Environmental Toxicology and Chemistry, 2025, 1–9
https://doi.org/10.1093/etojnl/vgaf082
Advance access publication: March 25, 2025
Brief Communication

Investigating the presence of nanoplastics in freshwater chironomids from glacial habitats using Raman spectroscopy

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Abstract

The detection of nanoplastics (NPs) in the natural ecosystems is challenging due to the size and the low concentrations of NPs. The aim of the present study is to investigate the presence of NPs in larvae of two chironomid species (*Diamesa zernyi* and *Diamesa tonsa*) colonizing two high-altitude glacier-fed streams (Mandrone and Amola streams, Trentino, Italy). The analytical method developed in this work combines enzymatic and oxidative digestion followed by a purification step in ethanol to enable on-chip identification through Raman spectroscopic analysis. To validate the extraction procedure, three pools of 100 mg (wet wt) each of *Diamesa zernyi* larvae from the Mandrone stream were spiked with polystyrene NPs of 500 nm in size at two different theoretical concentrations (10^7 and 10^9 particles/ml). Quantification of the particles in the residual matrix was performed using Single Particle Extinction and Scattering analysis. The results demonstrate good recovery rates, respectively, of 109 ± 28% and 82 ± 12% for the high and low concentration spiked samples. This methodology enabled the effective identification of plastic particles using confocal Raman spectroscopy. Successively, three pools of 100 mg (wet wt) of non-spiked specimens of *Diamesa tonsa* from the Amola stream were analyzed revealing the presence of polystyrene particles. Despite the low number of replicates from only one analyzed sampling site and the detection limits of the Raman spectroscopy, this approach represents the first reliable analytical extraction procedure to demonstrate the accumulation of NPs by aquatic insect larvae and, consequently, the potential environmental pollution of glacial streams from the Italian Alps.

Keywords: polystyrene, chironomids (non-biting midges), Raman spectroscopy, ice melt, Italian Alps

Introduction

The European Alps are experiencing a decline in freshwater biodiversity due to climate change, particularly due to the retreat of glaciers caused by rising temperatures during the summer months and a reduction in snowfall during the winter (Lencioni, 2018). However, climate change is not the only threat to alpine environments (Lencioni et al., 2023). These systems are also contaminated by pollutants transported from the atmosphere to the glaciers and released in ice-melt waters (Bizzotto et al., 2009; Villa et al., 2006, 2014). During the last years, the presence of organic, inorganic, and emerging contaminants has been detected in glacial habitats in the European Alps (Ferrario et al., 2017; Miner et al., 2017). The role of glaciers as temporary sinks of pollutants transported from the atmosphere has been demonstrated by the higher contaminant concentrations in glacial communities compared to those from nonglacial catchments (Bizzotto et al., 2009). Furthermore, it has recently been reported that high-mountain ecosystems are considered potential indicators of plastic pollution (Pastorino et al., 2023). The presence of microplastics (MPs; plastic particles with a size between 5 mm and 1 μm [Crosta et al., 2022]) and nanoplastics (NPs; plastic particles <1 μm in size [Materić et al., 2021, Kau et al., 2024]) has been confirmed in remote mountain systems, due to the long-range transport pathways of these particles through the movement of air masses (Corsi et al., 2022). Specifically, an average NP concentration of 46.5 ng/ml was found in melted surface snow from a remote area at 3106 m a.s.l. in the Austrian Alps (Materić et al., 2021).

Although the scientific community is increasingly focusing on plastic contamination of these remote areas, knowledge of the actual environmental levels of these pollutants and their relative risks to the biota of these sites is still scarce. The lack of standardized methodologies for detecting and quantifying NPs in biological matrices currently hinders a comprehensive

Received: December 09, 2024. **Revised:** February 10, 2025. **Accepted:** February 19, 2025

understanding of the environmental pollution level of NPs on living organisms (Mandemaker & Meirer, 2023; Masseroni et al., 2022; Zhou et al., 2023). The detection and quantification of NPs in environmental matrices pose significant challenges, primarily owing to their small size, low concentrations, and the pronounced matrix effects that can compromise analytical accuracy and reliability (Cai et al., 2021). Nanoparticles are smaller than the spatial resolution limits of the common identification approaches applied for MPs, such as Micro-Fourier Transform Infrared and Raman spectroscopy. Conversely, applying techniques commonly used for environmental contaminants, such as mass-based quantification, presents challenges due to the significant heterogeneity of environmental matrices and the complexities involved in extracting nanoparticles from them. Furthermore, NPs share a high degree of chemical similarity with the natural organic matter, restricting the analytical methods that can be used. For these reasons, the identification and quantification of NPs in complex matrices require new strategies to develop or analytical techniques to adapt. A possible solution to monitor NP pollution is the use of bioindicators, because of their capacity to take up and concentrate nanoscale particles enhancing their concentration (Valsesia et al., 2021).

In this context, chironomids (Diptera Chironomidae) might become valid plastic bioindicators in freshwater systems (Nel et al., 2018), being benthic organisms living in and feeding on sediments (Bertoli et al., 2022), without selecting what is in the sediment and so ingesting plastic litter if present (Stojanović et al., 2023). Specifically, chironomids of the genus *Diamesa* Meigen, 1935 are the main exclusive insect colonizing this habitat type (Lencioni et al., 2021). According to Materić et al. (2021), it is assumed that snow and ice on the glaciers feeding high mountain streams could be contaminated by MPs and NPs, mainly transported by the atmosphere. Consequently, the ice- and snowmelt waters are expected to be contaminated as well, as demonstrated for many other organic contaminants (e.g., pesticides, polycyclic aromatic hydrocarbons, and synthetic fragrances; Lencioni et al., 2023; Rizzi et al., 2022). Glaciers are temporal sinks for various contaminants, which are typically transferred to freshwater systems through meltwater in short and concentrated pulses. This phenomenon has the potential to pose a hazard to high-altitude biotic communities, because the melting of snow and ice often coincides with periods of intense biological activity and vulnerable stages of development (Morselli et al., 2014). However, contamination levels in streams may vary seasonally. In fact, the variability of the melt matrices plays an important role in pollutants concentration levels (Bizzotto et al., 2009; Villa et al., 2006). Early season meltwater comes from fresh snow, while late-season meltwater comes from old ice, which is less contaminated than recent ice (Chen et al., 2025), as evidenced by historical trends (Dahl et al., 2021). Once released into the freshwater glacial-fed system, the presence of NPs in the sediments of glacial rivers is predicted to be minimal, primarily due to the hydrodynamics of glacial-fed streams and the characteristics of NPs. Specifically, high water discharge and flow velocity impede the settling of fine particles onto the riverbed, sustaining NPs in a suspended state (Parrella et al., 2025). Consequently, the probability of accumulation in strata sediments and the consequent ingestion by benthic organisms is reduced (Dahms et al., 2020).

In this context, this study employed a novel analytical approach for the detection of NPs in biological samples to contribute to the development of more standardized and reliable NP detection techniques in biomatrices. This can help to expand the current knowledge of bioaccumulation of NPs in multiple biota species. In the present study, an analytical method already developed by Valsesia et al. (2021) to detect NPs in marine invertebrates (tunicates) was adapted to chironomid larvae from glacier-fed streams (Adamello-Presanella Alps, Italy). Two different purification approaches were tested on *Diamesa zernyi* Edwards specimens, and the efficiency of each was evaluated by measuring the recovery rates of polystyrene nanoparticles (PS 500 nm) through Single Particle Extinction and Scattering (SPES) analysis with a final readout by Raman spectroscopy. Moreover, the developed methodology was applied on non-spiked specimens of *Diamesa tonsa* Haliday larvae to investigate the occurrence of NPs in a pristine high-altitude cryosphere environment and to evaluate the role of these organisms as possible indicators of pollution in high altitude remote areas.

Material and methods

The analytical workflow used in this study to extract NPs from chironomid larvae collected in a remote alpine environment is reported in Figure 1. Through an iterative process of testing and refinement, the most effective method was identified and selected for application.

Sampling area and target species

Larvae were collected in two glacier-fed streams, Mandrone and Amola, within 15 m downstream from the glacier snout at 2583 m. a.s.l. and 2557 m. a.s.l., respectively (Adamello-Presanella Mts, Trentino, Italy; 46°N, 10°E). The two glacier-fed streams at the glacier front are cold (average summer water temperature 1–2 °C), turbid, and turbulent (Lencioni, 2018; Lencioni et al., 2021).

Among invertebrates, species of the genus *Diamesa* represent the main and even the exclusive taxon colonizing the kryal habitats of uppermost sectors of glacier-fed streams (Lencioni, 2018). For this work, *Diamesa zernyi* and *Diamesa tonsa* were selected as target species. The two species have the same ecological valency, being cold-adapted and restricted to high mountain freshwaters (Lencioni & Rossaro, 2005). *Diamesa zernyi* was the dominant species in Mandrone, while *D. tonsa* prevailed in Amola (Lencioni, 2018; Lencioni et al., 2021). *Diamesa zernyi* larvae were collected in August 2019 in the Mandrone glacier-fed stream and stored in the MUSE collections until analysis. This species was employed to optimize and validate the extraction and isolation protocol. *Diamesa tonsa* larvae were collected in the Amola glacier-fed stream in August 2022 with the purpose of applying the developed methodology on non-spiked specimens (Figure 2).

Larvae were collected using a pond net (30 × 30 cm, mesh size 100 μm; Scubla SNC, Italy) by kick sampling within 15 m downstream from the glacier front, according to Lencioni (2018). In the field, the material was poured into a polypropylene (PP) vessel from which larvae were sorted with steel tweezers, with bare hands. Larvae were transferred in PP bottles with stream water and transported alive to the laboratory in a refrigerated bag. After species identification under the stereomicroscope (MZ 7.5; Leica Microsystems, Germany; Rossaro & Lencioni, 2015), larvae were weighted and stored for further analysis. *Diamesa zernyi* were freeze-dried and stored at −20°C, while *D. tonsa* were preserved in ethanol 90%.

NPs extraction procedure

Three pools of 100 mg (about 40 larvae) each of IV-instar freeze-dried specimens of *D. zernyi* were utilized to develop the extraction protocol. The specimens were first transferred into a falcon tube containing 50 ml of Milli-Q water and they were vortexed to

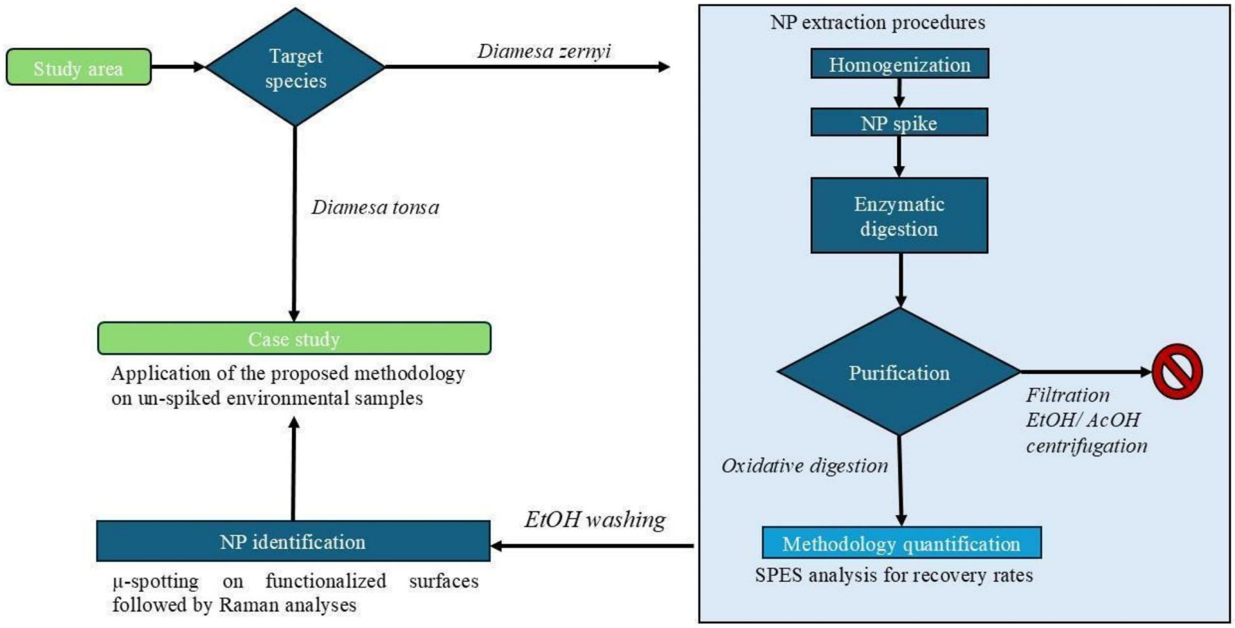

Figure 1. Analytical workflow representing the different steps performed in the development of an analytical method for the detection of nanoparticles (NPs) in biological samples. EtOH = ethanol; AcOH = acetic acid; SPES = Single Particle Extinction and Scattering analysis.

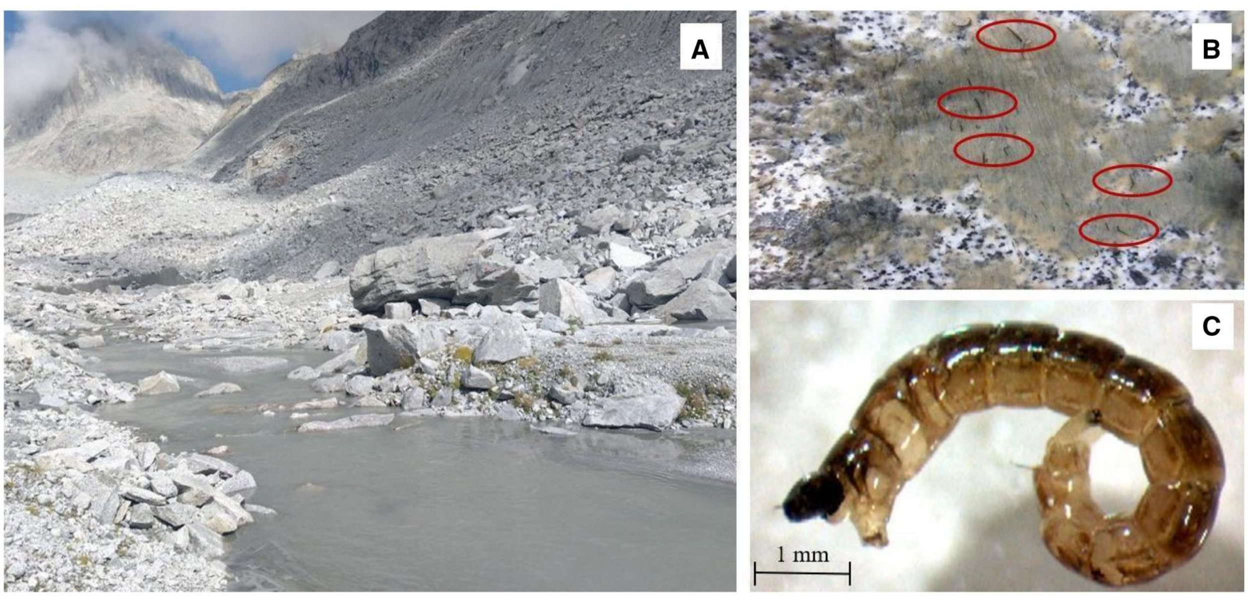

Figure 2. Amola glacier-fed stream and glacier front in 2022. (A). Chironomid larvae (in red ovals) attached to a stone covered by *Hydrurus foetidus* Villars (B). A IV-instar larva of *Diamesa zernyi* collected in the Mandrone glacier-fed stream (C). Photos by ©V. Lencioni.

eliminate any residual detritus from prior sampling. Afterward, the larvae were dried under a fume hood and subsequently weighed using an analytical balance (Pioneer, Ohaus Europe). For each test, 100 mg of larvae was processed.

The samples were first homogenized with 1 ml of cacodylate buffer using a Potter homogenizer (IKA, Eurostar 40, IKA-Werke). The resulting sample was transferred to a 25-ml glass vial, and the mortar walls were rinsed with an additional cacodylate buffer to ensure complete transfer to the vials. At this point, a known amount of commercial polystyrene beads (PS-NPs, 500 nm, Polysciences Europe, Germany) were added for the validation of the protocol.

The samples were digested using an enzymatic method, including modifications based on the protocol by Facchetti et al. (2020). In particular, the samples underwent three sequential enzymatic digestion steps, using lipase from *Aspergillus niger* (Sigma Aldrich, 200UI, powder), chitinase from *Streptomyces griseus* (Sigma Aldrich, 200UI), and papain from *Carica papaya* (Roche, 10 mg/ml, Basel, Switzerland) to digest, respectively, lipids, chitin, and proteins. Prior to use, papain stock solution needs to be activated for 1 hr at 50 °C in an activation buffer (1 ml) composed of 784 µL of sodium cacodylate solution (50 mM), 11 µL ethylenediaminetetraacetic acid (100 mM; Sigma-Aldrich), 55 µL of L-cysteine solution (100 mM; Sigma Aldrich), and 150 µL of papain stock solution.

First, lipase was dissolved in Milli-Q water (2 mg/ml) and added to the samples, corresponding to 2 mg of lipase per gram of initial *D. zernyi* biomass. The mixture was incubated in an oven at 50 °C for 3 hr with agitation. Subsequently, chitinase (1 mg/ml) was added, corresponding to 1 mg per gram of sample, and the mixture was incubated for 2 hr at 37 °C with agitation. Finally, 1 ml of activated papain solution was added, corresponding to 1.5 g of papain for 1 g of initial sample. The papain digestion was allowed to proceed overnight at 50 °C.

Before extraction, all laboratory equipment and materials were rinsed with prefiltered (0.22 µm) Milli-Q water. Additionally, wherever feasible, equipment manufactured from plastic was substituted with glass or metal parts.

Purification step and recovery rate

After enzymatic digestion, further steps were necessary to enable accurate identification of NPs through spectroscopic techniques. In this perspective, two different approaches to cleaning up the matrix were tested and the efficiency of each was evaluated by measuring the recovery rate through SPES analysis.

The recovery rate was assessed by spiking the larval suspensions after the homogenization step, with PS-NPs of 500 nm. Two theoretical spiking concentrations were tested: 10^9 and 10^7 particles per milliliter. The samples were then subjected to enzymatic digestion and then to the two different purification processes. For each of the tested concentrations, recovery rates were measured in triplicate (100 mg of homogenized larvae for each replicate), and a negative control was included. Further details regarding the optimization of extraction procedure by SPES analysis and quality controls are reported in online supplementary material Appendix A.

First tested approach: filtrations and washing steps

The first approach involved, after enzymatic digestion, two filtration steps using nylon membranes (10 and 5 µm) followed by purification with ethanol (EtOH, 96% v/v, AnalaR NORMAPUR, VWR International Srl) and acetic acid (AcOH, 90%, Sigma Aldrich). After diluting the digested larval suspensions, the suspension was filtered (10 µm and 5 µm nylon filter, Millipore), aliquoted into 2-ml Eppendorf tubes, and evaporated overnight at 60 °C. Subsequently, the solid residue was washed with absolute EtOH, 90% AcOH, and Milli-Q water (centrifugation at 16,000 rcf × 5 min). The final pellets, filters, and supernatants were analyzed by SPES.

Second tested approach: oxidative digestion

As an alternative method, the digested spiked larvae suspensions were transferred to 15 ml PP tubes, and a double volume of hydrogen peroxide (H_2O_2, 30% Sigma-Aldrich) was added with a final concentration of 20% v/v. The samples were then incubated in a heating block at 80 °C, shaking (300 rpm) for 2 hr, to complete the digestion process. Samples were gently centrifuged (2,000 rcf × 30 s) to remove any residue remaining from the digestion prior to SPES analysis.

Identification of NPs in biomatrix

Samples with best recovery were analyzed by electron microscopy and Raman spectroscopy. Before the analysis, it was necessary to carry out an optimization of spotting on surfaces with varying contact angle (CA): hydrophilic (θ ∼90°), hydrophobic (θ ∼105°), and super-hydrophobic (θ ∼ 35°). To reduce the detection area, an additional purification step involving EtOH was introduced into the extraction process before the Raman identification step. After gentle centrifugation (2,000 rcf × 30 s), 400 µL of the supernatant was diluted tenfold with pure EtOH and purified using Amicon centrifugal filters (Amicon Ultra-4, 100 kDa centrifugal filters, Millipore, Bedford, MA). The samples underwent two rounds of purification with EtOH, followed by two more rounds with Milli-Q water to remove any remaining alcohol.

An aliquot (1 µL) of the purified sample after both digestion steps was sputtered onto the three substrates and dried at room temperature. Subsequently, the chips were analyzed by SEM (FEI NOVA 600, Dual Beam at 2 kV) and then by Renishaw (inVia confocal Raman microscope, equipped with 532 nm laser) to determine the chemical structure of the particles. The spectra of spiked samples were collected in static mode (mean value 1500 cm^{-1}) using the 50× objective by averaging at least 5 spectra and an accumulation time of 5 s. The identification of the spectra was obtained by comparing the digested nanoparticles with the reference spectrum of PS.

Case study

Once the methodology has been established, it was applied on three pools of 100 mg each of wild non-spiked specimens of *Diamesa tonsa*, with the aim to investigate the occurrence of NPs in a pristine high-altitude cryosphere environment. Specimens of *D. tonsa* were removed from their storage alcohol suspensions and placed in a falcon tube filled with 50 ml of Milli-Q water. Samples were then vortexed to remove any residual detritus from the sampling activity. Samples of *D. tonsa* were analyzed using the previously optimized methodology. A drop of the resulting supernatant was placed directly onto a hydrophobic surface and allowed to dry at room temperature before being analyzed by Raman.

Raman spectroscopy was applied using the same mapping configuration as in the spiked tests. However, given that these samples were of unknown composition, the scan mode was switched from static to extended (600–3200 cm^{-1}) to cover a broader spectral range. While static scans are faster, they are limited in spectral range, whereas extended scans provide a more comprehensive analysis, although they require longer acquisition times. In addition, the default exposure time for the extended mode was set to 10 s, which is the minimum required for this range. Further details regarding the method optimization are reported in see online supplementary material Appendix B.

Results

NPs extraction protocol to chironomids

The SPES results demonstrate that the first approach, involving washing steps after enzymatic digestion, was inefficient, with a total recovery rate of only 30.5%. On the other hand, the second approach, involving two digestive processes, was much more efficient for the two spike levels (Table 1). The integrity of the NPs after the latest extraction protocol was verified with electron microscopy. The detailed results of recovery rates of the optimization procedures and quality controls are summarized in the online supplementary material Appendix A (Figure S1–S5).

For identification of NPs in biomatrix using confocal Raman spectroscopy, an aliquot of the extracted samples was deposited on substrates with varying hydrophobic properties. The CA of the substrate is critical in controlling droplet behavior, with higher angle values expected to inhibit diffusion and reduce the droplet footprint. Initial tests using PS 500 in ultrapure water were conducted with prominent results. However, spotting the spiked samples after both digestion protocols did not yield satisfactory results. The specific chemical composition of the larvae matrix prevented the observation of the Marangoni effect, which typically results in the suppression of droplet spreading. In addition, surface analysis with Raman spectroscopy was hampered by

Table 1. Recovery rates of spiked nanoplastics in chironomid specimens *Diamesa zernyi* with the proposed methodology ($n = 3$).

Theoretical spike conc. (part/mL)	Experimental spike conc. (part/ml)[a]	Detected NPs (part/ml)	Recovery rates (%)
10^9	$16.2 \times 10^8 \pm 0.14 \times 10^8$	$6.8 \times 10^8 \pm 1.7 \times 10^8$	109 ± 28
10^7	$6.2 \times 10^6 \pm 0.12 \times 10^6$	$5.1 \times 10^6 \pm 0.76 \times 10^6$	82 ± 12

[a] Experimental values obtained by Single Particle Extinction and Scattering analysis.

residual organic matter that caused signal saturation, making it impossible to obtain clear spectra of PS. To address this issue, an additional purification step using ethanol was incorporated into the extraction process prior to the Raman identification step. The three substrates were tested and, as can be seen in Figure S6, this change of substrate caused the particles to concentrate in a smaller area only when the hydrophobic surface was used, concentrating the particles in an area with a diameter of 0.48 ± 0.09 mm. This clearly improves the possibility of mapping and identifying the droplet's Raman spectral fingerprint and reduces analysis time. This effect is likely due to the role of ethanol in altering the chemical properties of the matrix, reducing its wettability on the hydrophobic surface.

Finally, all processed samples were analyzed by spectroscopic technique and, in parallel, by SEM. A Renishaw inVia Confocal Raman Microscope equipped with 532 nm laser excitation and white light imaging tool was used to create a montaged area for mapping. Raman data were collected across the entire sample surface. Spectral maps were generated at specimen points spaced nonuniformly, with a regular interval of 20 μm between points. This approach was chosen to balance the tradeoff between analysis duration and the spectral resolution of the maps, optimizing both efficiency and detail in the detection of particles across the sample surface. For further details of analytical methods used for Raman spectroscopy, please refer to see online supplementary material Appendix B.

After the acquisition, chemical image maps were obtained for each sample. In the case of PS spiked samples, data were analyzed using a direct classical least-squares method incorporating the reference spectra (Figure 3). The specific conditions for collecting map data are shown in Table S1. Raman imaging confirmed the presence of PS-NPs, particularly in samples with higher PS concentrations. Microscopic surface scans obtained by transmission electron microscope (JEOL JEM-2100), revealed prominent aggregation in samples dosed at 10^9 particles/ml, while in samples with lower PS concentrations aggregation was mostly absent (Figure S7). Additionally, surface scanning by confocal Raman with at step size 20 μm resolution introduces a probabilistic limitation in detecting specimen nanoparticles or smaller aggregates (Figure S8).

Identification of NPs in *Diamesa tonsa* specimens from Amola glacial river

Initially, in non-spiked *D. tonsa* samples, no polymeric particles were detected in any of the environmental samples. However, by reducing the mapping step size from 20 μm to 10 μm and thereby increasing the spatial resolution, a PS signal was successfully detected in one of the larval samples (Figure 4). This adjustment was critical for the identification of smaller or less abundant polymer particles within the samples. Nevertheless, this modification significantly increased the total scan time, necessitating the acquisition of smaller, more focused maps to manage time effectively while maintaining detection sensitivity.

Discussion

The presented methodology managed to identify the occurrence of plastic in chironomid wild samples, proving efficient for both freeze-dried and preserved in ethanol samples. The risk of contamination due to PP lab consumables used during the whole analytical process, including sampling, was considered minimal. Although it cannot be completely excluded, the probability of intentional ingestion of NP material by the animals is absent, as they were deceased after sampling. Furthermore, the samples underwent a washing step before homogenization to further reduce the risk of contamination. Finally, it is important to note that the presence of PP was not detected in any sample, including the process blank.

Concerning the extraction procedure, as widely reported in the literature (Cai et al., 2021), enzymatic digestion is confirmed to be nondestructive to extract particles from complex matrices. The subsequent step combines oxidative digestion with a final cleanup results crucial for NPs identification by spectroscopic techniques. The use of hydrophobic surfaces enabled the concentration of the samples in a small area of the surface, allowing the identification of NPs by Raman.

The methodology was tested exclusively on PS-NPs, and while recovery was evaluated only for this polymer, the proposed approach is not selective to it. It is important to highlight that the methodology is designed to detect all polymer-based particles. Future applications will focus on testing the methodology with other polymers to obtain quantitative data. However, these polymers must possess specific properties to serve as suitable models for recovery assessment via SPES. Considering these factors, the development of appropriate reference materials is crucial for validating nanoparticle extraction protocols (Mitrano et al., 2023).

The approach presented using a nondestructive technique (μ-Raman) allows the particles to be characterized from a physical-chemical point of view compared to other techniques tried and tested for the identification of NPs, such as pyrolysis gas chromatography combined with mass spectrometry (Pyr-GC-MS; Li et al., 2022; Xu et al., 2022). Furthermore, the proposed methodology proved to be able to see and identify NPs in complex matrices containing up to 10^7 particles/ml via confocal Raman due to the aggregation effect on the surface. However, it is important to note that, unlike Pyr-GC-MS, the presented methodology only provides qualitative identification of the presence of NPs. In this perspective, future application could involve a combination of spectroscopy and mass spectrometric techniques to gain a quantification of NP environmental concentrations in biota.

So far, it is hypothesized that environmental and geographical characteristics are the principal factors affecting the environmental concentrations of NPs in the surface snow of glaciers in remote areas (Maggi et al., 2006; Materić et al., 2021). Differences in meteorological conditions, airborne transportation, and river hydrobiology may lead to variations in water concentrations in the systems where *Diamesa* larvae live. From this perspective, it is crucial to highlight that the sampling effort, conducted in only two stations at the same time of year, does not allow for a representative scenario of the real state of pollution in the glacial habitat investigated. Further sources of variability may be attributed

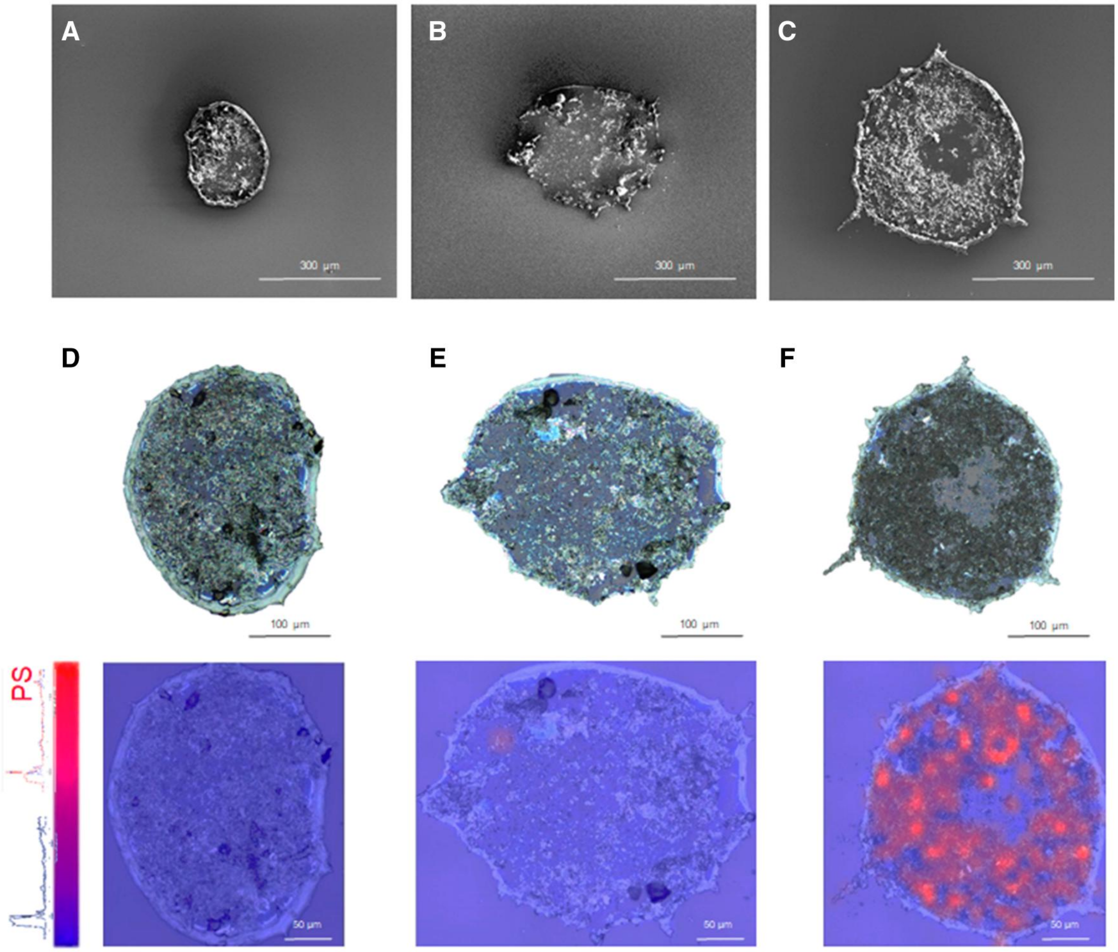

Figure 3. Scanning electron micrographs and white light montage with relative mapping with Raman images overlay of control (A, D), spike 10^7 particles/mL (B, E) and spike 10^9 particles/mL (C, F). The red dots in the Raman images (bottom) highlight if the polstyrene (PS) is present in the sample.

to the adopted sampling procedures, laboratory extraction techniques, and the subsequent identification methods employed. In this perspective, the absence of an internationally agreed detection methodology for NP in complex matrices may result in discrepancies in detecting these contaminants due to the use of different approaches.

Unfortunately, environmentally relevant concentrations cannot be precisely defined due to the limited conclusive evidence of NP bioaccumulation in wild organisms and their environments. This uncertainty underscores the rationale for testing at least two concentrations in the spiked samples. The higher concentration (10^9 particles/ml) was chosen to facilitate the development and validation of the methodology with the final Raman readout. In contrast, the lower concentration, two orders of magnitude smaller (10^7 particles/ml), was selected to evaluate the detection limits of the analytical techniques used in this study, aligning more closely with potentially realistic accumulated concentrations. Aware of the current limitations in determining the presence of NPs in complex matrices, this study aimed to ascertain whether the initial hypothesis of a low level of NP contamination in stream water fed by snow and ice melt is valid. In this perspective, the objective of the present study is not a precise description of NP distribution in the organisms of high mountain environments, but rather an evaluation of the feasibility of the presented methodology to identify NP in chironomids, as a first step to better determine the environmental level of NP pollution in a glacial habitat.

Conclusion

A protocol was developed to detect NPs in insects using confocal Raman spectroscopy. The study showed that this method is effective, with high recovery rates even at low concentrations, and can be used on both frozen and freeze-dried insect samples. Furthermore, a digestion protocol proved to be a promising approach to obtain more detailed information on NPs using other analytical techniques. Possible applications will include the quantification by Pyr-GC-MS, a current and robust approach used for the identification of microplastics in complex biological samples.

The study confirmed the initial hypothesis that chironomid larvae in glacial streams contain low levels of NPs, marking the first recorded evidence of NP bioaccumulation in alpine insects. This finding suggests that chironomids could serve as indicator species for plastic pollution in glacial ecosystems. However, further analysis of additional specimens is necessary to gain a more comprehensive understanding of the pollution status of glaciers. Notably, the successful application of the methodology to freeze-dried specimens also opens up the possibility of analyzing museum specimens from previous years, providing a valuable opportunity for retrospective pollution monitoring. Finally, an

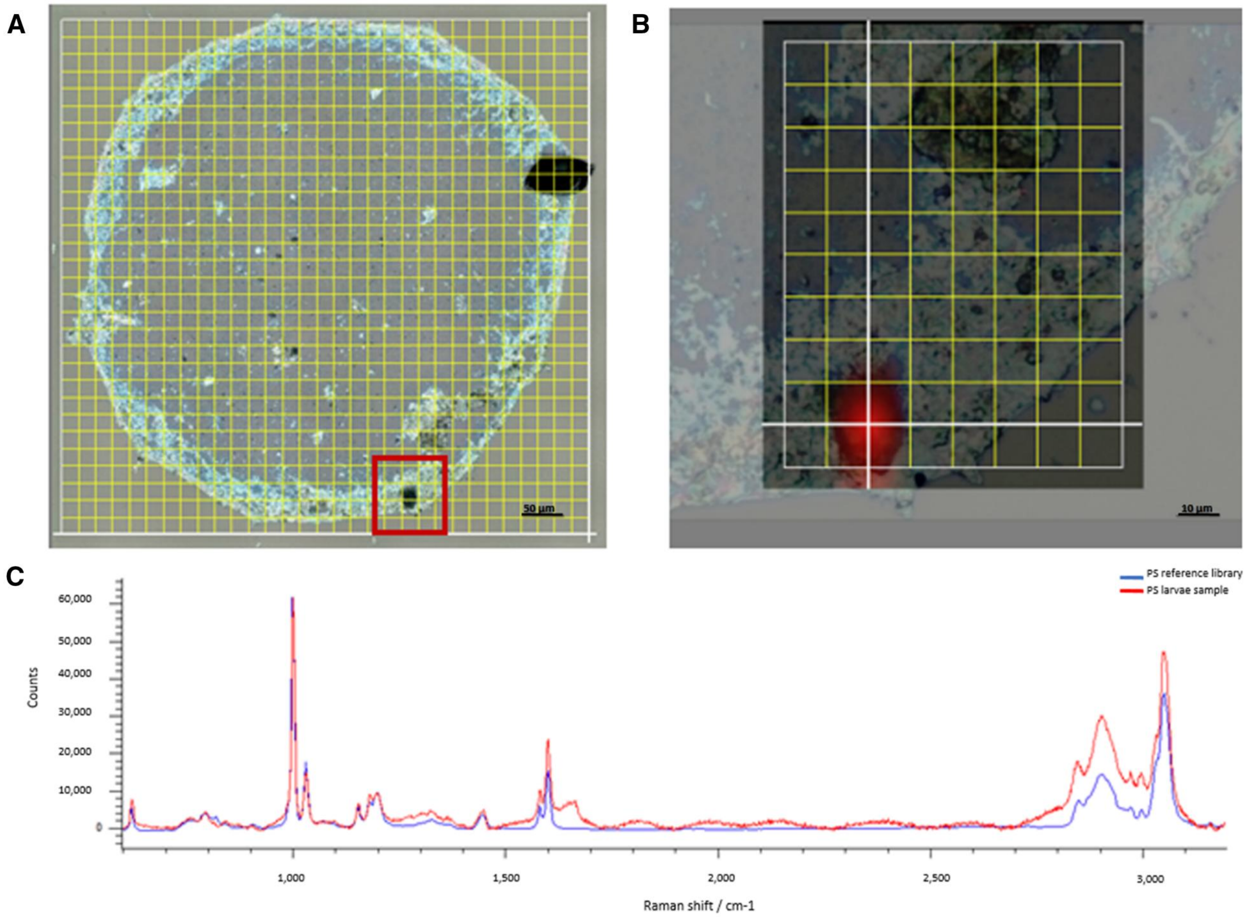

Figure 4. White light montage images of the entire area (20 µm step size, A), and of a small region of the same sample (10 µm step size, B). An overlay of the corresponding Raman image (red rectangle of section A), where polystyrene particles were identified, is shown in panel B. The associated Raman spectrum (C) was acquired in extended mode (600–3,200 cm^{-1}) with a 10 s exposure, 10% laser power and three accumulations (Renishaw Polymeric Materials Database). PS = polystyrene.

integrated approach should be developed, searching plastic particles in both abiotic (e.g., glacial waters and sediments) and other abiotic (e.g., algae) matrices to assess a more representative scenario of contamination of such areas.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

The authors confirm that the data supporting the findings of the present study are available within the article and its supplementary materials.

Author contributions

Andrea Masseroni (Data curation, Formal analysis, Investigation, Visualization, Writing—original draft, Writing—review & editing), Gabriella Schirinzi (Investigation, Methodology, Validation, Writing—original draft, Writing—review & editing), Sara Villa (Conceptualization, Project administration, Resources, Supervision, Writing—review & editing), Serena Pozzi (Investigation), Francesca Paoli (Methodology), Jessica Ponti (Investigation, Methodology, Validation), Andrea Valsesia (Methodology, Resources, Supervision, Writing—review & editing), and Valeri Lencioni (Conceptualization, Funding acquisition, Investigation, Writing—original draft, Writing—review & editing)

Funding

The research was co-funded by the Autonomous Province of Trento-MUSE and JRC. Schirinzi G.F. was supported by the European Commission CAS6 - NANOPLASTICS project (PRJ 32529).

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

The authors thank A. Franceschini and D. Debiasi (MUSE, Trento, Italy) and F. Latella involved in sampling activities; A. Mustoni and M. Armanini (Natural Geo-Park Adamello-Brenta, Strembo, Trento, Italy) for species collection permission.

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