Leopard	2	0.4874 ± 0.1462	0.9747	1597, 1386, 1722, 1656	PS, PE, PET, PA
34	Leopard	2	0.0325 ± 0.0098	0.065	1594, 1384	PS, PE
22	Leopard	0	0	0	-	None
27	Leopard	0	0	0	-	None
28	Leopard	0	0	0	-	None
32	Leopard	0	0	0	-	None

Note: Out of 34 collected samples, one sample belonged to a brown bear and was excluded from the statistical analyses and results because it did not belong to the target species (wolf and leopard

provided. Mann-Whitney U tests compared microplastic metrics between wolf (n = 11) and leopard (n = 16) samples containing microplastics (excluding 6 zeros). No significant differences were found in number or average size, but total microplastic area was significantly higher in wolves (Table 2).These findings suggest that while the number and average size of microplastics do not differ significantly between wolf and leopard samples, wolves exhibit a significantly higher total microplastic load in National Park.

Table 2
Mann-Whitney *U* test results for microplastic metrics in wolf and leopard scat.

Metric	Wolf (n = 11)	Leopard (n = 16)	U	p-value
Number of microplastics	Median: 8.0 Mean: 10.4	Median: 6.0 Mean: 7.5	67.5	0.227
Average particle size (mm^2)	Median: 0.0705 Mean: 0.227	Median: 0.0279 Mean: 0.062	58.5	0.108
Total microplastic area (mm^2)	Median: 0.85 Mean: 1.94	Median: 0.24 Mean: 0.28	44.0	0.028*

Note: *p < 0.05. Only samples with microplastics (n = 27) were included in comparisons.

3.2. *Type of microplastic*

Raman spectroscopy identified polystyrene (PS), polyethylene terephthalate (PET), polyethylene (PE), and polyamide (PA) (Fig. 2) in wolf and leopard samples, with characteristic peaks at 1384–1598 cm^{-1} for PS (aromatic ring vibrations and CH deformations), 1596–1725 cm^{-1} for PET (carbonyl stretching), 1357–1474 cm^{-1} for PE (CH_2/CH_3 deformations), and 1635–1655 cm^{-1} for PA (amide I stretching) [6]. In wolf samples, PS was the most frequently observed polymer, often accompanied by PE and PA, with spectral features showing attenuated bands indicative of surface degradation. Leopard samples contained PS, PE, PET, and PA, with PET and PA more commonly detected, and spectra exhibiting carbonyl peaks at approximately 1721 cm^{-1} suggesting environmental weathering [23]. Wolf samples predominantly contained PS and PE, while leopard samples showed a broader mix including PET and PA. This difference suggests varied exposure, with wolves likely

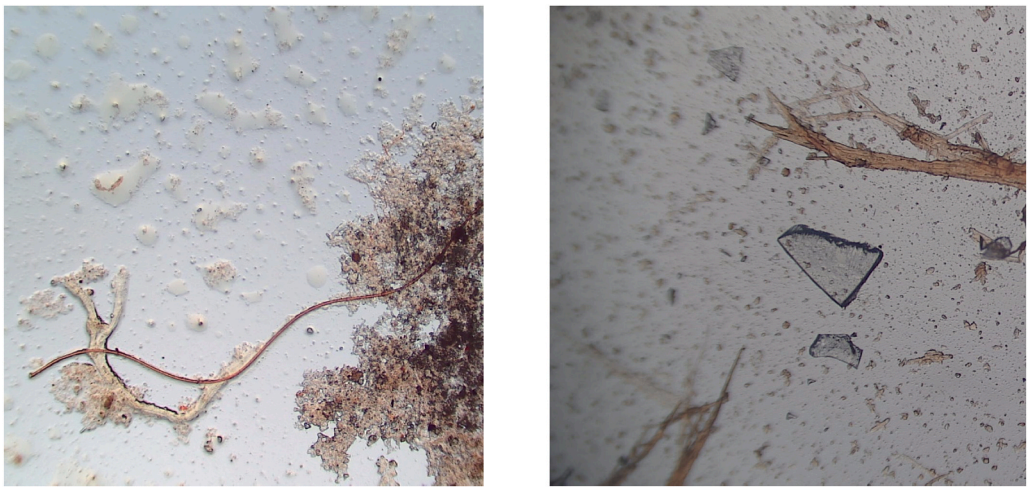


Fig. 2. (Right) Rectangular microplastic fragment resembling broken polyethylene terephthalate (PET) from a mineral water bottle, showing Raman peaks at approximately 1725 cm^{-1} (carbonyl stretch) and 1610 cm^{-1} (aromatic ring vibration), observed in leopard samples 5, 6, 11,12,15 16, 20, 21, 23, 24, 26, 30, and 31. (Left) Fibrous microplastic, polyamide (PA) from synthetic textile fibers, exhibiting characteristic Raman peaks at approximately 1639 cm^{-1} (amide I stretching) and 2922 cm^{-1} (CH_2 asymmetric stretch), observed in leopard samples 5, 6, 11, 16, 20, 21, 23, 25, 26, 30, and 31.

encountering simpler polymers and leopards exposed to more diverse microplastics (Table 1, Fig. 3).

Local anthropogenic activities around Golestan National Park provide direct evidence for the sources of these polymers. A major highway bisects the park into northern and southern halves, with an estimated ~1200 vehicles/day, generating tire wear particles (TWPs) rich in PS and PE through abrasion and roadside littering. Agricultural films, covering ~40 % of croplands in the 15 surrounding villages, fragment into PE-dominated residues that enter park soils via runoff and wind. Disposable packaging (predominantly PS) dominates local markets and tourist waste, while synthetic textiles and clothing fibers (PA, PET) from household laundry and livestock gear are prevalent in village effluents. Waste management is severely limited, with open dumping documented at all of village boundaries and along access roads, creating diffuse contamination gradients into the park. These locally documented sources, highway traffic, agricultural plastics, packaging litter, and informal dumps, directly align with the PS/PE dominance in wolf scats and the PA/PET presence in leopard scats, confirming trophic transfer from contaminated prey and environments [23,41].

3.3. Spatial variation in microplastic abundance

A total of 33 scat samples from Golestan National Park were analyzed to quantify microplastic abundance, expressed as the total microplastic area (mm^2).

All identified microplastics were in the size range of approximately 50 µm to 3.2 mm (Table 1). Due to the 0.8 µm filtration threshold and subsequent visual/Raman verification, particles in the nanoplastic range (<1 µm) were not captured in this study. The mean total microplastic area was 0.736 mm^2 (SD = 1.288, range = 0.000–5.765 mm^2). The mean distance to villages was calculated as the average distance from each scat sample to all 36 surrounding villages, yielding a mean of 27.444 km (SD = 4.683, range = 16.873–38.344 km). For distance to roads, data were available for samples, with a mean of 6.266 km (SD = 3.884, range = 0.730–14.710 km).

Shapiro-Wilk tests were conducted to assess the normality of the data distributions. The total microplastic area (n = 33) was non-normal (W = 0.609, $p < 0.001$). The mean distance to villages (n = 33) followed a normal distribution (W = 0.951, p = 0.123). The distance to roads (n = 32) was non-normal (W = 0.874, p = 0.002).

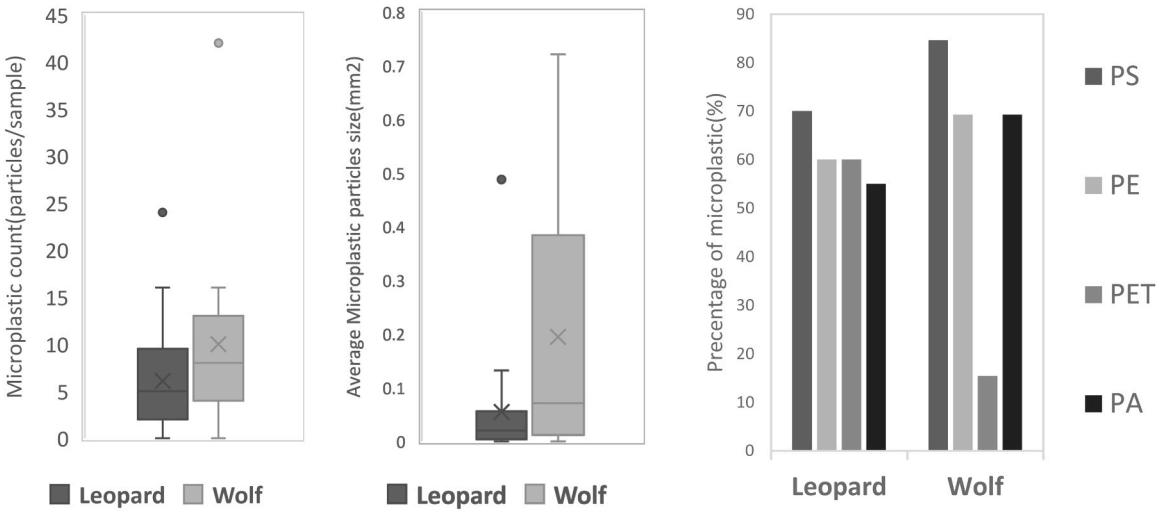


Fig. 3. Comparative analysis of microplastic contamination in Persian leopard and wolf samples: (a) Mean abundance of microplastics, (b) Average microplastic size, and (c) Identified microplastic types characterized by Raman spectroscopy.

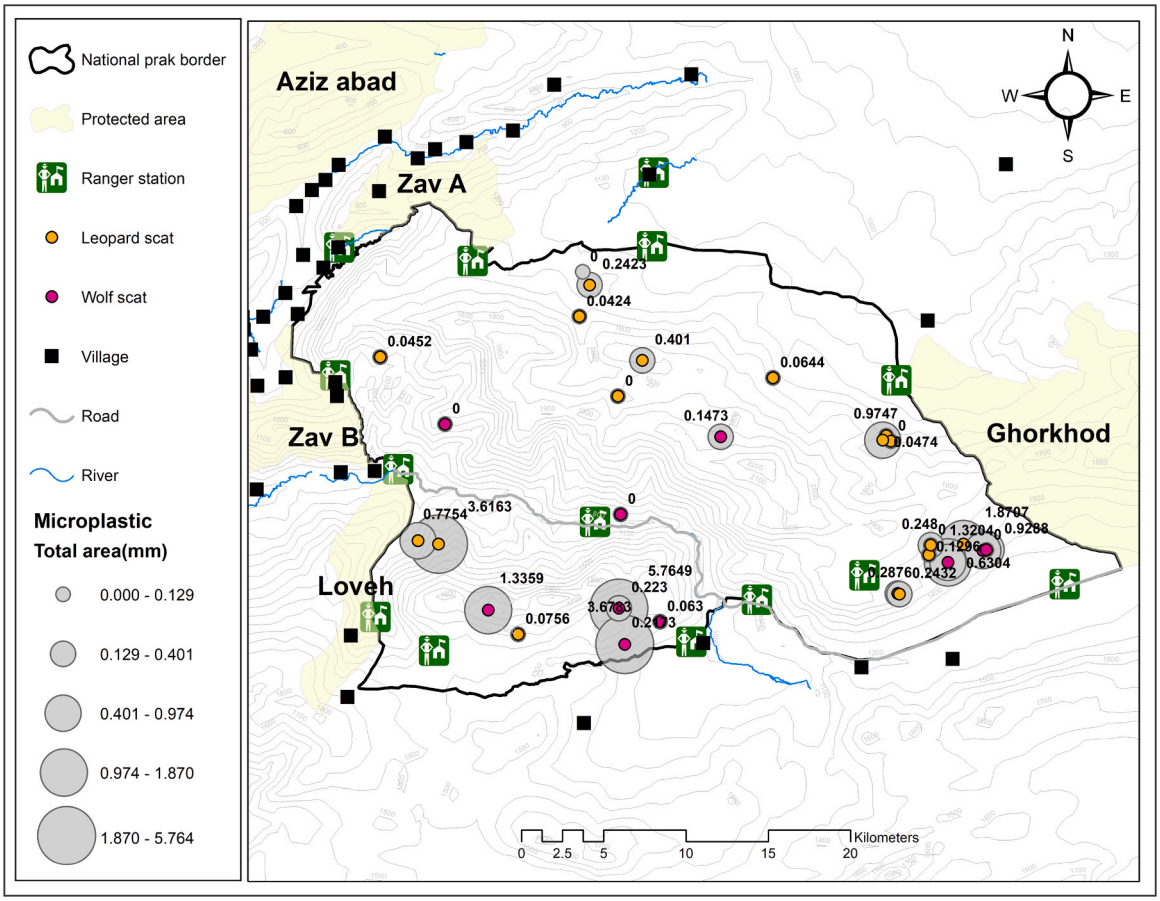


Fig. 4. Spatial distribution of microplastic abundance in scat samples collected from Golestan National Park, Iran. The size of circles represents the total microplastic area (mm^2), with larger circles indicating higher microplastic contamination.

Due to the non-normality of the total microplastic area, Spearman's rank-order correlation was employed to examine spatial relationships. A significant negative correlation was found between total microplastic area and mean distance to villages ($\rho = -0.351$, $p = 0.045$), indicating higher microplastic abundance in scat samples closer to villages. Additionally, a significant negative correlation was observed between microplastic abundance and distance to the nearest road ($\rho = -0.535$, $p = 0.002$), suggesting that proximity to roads is associated with increased microplastic contamination.

3.4. Livestock predation and proximity to anthropogenic sources

Dietary DNA metabarcoding of the 34 genetically confirmed scat samples revealed the presence of livestock prey in six samples. Domestic chicken (*Gallus gallus*) DNA was detected in three wolf scats, whereas cattle (Bos taurus) DNA was found in two leopard scats (one leopard scat contained both chicken and cattle DNA). Consequently, livestock prey occurred in 31 % of wolf scats (4 of 13) and 10 % of leopard scats (2 of 20)(Table 3).

Scats containing livestock DNA were collected significantly closer to anthropogenic features than scats lacking livestock remains. The mean distance to the nearest road was 4.6 ± 3.6 km for scats with livestock prey versus 6.4 ± 3.5 km for those without (Mann–Whitney U = 38, one-tailed $p = 0.044$). The mean distance to the nearest village was markedly lower in the livestock-positive group (16.8 ± 6.9 km) compared to the livestock-negative group (26.5 ± 7.3 km; Mann–Whitney U = 22, one-tailed $p = 0.0036$).

Table 3
Proximity of scat samples to anthropogenic features in relation to livestock prey DNA.

Group	Number	Distance to nearest road (km)	Distance to nearest village (km)
		Mean ± SD (Median)	Mean ± SD (Median)
With livestock DNA	6	4.6 ± 3.6 (3.7)	16.8 ± 6.9 (16.0)
Without livestock DNA	28	6.4 ± 3.5 (5.3)	26.5 ± 7.3 (26.8)
Mann–Whitney U (one-tailed p)		38 (0.044)	22 (0.0036)

3.5. Quality assurance and quality control

To ensure the reliability and accuracy of microplastic detection while minimizing contamination risks, a comprehensive quality assurance and quality control (QA/QC) protocol was applied throughout sample collection, processing, and analysis. Field blanks (n = 3 per major site) were prepared by opening sterile containers in situ without collecting material and processed identically to samples to control for environmental contamination. All laboratory work was conducted in a dedicated cleanroom environment equipped with HEPA filtration (Class 1000) to reduce airborne particulates, with daily monitoring maintaining counts below 1000 particles/m^3 (>0.5 μm). Personnel wore non-shedding cotton lab coats, powder-free nitrile gloves, and hair covers. Work surfaces were wiped with 70 % ethanol using lint-free cloths before and after each session. All reagents (e.g., 30 % H_2O_2, saturated NaCl) were pre-filtered through 0.4 μm polycarbonate membranes and stored in glass bottles. Equipment was acid-washed (10 % HNO_3) and baked at > 350°C prior to use. Sample processing occurred under a positive-pressure laminar flow hood, with samples covered by autoclave-sterilized aluminum foil to prevent aerial deposition.

Procedural blanks (n = 3) were processed in parallel with each batch of 11 scat samples using pre-filtered MilliQ water; no particles matching sample polymers were detected, confirming low procedural bias. For Raman spectroscopy, spectra were accepted only with > 80 % match to reference libraries [6]. Duplicate spectra were acquired for 20 % of particles, ensuring intra-sample variability < 5 %. Blind re-examination of 15 % of filters by a second analyst yielded 95 % concordance in particle counts and sizes, with discrepancies resolved by consensus [21, 32]. Limits of detection (LOD) were calculated as the mean + 3 SD of blanks.

To assess the efficiency of the extraction workflow, a recovery experiment was performed on 10 randomly selected samples using size-calibrated microplastic standards (50–500 μm). Application of the same digestion and NaCl-based separation procedure yielded a recovery rate of 87.4 % ± 4.1 %. This performance is consistent with previously reported efficiencies for oxidative digestion and density separation methods (≈84–95 %; [38,12]), confirming that the protocol ensures reliable particle retention with minimal procedural loss.

4. Discussion

The pervasive intrusion of microplastics into apex carnivores of Golestan National Park, a UNESCO biosphere reserve, vividly illustrates the vulnerability of protected terrestrial ecosystems to anthropogenic pressures, particularly in areas adjacent to human settlements and road networks. The park's central arterial road, coupled with numerous villages where residents engage in agriculture, animal husbandry, and tourism, serves as a potent vector for microplastic dissemination, transforming ostensibly protected habitats into sinks for synthetic debris. Our findings confirm significant microplastic presence in carnivore scats, with distinct polymer profiles and spatial gradients linked to these anthropogenic sources. This scenario reflects broader challenges faced by biosphere reserves globally, where roads and peri-urban settlements erode ecological buffers, channeling tire-derived particles, agricultural mulches, and household waste into remote interiors [35, 46]. For example, studies in agricultural soils have demonstrated the accumulation of microplastic fragments and fibers originating from sewage sludge and rural waste inputs, highlighting how mismanagement of village-sourced litter contributes to the persistence of plastics in terrestrial ecosystems [13]. Similarly, in urban wetlands, fishing cat *Prionailurus viverrinus* scats contained elevated levels of microplastics, indicating trophic transfer from prey species, paralleling patterns in Golestan's Hyrcanian forests, where ungulate foraging likely vectors village effluents into carnivore diets [47]. These parallels underscore that diffuse vectors, including windborne fibers and hydrological transport, bypass zoning protocols, accumulating in reserve cores and undermining their protective function [10,4].

Transit roads with heavy traffic represent a dominant source of microplastic deposition in Golestan. Tire abrasion and brake dust generate vast quantities of synthetic particles that are transported into soils and streams via stormwater. Our data indicate higher microplastic loads near the park's central artery, reinforcing traffic as a key contamination driver. This aligns with studies from high-use corridors, where tire wear particles (TWPs) comprise up to 30 % of road-adjacent sediments [63]. In western Thailand's forested reserves, dhole *Cuon alpinus* scats near rural trails revealed microplastic ingestion, likely from prey or village waste, a pathway potentially active in Golestan's wolves [60]. Similarly, in Hong Kong's Mai Po Nature Reserve, feral cat (*Felis catus*) scats near expressways revealed elevated microplastic levels, likely from prey exposed to roadside debris, a mechanism potentially active in Golestan's leopards and wolves patrolling road verges [56]. Golestan's road, frequently used by tourists, also disperses litter such as plastic packaging, compounding traffic-derived microplastics. Consequently, generalist carnivores scavenging roadkill or edge prey experience heightened exposure, consistent with our observed elevated microplastic burdens in wolves.

Surrounding villages form a rural pollution nexus, channeling microplastics from agricultural plastics, sewage, and informal dumps into the park's hydrology. Our results suggest that village proximity increases contamination, with textile-derived fibers being prominent in animal scat samples. Widespread plastic use in rural communities breaks down into soil-bound particles, which are ingested by herbivores and subsequently transferred to predators such as leopards and wolves.

Based on studies conducted in the protected areas of China's Qinling Mountains, microplastic pollution has been identified in threatened terrestrial wildlife, including carnivorous mammals [66]. These findings indicate a trophic transfer pathway where microplastics are transferred from contaminated prey to the bodies of predators. This pathway is relevant and crucial for Golestan's rodent-rich understory, which serves as primary prey for wolves and leopards. Furthermore, in China's Northeast Tiger and Leopard National Park, leopard *Panthera pardus* scats found near rural settlements revealed elevated microplastic fibers, likely from household waste contaminating prey. This is a pathway that could be potentially active in Golestan's leopards [24]. These cases highlight Golestan's "rural halo" effect, where villages emit primary and secondary microplastics, permeating various parts of the park and undermining its role as a biodiversity refuge.

The significantly closer proximity of livestock-containing scats to both the central highway and surrounding villages (28 % and 37 % reduction in mean distance, respectively; Table 3) provides direct quantitative evidence that predation and scavenging on domestic animals repeatedly draws gray wolves, and occasionally Persian leopards, into anthropogenic edges where microplastic sources are concentrated. This behavioural mechanism explains the higher total microplastic surface area observed in wolf scats despite comparable particle counts, as wolves exploit human-subsidised food resources near the surrounding villages and the heavily trafficked central highway, thereby increasing their exposure to locally derived polystyrene and polyethylene from packaging waste, agricultural films, and tire-wear particles. In contrast, the Persian leopard's markedly lower consumption of livestock and greater confinement to less disturbed core zones result in reduced contact with these point sources and a correspondingly lower microplastic burden, underscoring how subtle differences in foraging ecology and spatial behaviour within the same protected area can drive substantial variation in contaminant uptake among apex carnivores.

Spatial gradients in microplastic contamination, intensified by the interplay between Golestan's central road and surrounding villages, generate pronounced hotspots that challenge the integrity of the park's protected core. Elevated microplastic loads were observed in scat samples from edge zones, where runoff from roads and rural effluents converges along topographic gradients into forest interiors. This pattern aligns with findings from Peru's Pacaya-Samiria National Reserve, where jaguar *Panthera onca* scats near roads and settlements showed microplastic concentrations up to 2.2 times higher than in core zones, linked to runoff and human-derived debris [8]. Similarly, in the San Francisco Bay Area, coyote *Canis latrans* scats near urban-adjacent highways contained elevated polyethylene fragments, attributed to stormwater transporting litter into habitats ingested by small herbivores and rodents, a mechanism potentially active in Golestan's leopards [22]. These spatial patterns highlight how infrastructure and human settlements create contamination corridors, undermining zoning strategies, consistent with our data showing higher microplastic prevalence near Golestan's arterial road and village perimeters.

The ecological implications extend beyond mere presence. Microplastics' physical and chemical properties, such as adsorption of heavy metals or leaching of endocrine-disrupting additives, pose latent threats to Golestan's carnivores. In central Italy, barn owl *Tyto alba* pellets from sites near urban roads and settlements contained higher microplastic concentrations, predominantly polyester and polyamide fibers, ingested via prey from anthropized zones [43]. Arctic studies revealed microplastics, primarily polyester and polyethylene fibers, in polar bear (Ursus maritimus) scats, likely from prey ingesting litter in

contaminated coastal zones, a mechanism potentially active in Golestan's leopards reliant on ungulate prey [27]. Our detection of weathered polymers with carbonyl shifts indicates similar degradation processes that enhance their affinity for heavy metal uptake, as evidenced by research on microplastics in stormwater [1]. Furthermore, research on animal models has demonstrated that microplastic exposure can alter the fecal microbiome, suggesting broader impacts on digestive health [17]. These findings may highlight cascading effects on the health of apex predators such as the Persian leopard and gray wolf, underscoring the need for further research in this field. Collectively, these results emphasize the urgency of addressing microplastic infiltration, as pervasive contamination could disrupt trophic dynamics and long-term population viability in the park.

Mitigation requires innovative, multi-scalar strategies tailored to Golestan's rural-road interface, where roads and villages create persistent contamination corridors that undermine the park's core protections. Community-based recycling initiatives are essential for curbing plastic pollution, as research on rural waste management demonstrates their effectiveness in reducing emissions from agricultural and household sources [41]. Building on this foundation, local NGOs successfully conducted three roadside clean-up campaigns along the central highway in 2024, removing ~2.2 tons of plastic waste. However, persistent high traffic continues to deposit substantial roadside litter, necessitating sustained action to counter the ongoing influx observed in edge-zone scat samples. Practical measures include establishing village-level plastic agricultural film recycling cooperatives around the protected area to collect and repurpose ~40 % of cropland mulching films currently fragmenting into park soils, directly addressing the PE-dominant residues linked to prey contamination in wolves and leopards. Additional steps involve installing waste collection points and launching awareness campaigns in the 15 surrounding villages, targeting disposable packaging and textile waste through school programs and farmer cooperatives to mitigate the PS/PA/PET sources prominent in carnivore diets, while mandating biodegradable packaging for tourists entering the park to minimize single-use plastic litter from seasonal visitors. Creating physical barriers (e.g., fences or berms) along 8 km of high-risk highway segments can trap tire wear particles and litter before runoff enters forest interiors, complementing the spatial gradients identified in our results [33]. Furthermore, integrating non-invasive scat monitoring with existing camera-trap grids—as trialed in Russian Amur tiger reserves—could enable comprehensive tracking of contamination hotspots using Golestan's wildlife surveillance infrastructure, allowing adaptive management of the trophic transfer pathways highlighted in this study [62]. These locally grounded, community-involved actions provide feasible pathways to reduce microplastic influx, strengthen ecological buffers against the rural halo effect, sustain Golestan's role as a biodiversity refuge, and position the park as an international model for combating microplastic pollution and infiltration in protected areas.

CRediT authorship contribution statement

Pooriya Sepahvand: Writing – original draft, Investigation, Formal analysis. **Majid Moradmand:** Writing – review & editing, Formal analysis. **Ghasempouri Mahmoud:** Writing – review & editing, Supervision, Conceptualization.

Ethical standards

This research abided by the Journal of Hazardous Materials: Plastics guidelines on ethical standards.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.hazmp.2025.100026.

Data Availability

The data has been provided with DOI and are available at the http://doi.org/10.6084/m9.figshare.30146416

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