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Tracking anthropogenic microparticles in wildlife of an alpine insular environment

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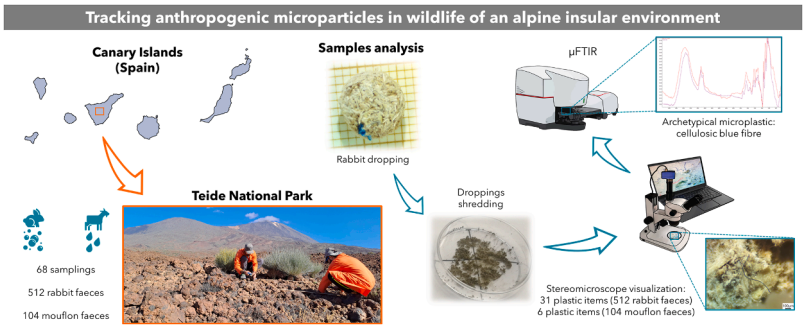
HIGHLIGHTS

- Anthropogenic microparticles in pristine areas and their biota is a growing concern.
- Rabbit and mouflon droppings were analysed from 68 sampling points in an alpine area.
- Sample mechanical shredding was more efficient than conventional chemical digestion.
- Four percent of the scats showed pollution, mainly cellulosic blue microfibres.
- Microparticles enter terrestrial food chains even in regions distant from anthropic areas.

GRAPHICAL ABSTRACT



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ABSTRACT

Despite the isolation of remote natural regions, it has been discovered that they are experiencing the accumulation of anthropogenic microparticles (i.e., microplastics or natural or semisynthetic cellulosic particles). Teide National Park (Canary Islands, Spain) is a high-mountain protected area known for its rich biodiversity. This study aims to assess the occurrence of coloured anthropogenic particles in the faecal matter of wild mammals, specifically rabbits and mouflons, residing in the park. With this purpose, faeces were collected from 68 systematically distributed sampling points. A stereomicroscopy-guided grinding process allowed a chemical-free and quick visual inspection of 616 individual excreta, revealing that 96% were particle-free. However, 37 anthropogenic particles were found, which correspond to 0.79 ± 0.20 items per gram of dry faecal matter. The

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archetypical particle was a cellulosic blue microfibre of 2721 ± 407 μm, though poly(ethylene-vinyl acetate) and polypropylene were also identified via micro Fourier-transform infrared spectroscopic analysis. Atmospheric deposition and touristic pressure may be the sources of the anthropogenic particles, as they were randomly found in 36% of the sampling points. These findings represent the first evidence of anthropogenic particle ingestion by wild rabbits and mouflons, signifying the introduction of microplastics into terrestrial food chains in a remote high-mountain environment.

1. Introduction

In 1907, Bakelite, the first synthetic plastic, was patented [1] and over a century later, global plastic production reached 400.3 million tonnes in 2022 [2]. Plastics have significantly benefited humanity, albeit their excessive production, improper disposal, and resulting pollution have become a global concern [3]. Once released into the environment, plastics break down as a consequence of biotic and abiotic factors to generate mesoplastics (5–25 mm in their largest dimension), microplastics (MPs, 0.1/1–5000 μm) and even nanoplastics (NPs, <0.1/1 μm) [4]. These ubiquitous pollutants may represent a threat to the conservation of the ecosystems since they can be inherently hazardous, or even adsorb organic [5], and inorganic contaminants [6,7], as well as host pathogenic organisms [8], thus exhibiting a potential Trojan-horse effect. Even more worrisome is that, due to their tiny size, they can easily enter food chains, hence constituting a threat to all the living organisms. Therefore, it is crucial to develop methods for detecting and understanding their presence in biota, from microorganisms and plants to animals and humans [9–12].

However, scientific research on MPs in wild mammals has been limited until now [13], despite the potential for such studies to provide insights into their presence in local food chains and their long-distance transport phenomena. As wild mammals generally exhibit an elusive behaviour, their faeces analysis can be used as a non-invasive fashion to evaluate aspects such as their exposure to pollutants. Indeed, this kind of studies has gained importance in the last years (see Fig. S1 of the Supplementary Material), since it is not required to be in contact with the animals [14,15]. For instance, colourful macroscopic plastic pieces have been observed in the scats of arctic fox (*Vulpes lagopus*) in Svalbard, highlighting faeces as bioindicators of plastic pollution [16]. Furthermore, a review of the presence of MPs in the digestive tracts and faeces of marine mammals revealed that MPs were detected in the faeces of pinnipeds (species from the Otariidae and Phocidae families) in 7 out of 10 different studies, with values reaching up to 267 fibres per excrement in the case of the South American sea lion (*Otaria flavescens*) [17].

Nevertheless, studies on the occurrence of MPs in the faeces of wild mammals from terrestrial ecosystems, including semi-aquatic mammals inhabiting freshwater environments, have received comparatively less attention, as evidenced by the publication of only seven previous works which have been summarized in Table S1 (methodology) and Table S2 (results) of the Supplementary Material. The determination of MPs smaller than 1 mm and/or a comprehensive study of MPs has not been the main goal of some of those studies [18–20], thus their conclusions should be considered in perspective. In summary, Table S1 illustrates that MPs have been found in faeces from Eurasian otter (*Lutra lutra*) in Italy [21] and Ireland [22]; European brown hare (*Lepus europaeus*) in Germany and Austria [18]; Asian elephant (*Elephas maximus indicus*) in India [19]; coypu (*Myocastor coypus*) in Italy [23]; European hedgehog (*Erinaceus europaeus*), wood mouse (*Apodemus sylvaticus*), field vole (*Microtus agrestis*), and brown rat (*Rattus norvegicus*) in United Kingdom [24]; and fishing cats (*Prionailurus viverrinus*) in Sri Lanka [20] (see locations in Fig. S2 of the Supplementary Material). By contrast, faeces of bank vole (*Myodes glareolus*), rabbit (*Oryctolagus cuniculis*) and pygmy shrew (*Sorex minutus*) from United Kingdom were also inspected and showed no MPs, probably due to the small sample size (n = 13, 5 and 2, respectively) [24]. Most of the research works were conducted in protected areas such as nature reserves and natural parks. Generally, a chemical treatment of the sample (e.g., with solutions of H_2O_2, NaOH, KOH or Fenton's reagent) was performed before being visualised via stereomicroscopy and analysed by means of Fourier-transform infrared (FTIR) spectroscopy.

As shown in Table S2, particles abundance ranged from 0.0072 items per sample in fishing cat [20] to 11.85 in Asian elephant faeces [19], although only particles ≥ 1 mm were recorded in both cases and the different sample weight hinders a more detailed comparison. Fibre was the dominant shape in 43% of the studies, followed by fragments (29%); films, spheres and sheets were also reported. Colourless, black, clear, green, blue, pink and white items were described. The occurrence of polyamide (PA) and polyethylene (PE) derivatives were reported in 57% of the studies, being thus the most abundant polymers. It was proposed that the faecal plastic pollution come from the involuntary ingestion of plastic rubbish together with edible plants and human wastes [19,23,24], or via predation of animals polluted with plastic in the case of carnivores [20–22], thus showing a trophic transfer of these pollutants.

Bearing this background in mind, the present study was devised aiming at the determination of anthropogenic particles (coloured MPs -100% synthetic particles- and coloured cellulosic particles -either natural or semisynthetic-) in faeces from the two wild herbivorous animals which inhabit Teide National Park (Tenerife, Canary Islands, Spain), i.e., European rabbit (*Oryctolagus cuniculus*) and mouflon (*Ovis gmelini*). *O. cuniculus* arrived to Tenerife during Spanish conquest in the 15th century, whereas *O. gmelini* was brought to Teide National Park in 1971 for hunting purposes [25]. The importance of this sensitive alpine insular enclave lies in multiple factors: a) its extraordinary natural richness: among all the Atlantic oceanic islands, Tenerife is the most diverse with 135 single-island plant endemisms [26]; b) its strategical geographical position: as the highest point above sea level in the Atlantic Ocean islands, it can act as anthropogenic particles sink due to meteorological phenomena [27]; c) its high human presence: in spite of being far from big cities and factories and subjected to several protective legislations, this area receives about 4 million tourists yearly, thus becoming the Spanish National Park with the greatest anthropic pressure [28]. Therefore, it was hypothesised that dry and wet deposition of mainly microfibres over the native vegetation of the National Park could provoke the accidental ingestion of such particles by herbivories, thus resulting in the entrance of plastic pollution in terrestrial food chain. In fact, in a previous work carried out by our group, an average concentration of 51 ± 72 items/L were found in snow fallen in the park in 2021 [27]. To address this thesis, an extensive sampling was designed to representatively cover the entire park area; after appropriate treatment of the samples, the stereomicroscopical visualization and characterization of the anthropogenic particles (attending to the common criteria shape, size, colour and composition determined by μ-FTIR spectroscopy) was expected to shed light on the pollution dynamics both in isolated high-mountain ecosystems as in terrestrial wild mammals.

2. Materials and methods

2.1. Sampling sites

Teide National Park (28°20'N 16°44'W, 28°09'N 16°29'W) covers a 189.9 km^2 area of Tenerife island (2034 km^2). The management of the park corresponds to the Canary Islands Autonomous Community (Spain) since 2010, and its natural, archaeological and ethnographic resources

has led to several international recognitions such as the World Heritage Site of United Nations Educational, Scientific and Cultural Organization (UNESCO) [29]. The National Park can be accessed from three paved roads (TF-21, TF-24, and TF-38) and it has a considerable trails network (41 official pathways for trekking). Moreover, there are many touristic facilities such as 24 viewpoints with parking, a cable car service to transport people from a lower base at 2356 m to an upper base at 3555 m, and 4 viewpoints at the top including Mount Teide peak (3718 m), which is the highest Spanish mountain and the third tallest volcano on Earth if measured from the oceanic floor [30]. Globally, the park exhibits a continental subalpine climate, with temperatures ranging from 3 to 15 °C and mean annual rainfall from 250 to 550 mm [31]. These characteristics has led to a noticeable biodiversity with 37 vegetal species as endemisms of the alpine zone of Tenerife, with more than the half being single-island endemisms. In the last decades, these unique plant species have been threatened by the non-native herbivorous mouflon (*O. gmelini*) and, specially, by the increasing population of European rabbit (*O. cuniculus*) [32]. Sighting of mouflons (see Fig. S3 of the Supplementary Material) and rabbits in the National Park is rare because they are both easily frightened animals, rabbits exhibit crepuscular habits, and mouflons population is low (1.6 individuals per km^2) [33]. However, due to the high population density of rabbits (up to 7.9 individuals per ha), rabbit latrines can be easily found throughout the park [32].

The 68 sampling locations in this research were inspired by a 1 km^2 grid previously designed to systematically perform soil samplings to study the spatial variability of soil properties in Teide National Park (see Fig. S4 and Table S3 of the Supplementary Material), maintaining their alphamerical codes [31]. These locations included 55 rabbit faeces sampling points, 4 mouflon faeces sampling points, and 9 points where both kinds of faeces were sampled. The sampling points were rationally selected to cover nearly the entire surface of the National Park, thereby minimizing the number of required sampling expeditions and mitigating deviations from the official trekking paths. The exact coordinates were recorded with a GPS eTrex® 20x (Garmin International, Inc., Olathe, Kansas, USA) (see Fig. S4 and Table S3). All samples were taken between 2035 and 3077 m (with an average height of 2256 ± 27 m), and no latrines were found at higher elevations. Samples were collected during 14 expeditions from 18th October 2022 to 19th December 2022 (see Table S4 of the Supplementary Material for further expedition details), and meteorological information related to that period (see Fig. S5 and S6 of the Supplementary Material) was obtained from the Spanish Meteorological Agency [34]. Briefly, temperatures ranged between 8.6–19.4, 1.6–17.0, and 0.7–14.9 °C during the study period in October, November and December, respectively. Similarly, accumulated precipitations were 0, 0, and 11.2 L/m^2, albeit it was a heavy rainfall three weeks before the beginning of the sampling (82.4 L/m^2 on 24th September). During the 63 days study period, the wind blew at an average speed of 23.6 km/h, and gusts of wind reached a maximum value of 112.0 km/h at the beginning of December.

2.2. Sample collection

Rabbit faeces were generally located in copious accumulations (latrines) and showed spherical shape, whereas mouflon faeces appeared in small and dispersed amounts and usually exhibited a drop-shape, bigger size and a more densely packed appearance (see Fig. 1 and Fig. S7, S8 and S9 of the Supplementary Material); moreover, mouflon faeces were generally further away from roads, paths and crowded areas. Faecal physicochemical features may differ between organisms depending on the specie, and within the same specie factors such as age, nutritional diet, weight and health status may have influence [14]. Thus, the animal of origin of the excrements was agreed through common consensus by two observers before being sampled, and in case of discrepancy faeces were not collected.

Faeces were collected using stainless steel tweezers and introduced in 40 mL amber glass vials with a screw top solid cap with polytetrafluoroethylene (PTFE) Liner (Supelco®, Sigma-Aldrich Corp., Merck KGaA, Darmstadt, Germany). Vials were stored at room temperature in the laboratory until they were analysed.

2.3. Sample characterization and preparation

2.3.1. Moisture and organic matter determination

For each sample (n = 64 and 13 for rabbit and mouflon dungs, respectively), two aliquots of 8 excrements each were placed into porcelain incinerating dishes, weighted in an analytical balance (Premium Balance Analytic PBA224l-1x, VWR International Eurolab S.L. Barcelona, Spain), and heated at 105 °C for 24 h in an oven (Conterm 80 L, J. P. Selecta, s.a.u., Barcelona, Spain) to determine the moisture content.

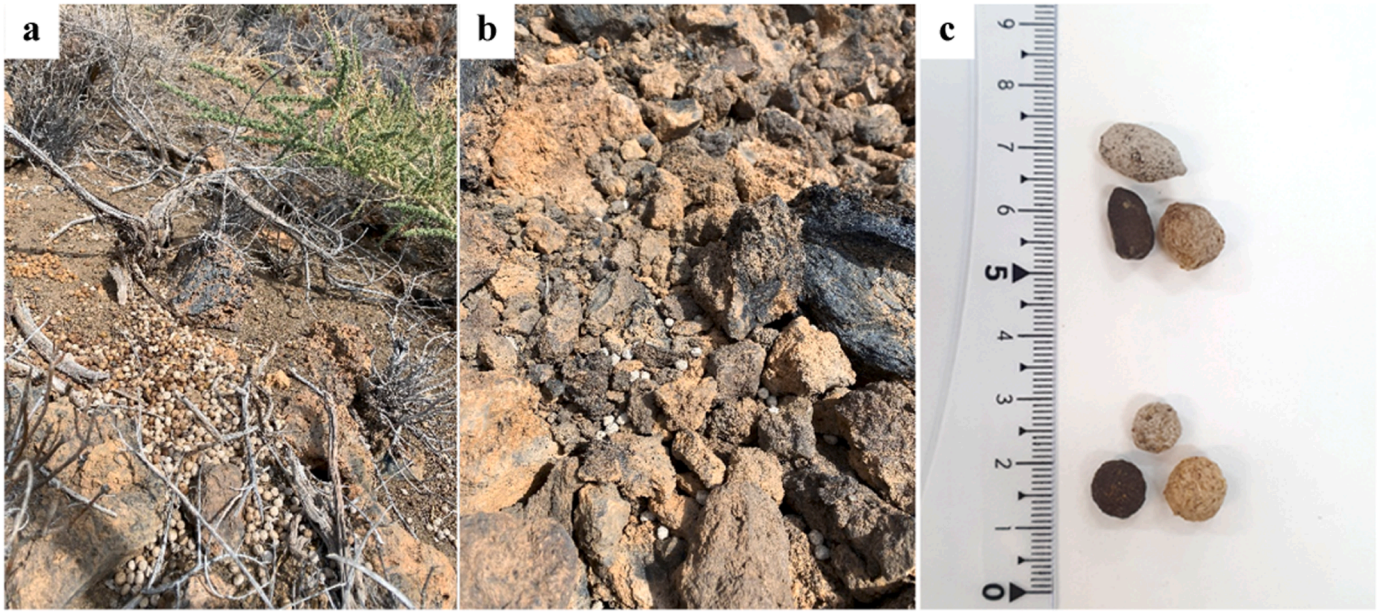

Fig. 1. Faeces samples from sampling point D12: a) rabbit latrine; b) mouflon droppings; c) appearance of representative rabbit (down) and mouflon (up) faeces in the laboratory.

Longer heating times did not relevantly affect the obtained value of moisture content (see Fig. S10 of the Supplementary Material). After that, 36 rabbit and 36 mouflon dehydrated droppings were randomly taken, heated at 200 °C until no smoke was released (30 min approximately) and then calcinated at 450 °C for 8 h in a muffle (CWF 11/13, Carbolite Gero Ltd., Hope, Derbyshire, United Kingdom) to determine the organic matter content.

2.3.2. Chemical treatments

Faeces from sampling point Q7 (see Table S3 and Fig. S4) were randomly chosen for the screening of chemical digestions. They were poured into a beaker and weighted. After that, the following general procedures were tested to chemically digest faeces samples by adding: a) a commercial H_2O_2 33% (w/v) non-stabilised technical grade solution (VWR International S.A.S., Rosny-sous-Bois, France) [35]; b) Fenton's reagent, i.e., an equal amount of a H_2O_2 33% (w/v) solution and a freshly prepared $FeSO_4{\cdot}7\ H_2O$ (Panreac Química S.L.U., Castellar del Vallès, Barcelona, Spain) 2% (w/v) solution which pH was adjusted to 3 by dripping diluted HCl [36]; c) a KOH (AnalaR NORMAPUR®, VWR International bv, Leuven, Belgium) 10% (w/v) solution [22]; and d) a H_2O_2 15% (w/v) and HNO_3 5% (w/v) aqueous mixture freshly prepared from commercial H_2O_2 33% (w/v) and HNO_3 65% (w/v) (Panreac Química S.L.U., Castellar del Vallès, Barcelona, Spain) [37]. When required, faeces were ground on a 50 mL agate mortar and pestle (VWR International Ltd., Leicestershire, UK), using a 16-size polyester (PES) orange paint brush (Innspiro On Line SL, Vinyoles, Barcelona, Spain) to move the sample to the beaker. When digestions were performed without stirring, beakers were placed in a Conterm 80 L oven (J.P. Selecta, s.a.u., Barcelona, Spain). By contrast, if continuous stirring was required during heating, an RCT basic magnetic stirrer with temperature control (IKA®-Werke GmbH & Co. KG, Staufen, Germany) was chosen; in those cases, temperature was carefully monitored during digestion processes to avoid rises above 70 °C that could compromise the chemical stability of the MPs [38].

To achieve a density separation of the particles, all those digestion protocols were followed by at least one flotation step that consisted of adding a freshly prepared NaCl (AnalaR NORMAPUR® ACS, VWR International bv, Leuven, Belgium) saturated solution to the digestion mixture, stirring 1 min at 600 rpm, and then allowing to settle down at room temperature for 1 h. Finally, the supernatant was vacuum filtered onto a stainless steel 53 μm mesh circular filter (AISI-316, Labopolis, S. L., Madrid, Spain) placed over a PTFE adapter disk which is, in turn, in the middle of a filtering apparatus Duran® (VWR International LLC, Radnor, Pennsylvania, USA) (see Fig. S11b of the Supplementary Material). That filtering system consisted on a 1 L filtering flask, a 250 mL funnel, and an adapter provided with a lateral exit which is connected with a flexible PVC hose Rauclair®-E 8/2 8 × 12 (REHAU Group, Muri bei Bern, Switzerland) to a membrane dry vacuum pump/compressor (VP 80, VWR International bvba, Leuven, Belgium); the system was kept together with an anodised aluminium clamp. After filtration, the filter was taken with stainless steel tweezers, carefully placed at a sterile Petri dish with no vents (VWR International bv, Leuven, Belgium), and dehydrated for 24 h at 40 °C in the oven to facilitate the subsequent visualization.

2.3.3. Mechanical treatment

Eight droppings per sampling point and animal type (i.e., 512 rabbit and 104 mouflon excrements) were individually placed into Petri dishes, weighted, and then ground while being visualized in a stereomicroscopy-supported process (see Fig. S12 of the Supplementary Material). Firstly, each dropping was spun several times using one straight and one lancet-shaped needle from a dissecting set (BMS 16645, BMS Microscopes b.v., Capelle aan den IJssel, The Netherlands) to meticulously observe its surface. This process allowed to discard any superficial deposited plastic contamination, considering only particles from the inside or embedded in the scats (see Fig. S13 of the Supplementary Material). Subsequently, the dropping was carefully shredded with the needles, looking for anthropic particles and separating the crumbled organic matter to the borders while monitoring the process through the stereomicroscope lens. The viewing time per excreta typically ranged between 5 and 10 min

2.4. Anthropogenic particles identification and characterization

A NexiusZoom EVO 0.65–5.5 Model NZ.1703-S stereomicroscope provided with a trinocular head and HWF 10x/23 mm eyepieces (Euromex Microscopen bv, Arnhem, The Netherlands) allowed the identification and counting of the particles bigger than 50 μm, and also acted as the visual support for catching the particles using stainless steel forceps with a straight tip (Dumont #5, Fine Science Tools Inc., North Vancouver, British Columbia, Canada); once caught, the particles were placed in an aluminium micro mount sample slide (ALUM EZ-SPOT MICRO MOUNT 5 L, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) located inside a Petri dish where they were stored until their composition was determined. Particle size was measured employing the LevenhukLite software version x64 after being photographed with a Levenhuk M1400 PLUS-14Mpx digital camera integrated into the stereomicroscope (Tampa, Florida, USA). The composition of the particles was confirmed via the acquisition of their infrared spectra with a Thermo Scientific Nicolet™ iN™10 microspectrometer. Thermo Scientific OMNIC™ Picta™ software version 1.8.259 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used to configure the acquisition parameters (reflection mode, cooled detector, 5 s of collection time, high spectral resolution, aperture of 50 x 50 μm, no focus during collection, and optimal Blackman/Harris signal filter) and to compare the obtained spectra with those from reference libraries (wizard_poly, HR Sprouse Polymers by Transmission, FLOPP and Bibliotheque Particules). Forty one percent of the identified particles were analysed by means of μ-FTIR, and the suggested composition was accepted only if a similarity percentage of 70% or higher was found with a reference spectrum.

2.5. Contamination control

Several measures were implemented during field and laboratory work to reduce as much as possible the self-inflicted contamination of the samples. Thus, orange laboratory coats Workteam® B6700 (Confecciones Mayton S.L.U., Ribaseca, León, Spain) were dressed during all the stages of the working process; as these coats were made of PES and cotton (65% and 35%, respectively), both constituent fibres were analysed via μ-FTIR spectroscopy and their corresponding spectra were saved as reference in our own library. Thus, if orange fibres with that composition were found in the sample, they were discarded (see Fig. S14 of the Supplementary Material). In any case, a lint roller (Mercadona S. A., Tabernes Blanques, Spain) was used to exhaustively clean both clothes as coats to remove superficial microfibres before working either at the field or at the laboratory. Moreover, any possible contamination derived from own microfibres was minimized during field work by sampling with wind in the samplers' face, and using aluminium foil to immediately cover the opening of vials containing faeces samples before closing the plastic cap. Before sampling expeditions, the aforesaid vials were abundantly washed first with tap water and then with Milli-Q water obtained from a Millipore Milli-Q Gradient A10 water purification system (Burlington, Massachusetts, USA), to be finally covered with aluminium foil and calcinated at 550 °C for 4 h. Milli-Q water was stored until necessary in glass bottles using aluminium foil between the opening and the plastic cap. The tweezers were gently rinsed with Milli-Q water and covered with aluminium foil between different sampling points.

Regarding the laboratory work, the surface of the working area was cleaned with an orange cloth (Mercadona S.A., Tabernes Blanques, Spain) made of an easily recognisable mixture of PES and PA microfibres

(82% and 18%, respectively) and moistened with Milli-Q water. As described above with the laboratory coat fibres, both constituent materials were registered (μ-FTIR analysis); similarly, PES microfibres spectrum from the orange brush employed to clean the mortar after samples grinding was also recorded. Hands were always washed with Milli-Q water and air-dried before handling the samples in an own-designed 0.33 m^3 methacrylate glove box (1 x 0.5 x 0.65 m length x width x height). It was placed, together with the stereomicroscope and the μ-FTIR system, in a 68 m^3 isolated part of the laboratory that simulated a cleanroom (5 x 4.25 x 3.2 m length x width x height), and in which two air purifiers (Rowenta PU3030, Groupe Seb Ibérica S.A., Barcelona, Spain) were in continuous operation. Before the chemical treatments of the samples, non-volumetric glassware was washed with Milli-Q water and heated at 550 °C for 4 h to remove any rest of organic matter including MPs; with the same purpose, the stainless steel filters were placed in borosilicate glass Petri dishes and heated at 450 °C for 4 h. In addition to retain anthropogenic particles from the samples during the filtration step, those filters were used for contamination control as procedure blanks (i.e., to filter the content of a beaker containing the corresponding studied reagents but with no faecal sample), and glove box/stereomicroscope blanks (i.e., whenever the sample was uncovered, they were placed to the side and also exposed to the air) (see Fig. S12); in all cases, they were then visualized to discard analogous pollutant particles. Commercial H_2O_2 and KOH solutions were filtered through 0.22 μm pore size Durapore® membrane filters made of hydrophilic polyvinylidene difluoride; analogously, NaCl solution was filtered using a 0.45 μm pore size MF-Millipore® filter of mixed cellulose esters (Merck Millipore, Massachusetts, USA).

2.6. Geospatial and statistical analyses

The geographic information system software QGIS (QGIS.ORG, Gossau, Switzerland) and the statistical software SPSS Statistics version 26.0.0.0 (IBM Corporation, Armonk, New York, USA) were employed for performing geospatial and statistical analyses, respectively. Fresh excrement weight, water and organic matter content showed a normal distribution according to the Lilliefors corrected Kolmogorov–Smirnov test and homogeneity of the variance according to the Brown–Forsyth test, so they were submitted to an analysis of variance (ANOVA) to inspect statistical significances. Anthropogenic particles concentration expressed as items per g of dry faecal matter did not show a normal distribution, but they did show homogeneity of the variance, so they were submitted to the non-parametric Kruskal–Wallis *H* test to look into statistical significances. Correlations were established based on Pearson correlation coefficients at the significance level $\alpha = 0.05$. All the given values are expressed as mean ± standard error.

3. Results and discussion

3.1. Faeces characterization

Gravimetric analyses reflected that water content of rabbit faeces ranged from 2.72 to 5.99% with an average value of 3.85 ± 0.08%. Mouflon faeces moisture content oscillated between 2.62% and 7.13% and showed a significantly higher mean value of 4.36 ± 0.35% ($p = 0.037$). Average fresh weight of individual excreta was also significantly higher for mouflon faeces (99.37 ± 9.54 vs 74.05 ± 1.59 mg, $p = 0.000$), albeit similar values of the organic matter content were found in the faeces of both species (91.96 ± 1.32 and 89.13 ± 0.76%, respectively, $p = 0.082$) (see Fig. S15, S16 and S17 of the Supplementary Material). Previous studies on MPs in excrements from similar species, i.e., European brown hare [18] and European rabbit [24], began with 0.5 g of fresh samples (n = 3) directly collected from the rectum after autopsy and with dry faecal samples ranging from 0.2 to 0.4 g (n = 5), respectively. However, data about the weight of individual excreta, as well as water or organic matter content, were not available.

3.2. Sample treatment optimization

Most of the studies involving terrestrial wild mammal faeces summarised in Table S1, employed a chemical pre-treatment of the samples. Thus, several chemical procedures were initially tested to achieve the digestion of organic matter from rabbit faeces randomly selected from sampling point Q7 (see Table 1). Initially, it was tried a previously optimized protocol for particles extraction from sediments, consisting of a digestion/flotation/filtration tandem [35]. Firstly, 10 g of rabbit faeces were submitted to a digestion step with 40 mL of H_2O_2 33% (w/v) to degrade the organic matter (a 1/4 sample/digester ratio); secondly, a flotation step with 100 mL of NaCl saturated aqueous solution allowed a density separation of the particles; and thirdly, a vacuum filtration step was used to transfer the supernatant through a filter to retain the particles (entry 1). However, this process was not able to eliminate the undigested plant remains that make up practically the entire sample (91.96 ± 1.32%, as indicated above). The low density of this vegetal material caused its migration to the supernatant during flotation, and the subsequent blocking of the filtration system above the filter (see Fig. S11). To avoid this sample loss, the faeces amount was reduced to 1 g and the sample/digester ratio was increased to 1/20 (entry 2). This modification prevented the filtration system from blocking, albeit the

Table 1
Faeces chemical digestion procedures tested in this work.

Entry	Sample amount (g)	Sample treatment[a]	Sample/ digester ratio	Observations
1	10	40 mL of H_2O_2 33% (w/v), 300 rpm, 2 h, 60 °C	1/4	Loss of material due to obstruction of the filtration system
2	1	20 mL of H_2O_2 33% (w/v), 300 rpm, 2 h, 60 °C	1/20	Supernatant was transferred in 3 portions to 3 different filters to avoid their obstruction. A 1205 μm blue cellulosic fibre was identified
3	0.5	40 mL of H_2O_2 33% (w/v), 300 rpm, 3 h, 60 °C; then 45 h, rt	1/80	[b]
4	0.5	20 mL of H_2O_2 33% (w/v) plus 20 mL of $FeSO_4{\cdot}7\ H_2O$ 2% (w/v) at pH 3, 300 rpm, 3 h, 60 °C; then 45 h, rt	1/80	[b]
5	0.1	Digestion with 10 mL of H_2O_2 33% (w/v), 300 rpm, 2 h, 60 °C	1/100	A 2070 μm blue cellulosic fibre was identified in the second filtration starting from non-ground sample[c,d]
6	0.1	5 mL of H_2O_2 33% (w/v) plus 5 mL of $FeSO_4{\cdot}7\ H_2O$ 2% (w/v) at pH 3, 300 rpm, 2 h, 60 °C	1/100	[b,c,d]
7	0.1	10 mL of KOH 10% (w/v), 48 h, 40 °C	1/100	[b,c,d]
8	0.1	10 mL of H_2O_2/HNO_3 15/5% (w/v), 24 h, 40 °C	1/100	[b,c,d]

[a] After the described digestion step, the same flotation/filtration protocol was performed by adding freshly prepared NaCl saturated aqueous solution, stirring at 600 rpm for 1 min, and allowing to settle for 1 h at room temperature prior to the vacuum-assisted filtration; [b] No plastic items found; [c] A variation of the protocol in which the sample was ground before the digestion was also tested; [d] 2 flotation steps were performed. rt: room temperature.

filter quickly filled with particles and had to be replaced twice with two new filters (see Fig. S18 of the Supplementary Material). No anthropogenic particles were firstly detected in any of the bulky filters when they were examined under the stereomicroscope, although after carefully rummaging with needles in the vegetal layer, a blue fibre was identified (see discussion about colourless microfibres in the next subsection).

Beriot et al. also experienced a similar problem with filtration as a consequence of the high organic matter content in some sheep faeces [39]. To minimize the undigested material amount and thus facilitate the visualization of the filters, a sample amount of 0.5 g and a 1/80 sample/digester ratio were established (entries 3 and 4). Moreover, Fenton's reagent was tested as digester, in line with the protocol described by Thrift et al. to digest terrestrial small mammal faeces (entry 4) [24]. Both suspensions showed again a turbid aspect (see Fig. S19 of the Supplementary Material), although only one filter was required during filtration in both cases. Again, it was required to crumble the vegetal material with needles under stereomicroscope to reliably discard the presence of anthropogenic particles. Based on these experiences, a single dropping (0.1 g approx.), a 1/100 sample/digester ratio and a second flotation step were finally fixed, and both H_2O_2 and Fenton's reagent were again tested; additionally, previously ground droppings were also submitted to those conditions (entries 5 and 6, see also Fig. S20 of the Supplementary Material). Only one blue fibre was identified after foraging in the filter with the needles (entry 5). Finally, as shown in entries 7 and 8 (see also Fig. S21 of the Supplementary Material), both ground and non-ground samples were submitted to a basic sample treatment as described by O'Connor et al. for the digestion of Eurasian otter faeces [22], and also to an acidic sample treatment adapted from that reported by Toto et al. for the digestion of non-wild rat faeces in a laboratory study [37]. Once again, disintegration of the vegetal material over the filter was mandatory to assure the presence or absence of anthropogenic particles.

In summary, in spite of having applied several digesting agents to different amounts of ground and unground samples, it was always necessary to rummage through the filter to check the presence of anthropogenic particles (see Fig. S22 and S23 of the Supplementary Material); thus, it was concluded that the chemical digestion and flotation steps could be directly replaced by a mechanical breaking up of the faeces, similarly as described by Katlam et al. and Ratnayaka et al. in their identification of > 1 mm plastics in Asian elephant [19] and fishing cat excrements [20], respectively. To broaden their scope of identifiable particles, it was established a stereomicroscope-directed physical grinding of the droppings in Petri dishes (see Fig. S12). Moreover, the suppression of the chemical digestion/flotation sequence translates into a greener and less time-consuming experimental procedure, and contribute to minimise the MPs contamination from chemical reagents [40].

3.3. Anthropogenic particles identification criteria

Following the optimization of the sample preparation protocol, a significant number of colourless fibres were observed in rabbit and mouflon droppings, displaying characteristics like brightness, sinuous shapes, and resistance to touch, with some featuring small blue bands (see Fig. S24, S25 and S26 of the Supplementary Material). Fortunately, μ-FTIR analysis confirmed their natural origin, aligning with the use of spectroscopic techniques to identify the source of environmental microfibres and prevent the overestimation of particles [41]. Abundant blue spotted microfibres were unequivocally identified as rabbit hair after comparing them to wild rabbit fur (see Fig. S24). Similarly, Smiroldo et al. ruled out eggs of amphibian, fish, and crustacean as spherical particles suspected of being MPs in the droppings of Eurasian otters [21]. While some colourless fibres were associated with an animal origin (see Fig. S25), most displayed a cellulosic composition (see Fig. S26), suggesting they could be mainly derived from frayed vegetal material due to the presence of microscopic plant tissues in the faeces. Although the presence of colour does not necessarily imply a synthetic origin of the fibre [42], it is common for man-made plastics and transformed natural (e.g., cellulose) or semisynthetic polymers (e.g., viscose or rayon) to yield non-naturally coloured fibres -anthropogenic particles- due to the addition of dyes and chemicals [43]. Given the specific characteristics of the samples and to avoid overestimating the concentration of anthropogenic particles, it was decided to exclude colourless particles and focus on coloured ones, a criterion previously used in anthropogenic particles analysis across various sample types, including air, sediments, and ocean water [43–46].

3.4. Anthropogenic particles abundance

In the case of rabbit excrements, and as previously indicated, 8 rabbit droppings were analysed per sampling point ($n = 512$). After the analysis, 489 of the droppings (95.5%) showed no coloured anthropogenic particles pollution, while 16 (3.1% of the total), 6 (1.2%) and 1 (0.2%) contained 1, 2 and 3 items, respectively. Bearing in mind the 64 studied sampling points, 42 of them (65.6%) were free of particles, while 21 (32.8%) exhibited 1 excrement with at least 1 particle, and only in sampling point L14 (1.6%) 2 different excrements had particles (see Fig. 2 and Table S3). A maximum value of 4.58 ± 3.21 items per g of dry faeces was precisely found in sampling point L14; only sampling points L14, R9 and K15 exhibited concentrations higher than 4 items per g of dry faeces. A value of 0.79 ± 0.16 items per g of dry faeces was found as average when considering all the sampling points (0.061 ± 0.012 items per excrement). It should be remarked that no particles were found when the 8 rabbit droppings from sampling point Q7 were shred; by contrast, two fibres had been identified from Q7 faeces when chemical digestions were performed (Table 1, entries 2 and 5). This finding is not necessarily contradictory, and may be attributed to the bigger amount of sample used in entry 2, or simply to the haphazard selection of excrements. Gallitelli et al. also described a random distribution of the number of anthropogenic items in the 30 studied coypu excrements, independently of the faeces wet weight [23].

Regarding mouflon, 101 (97.1%), 1 (1.0%), 1 (1.0%), and 1 (1.0%) droppings contained 0, 1, 2 and 3 anthropogenic particles, respectively. As shown in Fig. 2 and Table S3, 11 sampling points (84.6%) were particles-free, whereas the sampling point D12 showed 1 excrement containing 2 plastics, while 2 plastic polluted excrements were detected in sampling point R9, which corresponds with 5.7 ± 5.7 and 4.51 ± 3.41 items per g of dry faeces, respectively, being the average concentration 0.79 ± 0.54 items per g of dry faeces when the 13 sampling points were considered (0.058 ± 0.042 items per excrement). Noticeably, from the 9 points in which both kind of faeces were sampled, only R9 displayed plastic pollution in excreta from both rabbit and mouflon. The ingestion of anthropogenic particles by mouflons is in line with previous observations in which plastic materials were the dominant foreign bodies found after emptying the gastrointestinal tract of other ruminating mammals such as cattle, goats and sheep [47,48]. In the work of Beriot et al. [39], authors found 997 ± 971 items per kg of dry sheep faeces; they reported that sheep ingested plastics when feeding at the sheepfold, and also during free roaming on fields where plastic mulch was applied.

As shown in Table S2, Thrift et al. found no MPs in 5 faecal samples of rabbit (0.2–0.4 g of dry weight per sample) collected from British gardens, which is in accordance with their small sample size [24]. Moreover, they reported an apparently low value of 0.32 items per g of dry faeces from four different small mammals, but only positive samples were considered to calculate that amount; indeed, they reported that 43 from 261 samples (16.5%) were plastic polluted, whereas in our work only 26 excrements from 616 (4.2%) exhibited particles. The percentage of polluted faeces was also higher in Eurasian otters (57% of 53) [22], Asian elephants (32% of 75) [19], and coypus (100% of 30) [23] (Table S2). Regarding the number of items per excrement, findings from our work (0.06 items per excrement) are intermediate between those

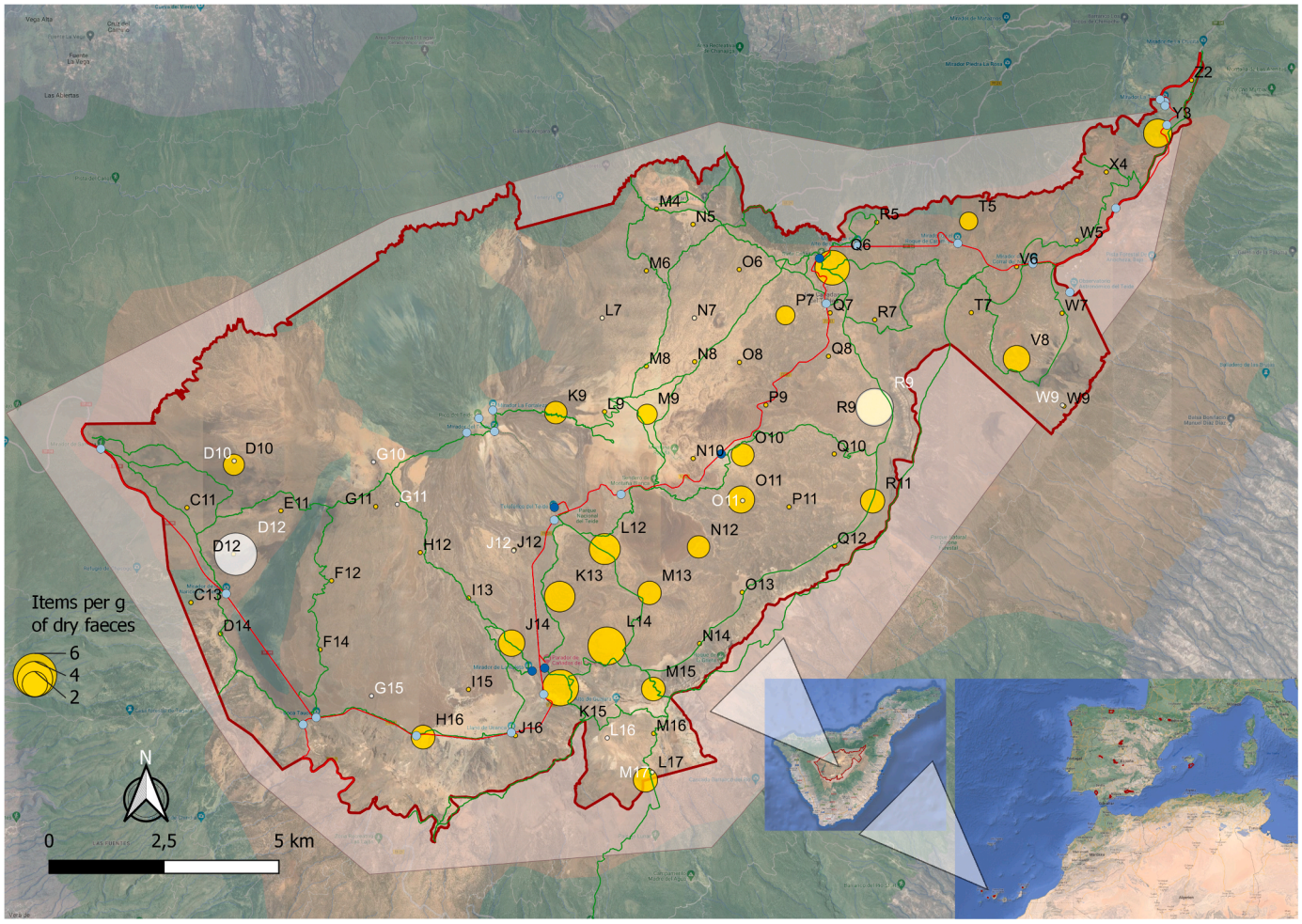

Fig. 2. Geospatial distribution of anthropogenic particles in rabbit (yellow dots) and mouflon (white dots) excrements in Teide National Park; yellowish dots in L7, N7 and R9 are consequence of the overlapping of equal sized yellow and white dot. Red dark line: National Park perimeter; pink area: peripheral protection zone; red line: paved roads; green lines: official pathways; blue dots: parking and viewpoints subjected to high anthropic pressure; dark blue dots: idem as before but with extremely high anthropic pressure. See Table S3 of the Supplementary Material for further details.

reported for fishing cats (0.0072) [20] and for Eurasian otters from Italy (0.02) [21], and those for Eurasian otters from Ireland (1.2) [22] (Table S2).

The nearly identical count of anthropogenic particles detected in faeces from both herbivores from Teide National Park, which stands at 0.79 items per gram of dry faeces, demonstrates the integration of anthropogenic materials into food chains and sides with the accidental ingestion of MPs previously proposed for other herbivores such as Asian elephants [19], coypu [23], and small mammals [24]. This phenomenon may be associated with dietary preferences, as herbivores consume plants that might have been exposed to such particles through dry and wet deposition. For instance, endemic bush flixweed (*Descurainia bourgeauana*, family Brassicaceae) is widely spread along the National Park and has been reported as an important component of the diet of both animals; additionally, other plants such as moralito (*Rhamnus integrifolia*, family Rhamnaceae) and tagasaste (*Chamaecytisus proliferus*, family Fabaceae) have also been described as dietary components when both herbivores feed in the near pine forests [25,49,50]. Moreover, Teide National Park is an elevated (sampling sites at 2256 ± 27 m) and particularly windy area (23.6 km/h as average during the sampling period), and both factors may contribute to the atmospheric deposition of anthropogenic particles, as reported for protected National Parks and wildernesses of the United States [44].

As outlined in Fig. 2 and detailed in Table S3, anthropogenic particles were found dispersedly in a third of the sampling points with no statistical differences between polluted points ($p = 1.000$). This finding is interesting because some of the sampling points were located relatively close to paved roads, pathways and viewpoints with parking, i.e., places with an expected high anthropic pressure, and consequently a presumed higher risk of plastic pollution. However, no correlations were found between particles abundance and distances from the sampling points to those locations or height (Table S5 of the Supplementary Material). Analogously, O'Connor et al. found no significant differences in MPs concentrations in Eurasian otter faeces from three different Irish regions, neither in excrements collected from areas of expected lower or higher exposure to MPs, i.e., upstream or downstream of urban wastewater treatment plants (UWWTPs), UWWTP biosolid application sites, and licensed waste facilities [22]. Contrastingly, Katlam et al. reported a spatial trend when described that the number of plastic particles in the excrements of Asian elephants was 2.4-fold higher in samples collected inside forests than in those from the forest edges subjected to higher human activities; this was explained by the evasive behaviour of elephants, which may quickly consume plastic polluted organic wastes in the edges before moving to deep forest to minimize human disturbance [19].

3.5. Anthropogenic particles characterization

Examples of differently shaped particles found in rabbit faeces are shown in Fig. 3, being the vast majority fibres (n = 29), although films

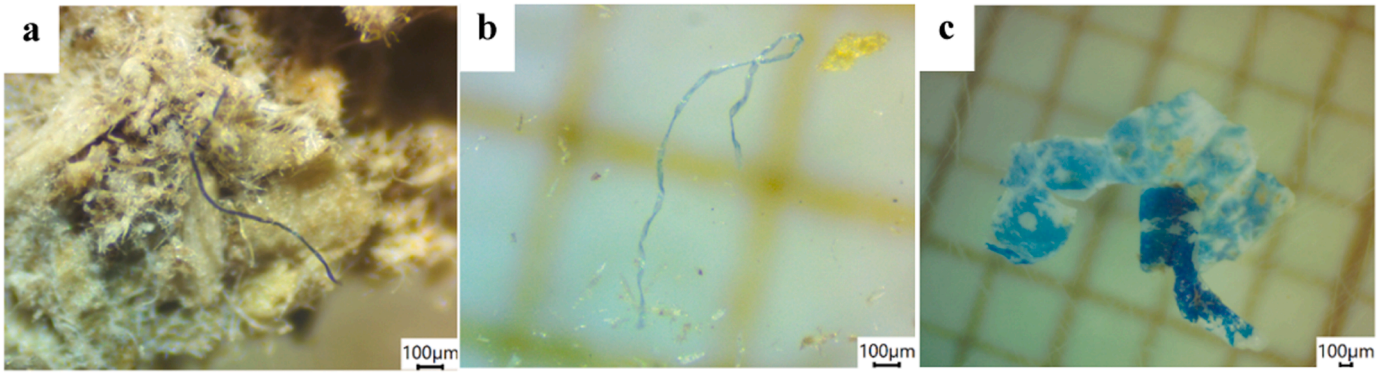

Fig. 3. Photographs of representative anthropogenic particles items found in rabbit faeces: a) a dark blue fibre from O10; b) a blue film from M13; c) a colourless and blue fragment from M9.

and fragments were also punctually identified. In mouflon faeces only 6 fibres were detected, probably because the sample size was 5-fold less abundant. Thus, the morphological distribution was globally (considering both animals) 94.6:2.7:2.7% fibre:film:fragment (n = 37).

Microfibres may shed from outdoor clothing and footwear [44,51], which sides with the high touristic activity registered in the National Park [28]. Microfibres were also the most abundant items found in the snow collected in the park in 2021 [27]. Moreover, in their study about the wet and dry atmospheric deposition of MPs in 11 protected and isolated areas across the USA, Brahney et al. found that microfibres were around 2-fold more abundant than non-microfibres [44]. They also reported that deposited non-fibrous particles ranged between 4 and 188 µm, with most of them showing sizes below the lower visualization limit considered herein (50 µm). Thus, the detected fragment (4134 x 2037 µm length x width) in sampling point M9 (Fig. 3c) may come from an in situ fragmentation of a bigger plastic material such as clothing, shoes or litter [52].

Fig. 4 shows the histograms with size and colour distribution of the anthropogenic particles found in rabbit and mouflon faeces, respectively; mean lengths were 2082 ± 285 and 6022 ± 1507 µm. A relevant amount (6% and 50%, respectively) of particles bigger than 5 mm was found in both matrixes, although the low number of particles in mouflon faeces hinders further comparisons. In rabbit faeces, higher amounts (16%) were found for particles in the 750–1000 and 2000–2250 µm range, albeit the majority (42%) of the particles ranged from 750 to 1500 µm. Similarly, in snow collected at Teide National Park, 31% of the total found fibres (n = 1775) ranged between those values, though in that work most of them showed a lesser size and were classified in the 250–500 and 500–750 µm groups (20% and 18%, respectively) [27]. Regarding colour distribution, dark blue was recurrently found in rabbit samples (84%), followed by pink (10%) and a mixture of colourless and blue (6%), probably because photodecolouration is enhanced in blue plastics [53]. Half of the fibres from mouflons were also blue, and curiously the other half corresponding to grey fibres came from the same dropping. Blue fibres were also the most frequently found (53%) in the National Park snow samples [27]. This prevalence of blue particles in the environment is linked to the bigger sunlight-mediated aging that suffer blue plastic due to the higher UV transmittance [53].

The composition of ingested anthropogenic particles may play an important role in their impact on the digestive system of exposed fauna and, consequently, their health. For instance, it has been reported that PES microfibres can become trapped in the gut of soil-dwelling earthworms (*Lumbricus terrestris*). This entrapment leads to a reduced assimilation of food and results in a decrease in faecal production proportional to the increasing concentration of microfibres [54,55]. Three different polymers were identified by means of µ-FTIR spectroscopy (Fig. S27, S28 and S29 of the Supplementary Material), being cellulosics over-represented both in the analysed rabbit and mouflon faecal particles (82% and 100% based on 11 and 4 particles analysed, respectively). In our case, due to the high similarities in the FTIR spectra between natural and semisynthetic cellulosic particles, which renders their classification challenging, we have categorised both groups as cellulosic. As previously mentioned, all of these particles are coloured, indicating an anthropogenic origin. Furthermore, a blue poly(ethylene-vinyl acetate) (PEVA) fibre and a colourless and blue blended polypropylene (PP) fragment were also found in rabbit faeces. This absence of synthetic

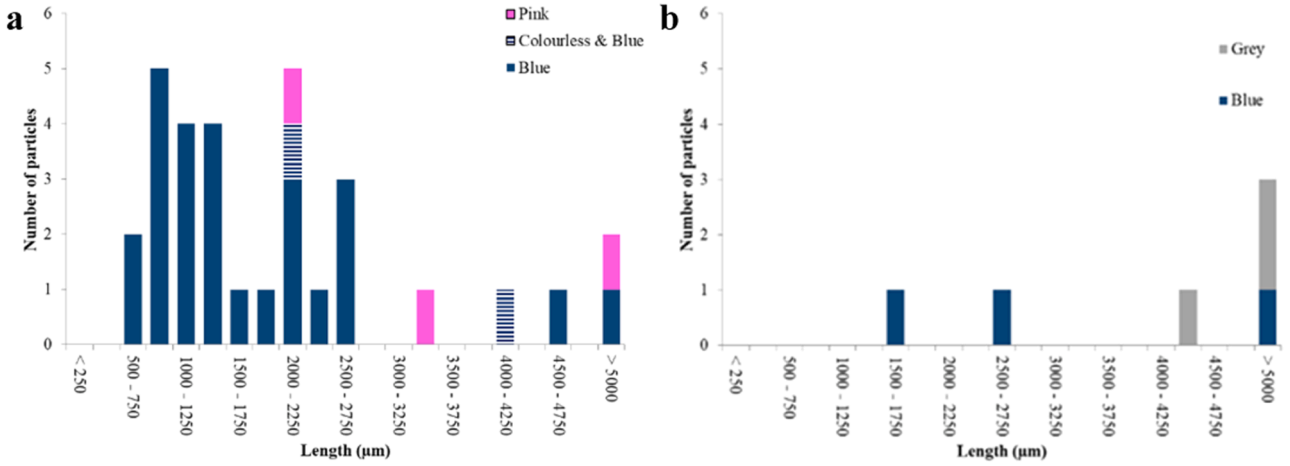

Fig. 4. Distribution of the size and colour of the anthropogenic particles found in rabbit (a, n = 31), and mouflon faeces (b, n = 6). Lower visualization limit established at 50 µm.

particles in mouflon faeces might be correlated with their rumen microbial community, since it has been proved that the rumen content from cattle (*Bos taurus*) is able to hydrolyse ester bonds in PES polymers, though more studies should be developed to confirm this issue [56]. Cellulosic particles from rabbit faeces were blue (78%) and pink (22%), whereas those from mouflon faeces were blue (50%) and grey (50%). This predominance of cellulosic particles could be expected since, as Finnegan et al. established, cellulosic microfibres are 2.5-fold more abundant than synthetic microfibres in the atmosphere [43]. Similarly, Villanova-Solano et al. reported a 2.1-fold prevalence of cellulosic microfibres regarding synthetic microfibres in Teide National Park snow [27]. Prior studies also carried out in Tenerife but focused on marine food chains, have revealed the presence of anthropogenic particles in sea urchins and fishes, accounting from 0.04 to 0.5 items per gram of tissue. Notably, these studies consistently identified prevalent cellulosic blue microfibres among the MPs [57–59]. This can be interpreted as regular exposure of the biota in the island ecosystems to these pollutant particles. In studies focused on wild marine mammals, cellulosic particles were only reported in the gastrointestinal tract of Indo-Pacific hump-backed dolphin (*Sousa chinensis*) but not in the faecal matter of pinnipeds. In these cases, fragments and fibres were the most common shapes, while white and blue were the most abundant colours, with PE and PA being the most recurrent polymers [17]. Regarding the faeces of terrestrial mammals, only Smiroldo et al. [21] and O'Connor et al. [22] reported explicitly the detection of cellulosic particles (see Table S2). Other authors just omitted them (especially those colourless) due to their possible natural origin [43]. For its part, two black PEVA fragments were found in hedgehog faeces [24], and PP was detected in hedgehog [24], European brown hare [18], Eurasian otter [22] and fishing cat [20].

4. Conclusions and further work

In spite of the legislative measures led to protect the rich natural legacy of Teide National Park, anthropogenic particles were recently detected in its snow, and now for the first time in the faecal matter from the two herbivorous inhabitants by using a novel, quick and reagent-free protocol. The concentration determined in this work is lower than those found in faeces from other terrestrial wild mammals, but enough to lay bare the omnipresence and the inherent mobility of anthropogenic microfibres, which can traverse long distances carried by air and then entering even remote ecosystems through dry or wet deposition. Once there, inhabitants are exposed to this pollution through diet, as demonstrated herein based on the dropping analysis. The prevalence of materials identified as cellulosics suggests the need for a more in-depth characterization of these components to distinguish between a natural or semi-synthetic origin, thereby avoiding overestimating plastic pollution levels.

Further research is required to evaluate the impact of these contaminants in mammals, as well as in other environmental compartments within isolated regions. Given the widespread distribution of rabbits across Tenerife and beyond, the innovative stereomicroscope-guided grinding protocol optimized herein offers a valuable tool for detecting coloured anthropogenic particles in fecal samples from various locations. This extends our ability to establish meaningful comparisons, assess the viability of feces as bioindicators of anthropogenic particle pollution, and enhance our understanding of the presence and consequences of these particles within terrestrial food chains worldwide.

These findings underscore the critical need for ongoing vigilance and conservation efforts within Teide National Park and similar natural sanctuaries. The broader environmental implications extend beyond this park, serving as a reminder of the global challenge posed by anthropogenic particles in natural ecosystems, highlighting the paramount importance of preserving these pristine environments for biodiversity conservation.

CRediT authorship contribution statement

Sergio J. Álvarez-Méndez: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data curation, Writing Original-Draft, Writing -Review & Editing, Visualization. **Francisco J. Díaz-Peña:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Data Curation, Writing Original-Draft, Writing -Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **Santiago Gómez-Escabia:** Formal Analysis, Investigation, Data curation, Writing Original-Draft, Writing -Review & Editing. **Javier González-Sálamo:** Methodology, Validation, Formal Analysis, Writing Original-Draft, Writing -Review & Editing. **Javier Hernández-Borges:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Data Curation, Writing Original-Draft, Writing -Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Environmental Implication

The main goal of this work is the determination of anthropogenic particles in rabbit and mouflon faeces from a relatively isolated and protected alpine area in Tenerife, the highest island in the Atlantic Ocean. Currently, microplastics and natural or semisynthetic cellulosic particles constitute a concerning group of emerging contaminants that are causing important environmental problems, also to biota. Therefore, it is crucial to monitor their occurrence in all the environmental compartments to characterise their physical features and chemical composition, thus delving into their sources, distribution patterns and fate, especially when enter food chains.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francisco J. Díaz-Peña reports financial support was provided by Canarina Foundation. Javier Hernández-Borges reports financial support was provided by Canarina Foundation. Javier González-Sálamo reports financial support was provided by Canarian Agency for Research Innovation and Information Society. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

I have shared the link to my data at Attach File step.

Acknowledgements

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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.jhazmat.2023.133291.

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