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Fluorescence microscopy versus Raman spectroscopy for direct identification of small (< 2 µm) microplastics in soils

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Abstract

Research on microplastics (MP) in soils is much complicated due to the lack of dedicated (extraction) methodologies and the strong matrix interferences for MP detection, and there is almost no research on the dynamics of the smallest MP in soil. Here we compared the possible detection of the smallest MP fraction (1-2 µm) by µ-Raman spectroscopy and fluorescence microscopy in matrices of highly varying complexity. Samples of pure quartz sand, soil with removal of native soil organic matter (SOM), and soil with native SOM still present were amended with fluorescent polystyrene (PS) microparticles (diameter 1.65±0.04 µm) in different concentrations ranging from 0.1 to 0.001%, and after mixing and compaction both the Raman spectra and fluorescence microscopy images were obtained. Characteristic PS Raman peaks (main peak at 1009 cm^{-1}) were visible in quartz sand (all concentrations) and soil without SOM (highest concentration only), but not in the other situations, whereas fluorescence microscopy clearly visualized the MP at all concentrations in all matrices. The possibility of direct and unambiguous fluorescent MP detection in real soil also circumvents the need for lengthy extraction procedures, and opens up new avenues for studying mechanistic aspects of the smallest MP fractions in soil.

Introduction

Plastics are ubiquitous and persistent pollutants in both aquatic and terrestrial environments. Plastics in soil degrade into increasingly smaller particles, yielding microplastics (MP) and eventually nanoplastics (NP), and even though little is known with certainty about the ecosystem and health impacts of plastics, there is increasing concern about serious negative effects of the smallest fractions (MP and NP) on e.g. soil fertility (Kleunen et al., 2020), soil biology (Correia Prata et al., 2020; Bläsung & Amelung, 2018) and human health, including the disruption of immune function (Yooeun & Youn-Jo, 2018) and neurotoxicity (Rahman et al., 2021). To date the research on MP has mostly failed to recognize 'the importance of going small', and this has even more been the case in soils, where most research focused on the largest MP fraction (> 0.1 mm), with only very few studies focusing on the fraction < 20 µm (Du et al. 2020a,b) thus effectively missing the fraction with the highest bioavailability and reactivity. The smallest MP fraction (<10-20µm) can be identified using micro-Raman spectroscopy (µ-Raman, detection limit of ~1µm), but the strong matrix interactions in soils and sediments from mineral and mainly natural organic matter (Surtees, 2015; Xing et al., 2016) necessitate lengthy and drastic fractionation and separation steps. Also, the weak scattering with µ-Raman results in exceedingly long scanning times (tens of hours per mm^2, Zada et al. 2018), necessitating to limit the analysis to minute sample subareas. Therefore, there have been no reports of detection of the smallest MP (1-2µm) in natural soils, rendering mechanistic studies on the dynamics of these smallest MP in soils virtually impossible. One possibility to circumvent this issue would be to apply fluorescently labeled MP (with the fluorophore embedded in the polymer matrix) in experiments and subsequently try to visualize these using fluorescence microscopy, which might also make the extraction steps redundant, but has so far not been attempted. Here we studied the possibility of directly (i.e. without prior extraction steps) identifying MP that are covalently labeled with a fluorophore embedded in the polymer matrix, of the smallest size fraction (1-2µm) at different concentrations in matrices of varying complexity, comparing fluorescence microscopy to Raman spectroscopy.

Material & Methods

Sand and soil samples were spiked with fluorescent (fluogreen, Ex/Em 502nm/518nm) monodisperse polystyrene (PS) microparticles (mean diameter ± standard deviation of 1.65± 0.04)) (Microparticles GmbH, Berlin, Germany) and scanned using Raman and fluorescence spectroscopy. The MP were added to matrices of increasing complexity, namely i) pure quartz sand (purchased from a local supplier (SCR-Sibelco N.V., Antwerp, Belgium), further abbreviated as "sand"; ii) a loamy sand soil from an agricultural field at the Institute of Agricultural and Fisheries Research (ILVO) in Merelbeke, Belgium (WSG84 coordinates 50.99141/3.78118) sampled at a depth of 40-60 cm and from which all native soil organic matter (SOM) was removed by incineration in a muffle furnace for 4 hours at a temperature of 550°C, and further abbreviated as "INC_soil"; and iii) the same loamy sand soil without any pretreatment (thus still containing the native SOM equivalent to a soil organic carbon (SOC) content of 0.126%), further abbreviated as "UNT_soil". The fluorescent MP's were in an aqueous solution of 2.5% and were added to the sand and soil samples in concentrations of 0.1, 0.03, 0.01, 0.003 and 0.001 mass%, resulting in a total of 30 samples (3 matrices × 2 MP types × 5 concentrations). First, the sand/soil samples (0.101 mg) were filled into steel screw rings (diameter of 8.25 mm, height of 1.1 mm, volume of 58.8 mm^3). Then, the MP's were administered to the soil as an aqueous solution using a pipette, after dilution of the initial solution with demineralized water to appropriate concentrations to allow homogeneous addition. Then the samples were carefully mixed with a needle and gently compressed with a nail head to reach a bulk density of approximately 1.7 Mg m^{-3}. After airdrying, the samples were placed upon a calcite slide, the metal rings were carefully removed without disturbing the soil structure, and a Raman area scan (50*50 µm) was performed with an integration time of 0.5 second and 50 points per line for each sample. The Raman scattering spectra were recorded using a WITec Alpha 300 microscope with both upright and inverted modes, using a laser diode (785 nm, Toptica XTRA II). A UHTS 300 spectrometer with a CCD camera (ANDOR iDus 401 BR-DD) cooled to a temperature of −70 °C was used to collect Raman Stokes signals under a 40x objective lens (Nikon). Laser power was measured at the beginning of each run using a power sensor (Thorlabs PM100D). Control Five software suite was used to process and analyse the scattering signals. From the area scans, heat maps were produced for the fingerprint signal of PS at 1009 cm^{-1}, with a width of 20 cm^{-1}. The Raman microscope was custom-modified (green LED excitation and a CCD camera) for simultaneous recording of fluorescence microscopy images on the same areas of the sample, where Raman microscopy images were recorded.

Results and discussion

The characteristic Raman peaks of PS (high intensity peak around 1009 cm^{-1}, and minor peaks around 600, 800, 1200, 1500 and 1600 cm^{-1}, Figure 1) were very clearly distinguishable in pure quartz sand at all MP concentrations, and correspond to the ring deformation mode (614 cm^{-1}), ring breathing mode (1000 cm^{-1}), C−C stretches (1150−1200 cm^{-1}), CH_2 scissoring (1446 cm^{-1}), and ring-skeletal stretch (1597 cm^{-1}) (Bridges et al. 2004). In the INC_soil only the highest intensity PS peak (1009 cm^{-1}) could be identified against the background signal, and only at the highest concentration of 0.1%. The fluorescent microscopy image and the Raman heatmap (based on the PS peak at 1009 cm^{-1}) for quartz sand (0.001%) and INC-soil (0.1%) are compared in Figure 2. In the UNT_soil no PS peaks could be identified against the background signal of the soil at any of the concentrations. Fluorescence microscopy allowed to visualize and quantify the individual fluorescent MP at all concentrations and in all matrices. The Raman scans take 20 min for the 50 × 50 $µm^2$ area, whereas the fluorescence image is acquired instantaneously once the sample is in focus.

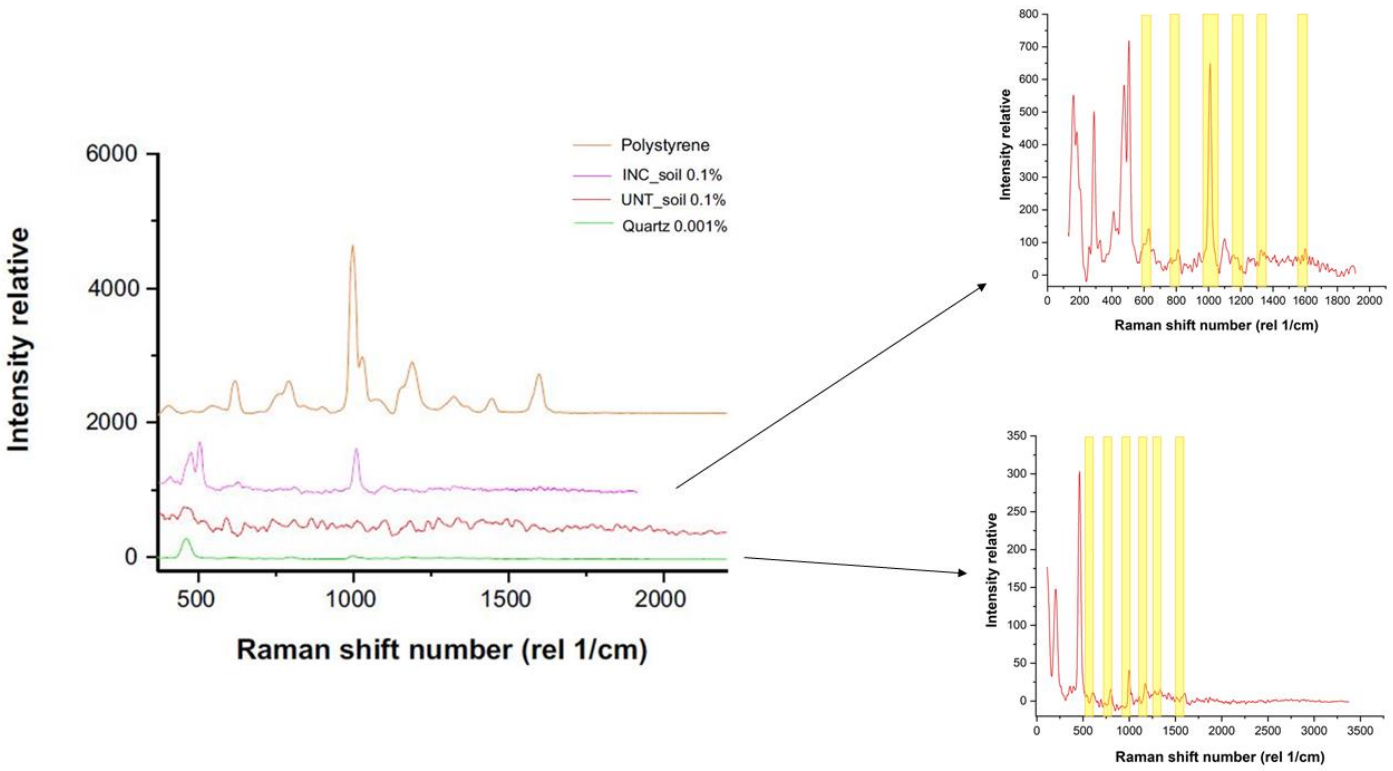

Figure 1: Intensity peaks (CCD) of relative frequencies for PS microparticles (without background), UNT_soil (concentration of 0.1%), INC_soil (0.1%) and sand (0.001%) (left) and the individual spectra for MP + INC_soil (0.1%) and MP + sand (0.001%) (right)

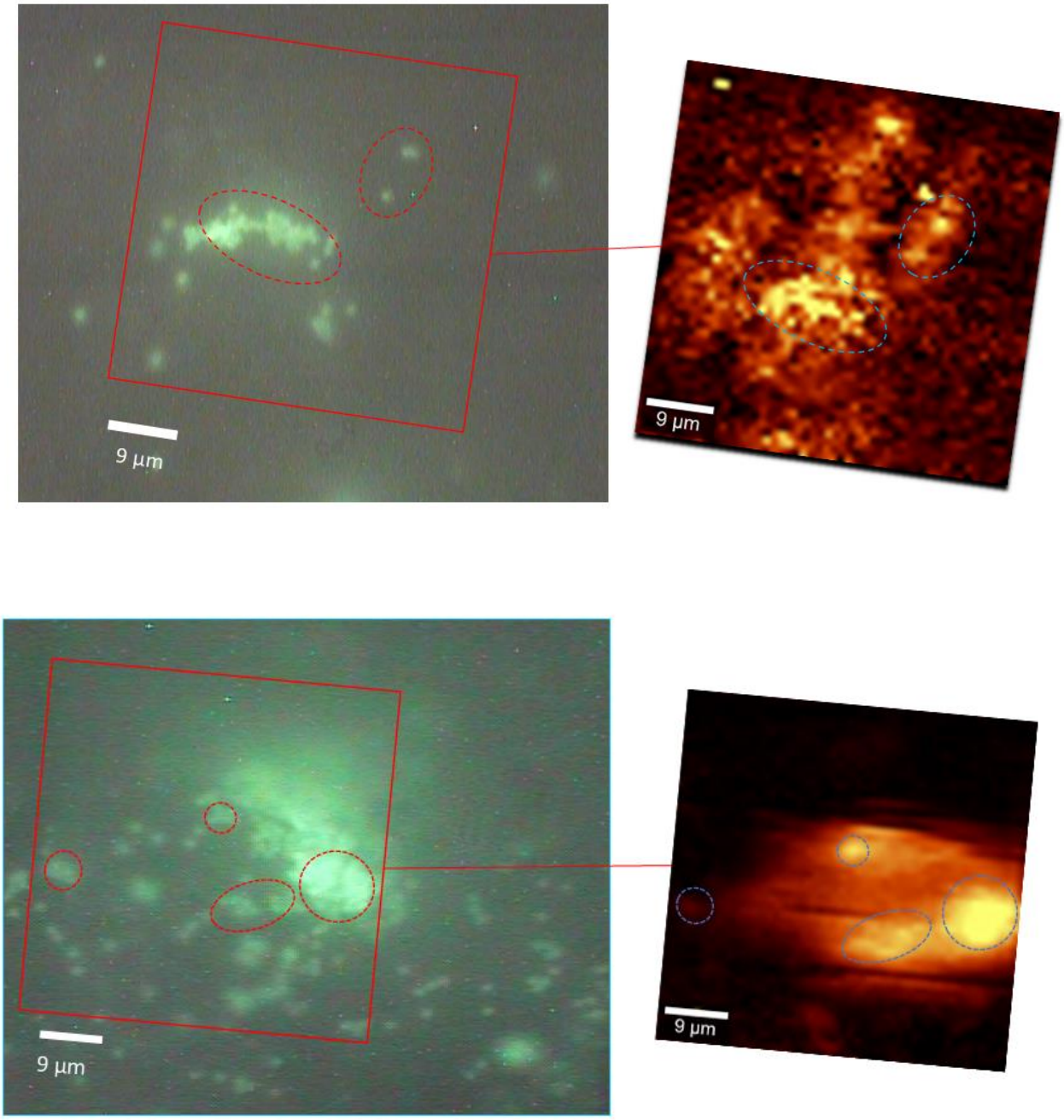

Figure 2: Fluorescent images from pure sand (0.001%) (top) and INC_soil (0.1%) (bottom) and the corresponding heat maps of Raman area scan (based on PS peak at 1009 cm^{-1}). The dotted circle indicates areas with high concentration of MPs that were also identified by Raman spectroscopy.

Studies using fluorescence microscopy have used a posteriori labeling of MP extracted from soil (mostly using Nile red dye) but this technique suffers from a number of drawbacks (e.g. unspecific and incomplete labeling), and is mostly seen as a preparatory technique for further spectroscopic identification of MP. We demonstrated for the first time that direct visualization of the smallest MP fraction in a complex environmental matrix is possible, even at low concentrations, when these MP are labeled fluorescently

(fluorophore embedded in the polymer matrix) and identified using fluorescence microscopy, while Raman spectroscopy is not suitable for detecting MP of this size directly in real soils. The correspondence between the fluorescent images and Raman heatmaps of the same area exhibited some differences which are due to the "rough" soil surface when considered on the µm scale. The MP were added homogeneously and samples were mixed and then recompacted, but obviously this does not result in a completely flat surface, causing MP at the surface to be present at different focal lengths. This can thus also lead to some discrepancies in particle detection between the Raman scan and the fluorescence microscopy. In any case, the fluorescent image should be viewed as the reference, given that the Raman spectra are subject to interference and were thus not visible in almost all cases in the real soil. From the difference in Raman performance between pure quartz sand and soil, it follows that the interference of mineral soil constituents (INC_soil) but more so of native SOM (UNT_soil) preclude the direct detection of MP in soil. The difference between INC_soil (where all SOM has been removed) and the pure sand lies mostly in the presence of silt and clay particles in the former, given that the sand fraction in this soil is largely made up of quartz as well. It are thus these finest mineral fractions that interfere with the Raman signal, allowing MP detection only at the highest concentration (0.1%). Clearly the native SOM causes a much larger influence on the Raman signal of the MP than do the silt and clay fractions, given that the MP could no longer be detected by Raman in the UNT_soil even at the highest concentration, despite the very low SOM content (equivalent to 0.126% SOC, which is about one order of magnitude lower than in normal surface soils). This causes a form of background noise with much fluorescence associated with the SOM and very little Raman scattering (Yang & Wang, 1997), preventing detection of any PS peaks.

Given the strong matrix interference, it is clear that extraction/isolation of the MP from soil or sediment is an indispensable step prior to MP detection by Raman spectroscopy. However, MP extraction procedures are labour intensive and may alter the nature of the extracted MP (Bläsung & Amelung, 2018). Fluorescent labeling allowed to visualize the MP at all concentrations in all three matrices, without any pretreatment step. Obviously, detection of fluorescent MP using fluorescence microscopy is equally possible directly on filters following extraction/isolation steps in case this is needed. This is an enormous step forward for the study of MP dynamics in soils, where the smallest (<20 µm) MP fractions have so far been neglected because of these methodological obstacles for detection. Obviously fluorescent detection of MP is not possible for plastics that have not been added intentionally to a soil. However, intentional MP addition to soils can readily be done in mechanistic experiments where typically the fate of MP is monitored as a function of time. Examples of such experiments include the incorporation and (concomitant) protection of MP in (micro)aggregates, degradation of MP in soil, leaching of MP to deeper soil layers or groundwater, which so far have not been possible for the small MP fractions. Addition of fluorescent MP also eliminates the problem of background pollution of MP already present in the soil during such experiments. Here MP were added in a large range of concentrations which would go from extremely polluted soils (highest concentrations) to the "normal" background MP concentrations expected in agricultural and natural soils. However, data on soil MP concentrations in literature refer to the larger MP fractions only, and there are virtually no data on concentrations of the smallest MP size fractions.

In conclusion, use of fluorescent MP in combination with fluorescence microscopy allows to directly visualize MP of the smallest size fraction (1-2 µm) even in soil with native soil organic matter. In case of extraction of MP from soil followed by MP identification, fluorescence microscopy should be even more powerful, but this extraction step appears even not to be needed, in contrast to detection by µ-Raman spectroscopy where MP extraction is imperative. The use of fluorescent MP added to soil in mechanistic studies can open entirely new research avenues for studying MP behaviour in soils.

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