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Article

Upgraded Protocol for Microplastics' Extraction from the Soil Matrix by Sucrose Density Gradient Centrifugation

Tara Grujić [1,*], Elmira Saljnikov [2], Slobodan Stefanović [3], Vojislav Lazović [1], Snežana Belanović Simić [4] and Žaklina Marjanović [2,*]

[1] Institute of Soil Science, Teodora Drajzera 7, 11000 Belgrade, Serbia; soils.lazovic@gmail.com
[2] University of Belgrade–Institute for Multidisciplinary Research, Kneza Višeslava 1, 11000 Belgrade, Serbia; esaljnikov@imsi.bg.ac.rs
[3] Faculty for Applied Ecology "Futura", Metropolitan University, Požeška 53a, 11000 Belgrade, Serbia; slobodan.stefanovic@futura.edu.rs
[4] Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, 11000 Belgrade, Serbia; snezana.belanovic@sfb.bg.ac.rs
* Correspondence: soils.grujic@gmail.com (T.G.); zaklina@yahoo.com or zaklina@imsi.bg.ac.rs (Ž.M.)

Abstract

As land plastic pollution has piled up in recent decades, small products of its degradation, microplastics (MPs), have emerged as a rapidly growing problem in soil environments. The first step in MP evaluation from soils is the extraction of MP particles, and it appears to be a particularly difficult substrate to work with. Aggregate formation and the presence of other organic particles of similar sizes appeared as challenges in constructing an efficient and trustworthy protocol for MP extraction from soils. Density separation-based methods are usually applied and finding efficient cost- and environment-friendly solutions is of high importance, while data comparability is a key factor in increasing general knowledge on the topic. Here, we propose an environmentally friendly protocol based on density separation using sucrose solution. Its efficiency for MP extraction from soil was tested and validated. Protocol validation showed that its use in the extraction of PE was efficient for all examined MP sizes (>32 µm) and PVC >500 µm with high recovery rates, while the extraction of PVC >125 µm is justified since sucrose solution is practically cost-free and completely environmentally safe. Result validation indicates that the proposed protocol has high potential for MP extraction from difficult soil samples.

Keywords: soil plastic pollution; density separation; microplastic extraction

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1. Introduction

The increase in plastic production in recent decades has led to an accumulation of plastic waste in the environment, making it one of the leading environmental problems worldwide. Overall, 33 billion tons of plastic are estimated to be produced by 2050 [1], which is alarming considering that currently only 6–26% of plastic waste is being recycled or reused [2]. Due to the influence of external factors (ultraviolet radiation, temperature, humidity fluctuations, etc.), microcracks appear on the surface of plastic waste, leading to erosion of the material and thus the formation of small plastic particles. Particles between 100 µm and 5 mm in size are classified as microplastics (MPs) [3]. Studies on the impact of MPs on aquatic ecosystems began in the 1970s [4]. However, although terrestrial ecosystems are the primary carriers of MPs [5], much less has been discovered about the fate, transport, and impacts of MPs in the soil environment since the first research started a little over

10 years ago [6]. MP pollution in soil represents a growing danger, primarily due to the slow rate of degradation in ambient conditions and the lack of effective detection and remediation techniques (primarily in situ) in environmental systems. Due to their small size and capacity for bioretention, these particles are more or less mobile and thus their harmful influence spreads spatially and can have potentially far-reaching consequences [7]. The presence of MPs in soil changes the biotic component of terrestrial ecosystems as well [8]. The interaction of soil microorganisms with MPs leads to changes in their metabolic pathways through changes in their enzyme activity [9], which is why MPs can have adverse effects on the growth, reproduction, nutrition, survival, and immunity level of soil biota [10–12]. MP particles also change the structure and water–air regime, thereby changing the conditions of the environment, i.e., the moisture conditions as well as the content and flow of O_2 [13]. In this way, MPs indirectly affect the vital activities of soil biota, soil functions, and biodiversity, while changes in soil structure usually occur when large amounts of MPs are present [6]. MPs have also been found to affect pH [14,15], soil structure and thus water retention, porosity, electrical conductivity [16–18], and evaporation [19], and prevent downward water movement, resulting in water wastage [20]. Hydrophobic surfaces of MPs bind organic matter, e.g., lipids, proteins, and nucleic acids [21]. In this way, MPs interfere with the carbon and nitrogen cycle in the soil, soil microbial activity and nutrient transfer, energy flow of terrestrial ecosystems, global CO_2 production, climate, plant communities, crop production, and biodiversity which has been documented by several studies [7,8,18,22–27]. MPs adsorb pesticides, heavy metals, organic pollutants, pathogens, and resistance genes [28,29]; also, plastic additives used to increase material performance cause soil pollution [30,31]. The further decomposition of MPs results in nanoplastics that can enter plant cells, thus reaching humans through the food chain [32].

1.1. Isolation of Microplastic Particles

One of the first challenges researchers dealing with MPs in the soil came across was the isolation of MP particles from the soil matrix. There is no standard methodology for the extraction of MPs from solid samples, and there are currently numerous protocols that are being applied [33]. This can complicate or even prevent the comparison of research results that are conducted worldwide. Soil particles can form stable aggregates around MPs, which can lead to the underestimation of MP pollution. Finding a method for MP isolation that includes aggregate dispersion and the removal of adherent and other substances from the soil matrix without causing additional fragmentation of MPs is crucial, but has turned out to be complicated [34]. Fan et al. [33] retrieved 882 articles on MPs in the soil and concluded that a step forward must be made in terms of detailed and thorough reporting on MP extraction methods and technologies. Authors have also pointed out the emergence of new extraction and detection techniques such as pressurized fluid extraction, laser direct infrared, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry, which are still rarely mentioned and also technically very demanding [33]. There are many other methods for extracting MPs from the soil matrix that can be found more often in the literature [34–42], but none of them are simple, cheap, environment-friendly, and reliable at the same time. The simplest method is manual extraction, but it is also very time-consuming, size-limited, and prone to misidentification [34]. Electrostatic separation was described by Felsing et al. [38] as an effective method with high recovery rates for pristine MP particles (90–100%) unaffected by organic matter content, the density of the plastic polymer, particle shape and age etc., but it can only be performed on dry samples, which limits the possibilities for aggregate dispersion. The method was verified using pristine MPs sized 63 μm–5 mm, and high recovery rates of 90–100% were reported [38]. Its suitability for cohesive soil samples still needs to be examined, since the potential inability of the method to retrieve samples "caught" within soil aggregates can lead to underestimation, especially for small-sized particles. Froth Flotation and Magnetic Extraction rely on plastic surface hydrophobicity and are highly dependent on polymer type, leading to low recovery rates [40,42]. Claessens et al. [35] developed an elutriation method for MP extraction from sediments, which was later developed by Hengstmann et al. [39]. The authors introduced an elutriation with a glass tube method that separates MPs from the soil matrix by differences in buoyancy according to Stokes' law [39]. The downside of this method is the frequent occurrence of sand as a bycatch; due to this and the relatively low recovery rates, it has not been widely used. Vertical Density Gradient Separation was developed for the recycling industry, which is a method that uses a liquid containing colloidal ferromagnetic particles along with a magnetic field around it [36]. This method, although promising, must be further examined, and it might be financially unjustifiable.

The second-most-applied method according to the available literature is oil separation. It was developed by Crichton et al. [37], and it is an example of a simple, cheap, and safe method in terms of environmental and human health. Authors reported a 90–100% recovery rate for seven tested pristine polymers, which was confirmed in the study that was conducted by Mani et al. [43], where four types of pristine MPs were extracted from spiked complex environmental samples (fluvial and marine-suspended surface solids, marine beach sediments and agricultural soil) using castor oil with an even higher average recovery rate of 99% ± 4%. However, according to Yang et al. [41], this method also has a size limitation (<200 µm), and oil residues can interfere with spectroscopic identification methods that often follow the extraction step. These authors also evaluated MP separation from eight types of agricultural soil using oil extraction and density extraction methods. Based on their results, oil separation is recommended for high-density MP, while density separation is recommended for low-density MP extraction. Table 1 shows the advantages and limitations of the described methods according to the available literature.

Table 1. Advantages and limitations of methods for the extraction of MP from the soil matrix.

Method	Advantages	Limitations	References
Manual extraction	Simple Cheap	Time-consuming Size-limited Misidentification	Möller et al. [34]
Electrostatic separation	High recovery Simple Fast Unaffected by plastic properties	Must be verified for small-sized MPs Sample preparation Potentially inapplicable for cohesive samples	Felsing et al. [38]
Froth flotation	Large sample	Time-consuming Low recovery Polymer-dependent	Imhof et al. [42]
Magnetic extraction	Separates small-sized MPs Cheap	Time-consuming Low recovery Polymer-dependent Sample damage	Grbic et al. [40]

Table 1. *Cont.*

Method	Advantages	Limitations	References
Elutriation	Fast Simple Cheap	Low recovery Sand bycatch	Claessens et al. [35] Hengstmann et al. [39]
Vertical density gradient separation	Applicable within the recycling industry	Must be further examined Potentially financially unjustifiable	Hu et al. [36]
Oil separation	High recovery Simple Fast Cheap	Size-limited Lower recovery for low-density MPs Interference with spectroscopic identification methods	Crichton et al. [37] Yang et al. [41]

1.2. Density Separation

So far, it appears that the most frequently applied method for MP extraction from soil is density separation. Currently, there is no standard analytical method for MP extraction from soil. European standardization committee CEN/TC 444 formed a Task Group under WG6 in 2022 to develop a standard for the sampling, sample pre-treatment, and determination of microplastics in solid matrices. The analytical part of the upcoming standard is expected to include the density separation step as one of the key points of the method. This method uses high-density salt solutions as the extraction media for MP separation from the soil matrix. Since the density of most common plastic polymers varies between 0.8 and 1.6 g/cm^3, and densities of soil mineral particles are greater than 2.0 g/cm^3 [44,45], the optimal density of the extraction solution should be between 1.6 and 1.8 g/cm^3 [46]. Previous studies have reported the application of many different solutions, as well as different numbers of samples and technical setups. Since there is no standard protocol so far, researchers dealing with MPs in soil develop new or modify existing methods that correspond to their technical possibilities and research needs. When it comes to the selection of the density solution, the decision should be primarily based on which polymer is being extracted, but toxicity and price are also to be considered. Only polymers of lower density than that of the separation solution will be extracted. Based on previous studies, the most common types of MP polymers found in soil are PE (0.88–0.96 g/cm^3), PP (0.89–0.92 g/cm^3), and PS (0.96–1.05 g/cm^3) [33]. It is noted that the three most common polymers found in soil are all low-density. Lee et al. [47] reviewed forty studies published from 2017 to 2022 dealing with MPs in soil and freshwater sediments, all from real environmental samples, with a focus mostly on sample pretreatment as well as extraction and characterization methods. The authors found that the most commonly used extraction solution was NaCl (1.2 g/cm^3), since it was used exclusively in thirteen out of forty studies. Additionally, two other studies used only water (1.0 g/cm^3) as a separation medium. This means that the extraction media used in 37.5% of reviewed studies were not applicable for the separation of high-density polymers such as PVC (1.4 g/cm^3) and PET (1.3–1.4 g/cm^3), which indicates that the presence of denser MPs in the soil environment may be underestimated. This is also supported by the fact that PET and PVC comprised 17.9% of the world's total plastic output by 2019 [48]. Some authors still recommend NaCl since they believe that PVC and PET represent only a small percentage of soil MP pollution [49]. NaCl remains a common choice of extraction medium as it is cheap, easy to acquire, and safe for human health and the environment. $CaCl_2$ (1.3–1.5 g/cm^3) and NaBr (1.4–1.6 g/cm^3) have also been evaluated as good options for density separation since they are applicable for denser polymers, inexpensive, and are environmentally friendly. The downsides of using these salts are that $CaCl_2$ can cause an aggregation of organic matter, which may lead to difficulties in quantification and qualitative assessment [49], and slightly lower recovery has been reported for NaBr density separation compared to other high-density solutions [50]. K_2CO_3 (1.54 g/cm^3) is another non-toxic and cost-effective option, with a high reported recovery of approximately 90% for PVC [51]. Sodium polytungstate—SPT ($3Na_2WO_4 \cdot 9WO_3 \cdot H_2O$)—is a medium of adjustable density, which can reach up to 3.1 g/cm^3; it is also of low toxicity and relatively high cost, but can be reused multiple times, with a reported recovery of 86–99% for PET [52]. Lithium metatungstate—LMT ($Li_2O_{13}W_{4-24}$)—can also be used as a separation medium; it has a density of 1.62 g/cm^3, is relatively low in toxicity and high in cost, and reportedly provides a recovery rate of 81% for PE pellets extracted from sediments [53]. When it comes to unknown samples from the environment, when researchers do not know what type of pollution they can expect, it is always safer to choose a denser separation solution. ZnBr and $ZnCl_2$ can be made at different concentrations to achieve the desired density, which is usually between 1.4 and 1.8 g/cm^3. Maes et al. [54] recommended 1.37 g/cm^3 $ZnCl_2$ since they concluded that this concentration provided a good compromise between recovery and interference from mineral bycatch. The ability to adjust the density of the solution according to the research question is a great advantage of Zn solutions; however, solutions containing Zn are hazardous and corrosive [55]. Additionally, $ZnCl_2$ can react with carbonates, which leads to foaming and significantly complicates further procedures [56]. NaI (1.8 g/cm^3), with its high density, is suitable for the extraction of a wide range of polymers, but due to high costs and considering environmental hazards and its embryotoxicity to aquatic organisms, other solutions are often recommended [50]. All the methods used so far for density separation are presented in Table 2.

Table 2. Density solutions used for MP density separation and their recovery rates according to the literature.

Solution	Density [g/cm^3]	Polymer(s)	Recovery	Reference
H_2O	1	PP, PE	40–90%	Quinn et al. [57]
NaCl	1.2	PS, PP, PE, PA, PC, ABS, PMMA	69–98%	Liu et al. [58]
NaBr	1.4–1.6	PA, PP, PE, PET, POM, PVC, PC, ABS, PMMA, PS	85–100%	M. Liu et al. [50]
NaI $^{\mathbf{hc}}$	1.6–1.8	PVC	98–100%	Cauwenberghe [59]
$CaCl_2$	1.3–1.5	MPs	93–98%	Scheurer and Bigalke [49]
$ZnCl_2$ $^{\mathbf{hc}}$	1.37	PA, PE	85–98%	Maes et al. [54]
$ZnBr_2$ $^{\mathbf{hc}}$	1.7	PE, HDPE, PS, PVC, PET	95–100%	Quinn et al. [57]
K_2CO_3	1.54	PVC	90%	Gohla et al. [51]
SPT	up to 3.1	PET	86–99%	Uguagliati et al. [52]
LMT $^{\mathbf{c}}$	1.62	PE	81%	Masura et al. [53]

h—Potentially hazardous for human health or/and environment; c—costly. Modified from Fan et al. [33].

When deciding on a separation solution, a compromise between efficiency, cost, and toxicity is to be made. There have been developments in reducing costs by optimizing the consumption of expensive, higher-density solutions without decreasing efficiency. According to Kedzierski et al. [60], NaI can be reused up to ten times with the same efficiency. The recovery of 68–99% on various MPs was reported using a 1.50 g/cm^3 NaCl and NaI mix, which can be recycled at least five times by filtering it [61,62]. Several multi-stage separation protocols can be found in the literature that imply several consecutive density separation cycles going from low- to high-density solutions, which can increase efficiency and reduce the use of denser solutions [63–71].

Even though there are many protocols for MP extraction from the soil matrix based on density separation, most can be summarized in a few steps: sample pretreatment, density separation, and soil organic matter removal [47]. Since the upcoming standard that is being developed by the European standardization committee CEN/TC 444 is also expected to include density separation, which is the most widely used method, we decided to test a separation medium that we are familiar with. Some members of our project team have experience in the isolation of arbuscular mycorrhizal fungi (AMF) spores through wet sieving and sucrose density gradient centrifugation, as described in Oehl et al. [72]. Sucrose solution is basically cost-free and completely environmentally safe, and it has been widely used for the extraction of AMF spores from the soil matrix by density separation for fifty years [73]. This is why we decided to modify the protocol [72] and test its applicability for MP extraction.

2. Materials and Methods

The protocol described here was developed to suit the needs of the first research on MPs in the soils in the territory of Serbia, through “Evaluation of the Microplastic in the Soils of Serbia-EMIPLAST-SoS” financed by the Science Fund of the Republic of Serbia. Its capability to isolate MPs from soil was tested and validated. All soil samples used in this study were alluvial soils and, according to texture class, could be classified as “light clay” with a clay content between 30 and 45%, which was determined by the pipette method according to the modified International “B” method [74].

2.1. Pretreatment

For soil disaggregation, 30 g of air-dried soil was suspended in 500 mL of deionized water and 50 mL of 1 M sodium pyrophosphate and left overnight followed by occasional manual shaking. The soil suspension was wet-sieved through a set of sieves (1000, 500, 125, and 32 µm) at a shaking amplitude of 85 for 5 min or until the discharge of clean water using a Retsch vibratory sieve shaker (AS 200 basic B, 2018).

2.2. Density Separation

The contents of each sieve from the wet-sieving step were transferred to 50 mL centrifugation tubes, divided so that the amount of sample did not exceed 5 g per tube, and filled with sucrose solution (1.73 g/cm^3) made by dissolving 2039 g of sugar in 1000 mL of distilled water. Centrifugation was performed at 1500 rcf for 5 min. After centrifugation, the resulting supernatant was passed through the smallest sieve (32 µm), thoroughly washed with water to remove all the sugar, and transferred to deep glass dishes.

2.3. Soil Organic Matter Removal

Then, 50 mL of 30% H_2O_2 was applied to each sample, and they were then incubated for 72 h at 40–45 °C. At the end of the 72 h period, samples were again washed through the smallest sieve (32 µm) and collected for quantification.

2.4. Validation

Method accuracy was validated by spiking the samples with known amounts of MPs and determining their recovery. Recovery was calculated as follows:

$\mathrm{REC} = \mathrm{Rmp} \times 100/\mathrm{Smp}$
REC = recovery; Rmp = recovered MP; Smp = spiked MP.

Method precision was validated by determining the relative standard deviation (RSD) of recovered MPs. We considered an RSD lower than 5% satisfactory, and it was calculated as follows:

$\mathrm{RSD} = \mathrm{SD}/\mathrm{MEAN} \times 100$
$\mathrm{SD} = \sqrt{\sum(xi - \overline{x})^2/(n-1)}$
RSD = relative standard deviation; SD = standard deviation; MEAN = mean value.

Spiking of quartz sand with MPs. Density separation using sucrose solution was first validated separately by spiking quartz sand (as an adequate exchange for natural soil particles) with a known amount of pristine PE and PVC. Density separation was also validated for four size groups based on our sieve sizes: 1000–5000 µm, 500–1000 µm, 125–500 µm, 32–125 µm. Samples were spiked with each polymer type and size group separately in triplicate. MP particles of size > 500 µm (1000–5000 µm and 500–1000 µm) were counted (20 particles per sample) and MPs of size < 500 µm (125–500 µm, 32–125 µm) were measured on an analytical balance with a precision of 0.00001 g and samples were spiked with quantified MPs. The centrifugation step was performed as previously described.

Spiking of soil columns with MPs. In order to simulate and validate MP extraction from real samples, we sampled undisturbed soil columns at project control sites in alluvial forests in the Sava, Danube, and Morava River basins in triplicate (30 cm deep with 3.5 cm radius). Columns were installed and secured, and 5 g of 1000–3000 µm-sized PVC and 1.5 g of 500–1000 µm-sized PVC were applied on the surface of each column. The columns were watered once a week for 3 months (31 May–31 August). After the three-month experimental period, MPs that did not penetrate soil columns were collected, divided into two size groups by sieving through a 1000 µm sieve, and measured on an analytical balance (RADWAG AS 160/C/2, Radom, Poland). This procedure was used to determine how many MPs were within each column. MP particles that penetrated soil columns were extracted from the matrix and quantified. MPs were isolated using the described protocol with one modification; specifically, the organic matter removal step using H_2O_2 digestion was skipped. OM was separated by density separation using 1.2 g/cm^3 sucrose solution, which was made by dissolving 500 g of sugar in 1000 mL of distilled water.

3. Results

3.1. Validation Results

3.1.1. Spiking of Quartz Sand with MPs

The recovery of MPs from spiked sand samples using centrifugation by sucrose density gradient is shown in Figure 1.

The average recovery of PE was >90% for all size groups; however, there is a trend of a slight decrease in recovery with a decrease in particle size for PE < 500 µm. This trend is more expressed in the case of PVC recovery, as shown in Figure 2.

3.1.2. Spiking of Soil Columns with MPs

The amount of MPs that penetrated the soil columns is shown in Table 3.

Table 3. MPs that penetrated the soil columns.

		Sava			Danube			Morava		
Replicate		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
MP [g]	1000–3000 µm	2.0457	2.2122	2.2800	2.5873	2.4185	2.6097	2.8718	2.7567	3.0712
	500–1000 µm	0.9708	0.9462	1.0192	1.0403	1.0119	1.0542	1.0659	1.1145	1.1358

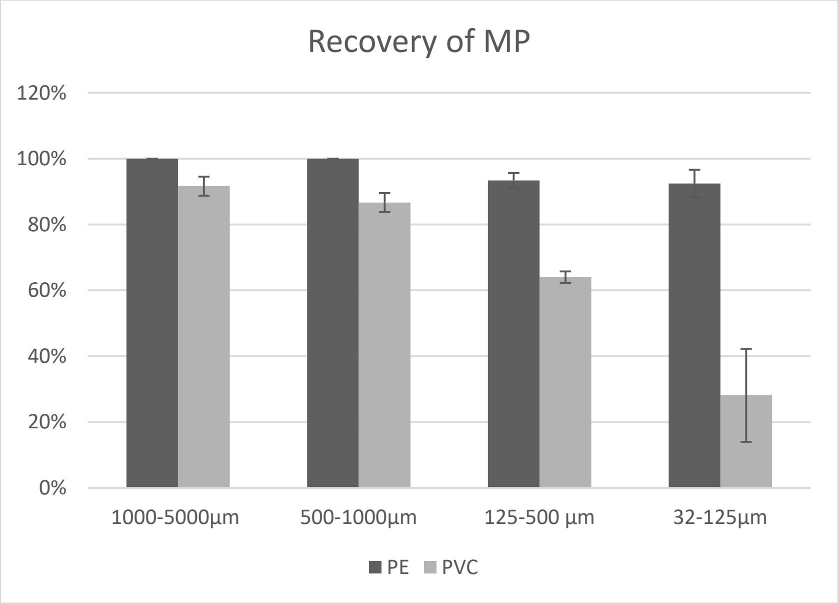

Figure 1. Recovery of MPs according to polymer type and size. Average recovery depending on type of polymer and size is as follows: 1000–5000 µm PE—100 ± 0%; 500–1000 µm PE—100 ± 0%; 125–500 µm PE—93.41 ± 2.24%; 32–125 µm PE—92.45 ± 4.20%; 1000–5000 µm PVC—91.67 ± 2.89%; 500–1000 µm PVC—86.67 ± 2.89%; 125–500 µm PVC—64.04 ± 1.73%; 32–125 µm PVC—28.13 ± 14.13%, as shown in Figure 1. RSD was satisfactory for all size groups and both polymer types, except for 32–125 µm-sized PVC.

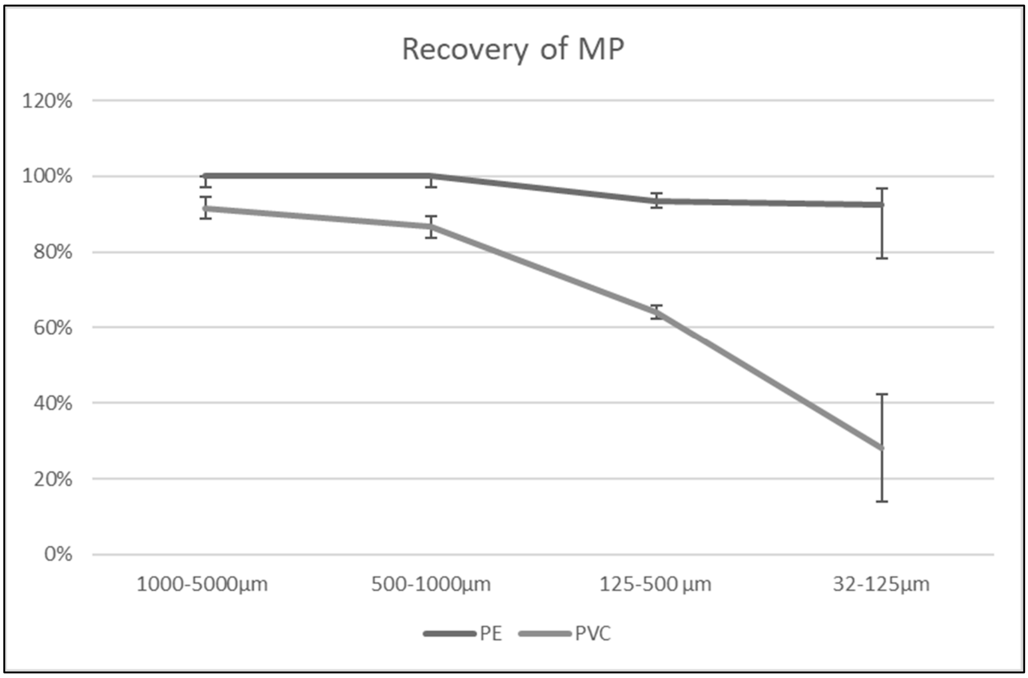

Figure 2. MP recovery by size and polymer type.

The average recovery of 1000–3000 μm-sized particles was between 91.05 ± 2.41% in columns from the Danube River basin site and 94.63 ± 1.35% from the Morava River basin site; the average recovery of 500–1000 μm-sized particles was also around 88.85 ± 3.65% from Danube site columns and 90.74 ± 1.35% from the Sava River basin site, as presented in Figure 3 and Table 4. RSD was satisfactory for both size groups.

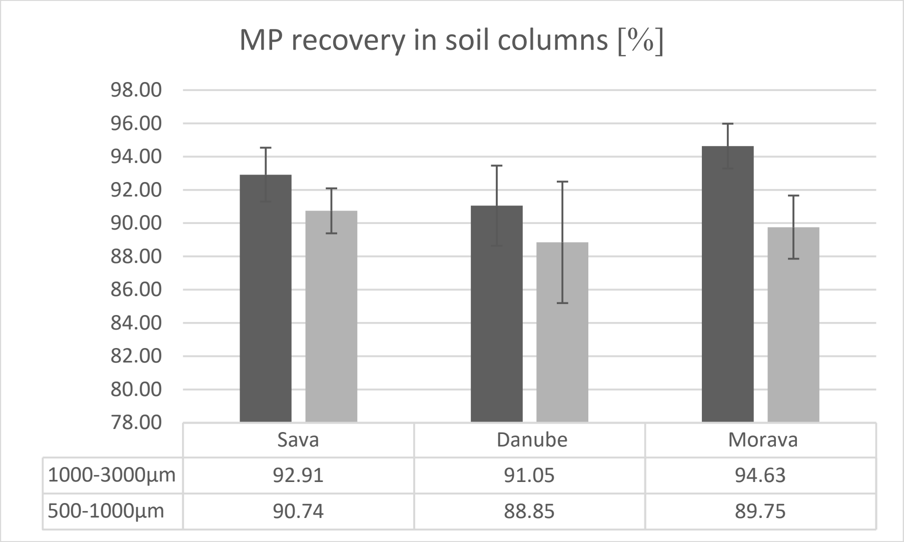

Figure 3. MP recovery from spiked soil columns.

Table 4. Isolated MPs [g] and their recovery [%] and RSD [%].

		Sava			Danube			Morava			RSD	Average
Replicate		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**[%]**	**Recovery [%]**
1000–3000 μm	[g]	1.9280	2.0658	2.0774	2.3038	2.1856	2.4459	2.7376	2.5659	2.9327	**2.40**	**92.86 ± 2.23**
	[%]	94.25	93.38	91.11	89.04	90.37	93.73	95.32	93.08	95.49		
500–1000 μm	[g]	0.8682	0.8718	0.9239	0.8884	0.8953	0.9769	0.9334	1.0104	1.0340	**2.58**	**89.78 ± 2.31**
	[%]	89.43	92.14	90.65	85.39	88.48	92.66	87.57	90.66	91.03		

4. Discussion

4.1. Pretreatment

MPs can be “trapped” within soil aggregates, and the release of these particles requires aggregate disintegration and dispersion, which can be difficult, especially for cohesive soils with high clay content. Since soil for MP analysis cannot be ground because it can lead to further MP fragmentation, chemical dispersion agents such as aqueous sodium hexametaphosphate solution [70,75,76] or ultrasonication [77,78] can be applied. Even though their interference or the enhancement of microplastic fragmentation has not been assessed yet [55], we believe that failure to isolate particles trapped in aggregates would have a greater impact on overall results and estimates. This is why we opted for chemical disaggregation using 1 M sodium pyrophosphate, followed by wet sieving. The choice of sieves (1000, 500, 125, and 32 μm) was taken from the protocol for isolation of the spores of arbuscular mycorrhizal fungi (AMF) described in Oehl et al. [72], which was used as a starting procedure for developing the protocol for MP isolation. Therefore, we narrowed the MP size 5 mm to 32 μm. Sieve sizes and sample amounts can be adapted to the research needs and technical possibilities of the laboratory.

4.2. Density Separation

The AMF spore isolation protocol originally assumes the usage of a 1.35 g/cm^3 water–sucrose solution for density separation and subsequent centrifugation at 900 rcf for 2 min. Since this would not be applicable for the separation of some high-density MPs, this step was modified for purposes of MP separation. The solubility of sucrose is 2039 g per 1000 g of water at 20 °C, and such a solution has a density of 1.73 g/cm^3 [79]. The solution was prepared as described by Hagen and Tadokoro [79]. Since granulated sugar is a high-purity sucrose (about 99.9%), the separation solution was made by melting this cheap and easily available substance in hot deionized water. The sucrose used in this study was a commercial-grade sugar purchased from a local store, derived from sugar beet. The solubility of sucrose at 40 °C increases to 2381 g per 1000 g of water [79], which is why it was enough to heat deionized water up to 40 °C to dissolve 2039 g of sugar in 1000 g of water without difficulties. When all the sugar was dissolved and the solution was homogenized, it was left to cool down before using it for density separation. The centrifugation technical setup was also modified to be applicable for MP density separation. Grause et al. [80] examined the dependence of recovery and purity on centrifugation time and speed. Authors reported that a high recovery was reached after only 1 min, but with low purity. It was concluded that the optimal centrifugation time is between 5 and 20 min to provide enough time for mineral particles to settle. It was also reported that high recovery can only be achieved at high speeds, and centrifugation at 3700 rpm was recommended [80]. This was adapted to the technical setups and capacities of our laboratory, so we opted for centrifugation at 1500 rcf for 5 min.

A potential limitation of using sucrose solution as a medium for density separation could be possible interactions with MP particles under suspended conditions. Specifically, sucrose solution may promote the formation of aggregates, which is an issue that has already been observed with the use of $CaCl_2$ as a separation medium [49]. Aggregates could become large enough to settle more rapidly, thereby affecting the recovery and separation efficiency. This should be further investigated.

4.3. Soil Organic Matter Removal

The density of soil organic matter depends on the degree of decomposition and ranges between 0.9 and 1.5 g/cm^3 [81]. Since the density of soil organic matter (SOM) is similar to the density of MPs, it also separates from soil mineral particles during centrifugation. The presence of SOM can interfere with the quantification and characterization of extracted MPs, which is why many existing protocols include SOM removal steps using, e.g., acidic or alkaline digestion, oxidation with H_2O_2, or enzymatic digestion. The use of strong acidic or alkaline solutions can be destructive and compromise MPs [82], while non-destructive digestives such as 10% KOH can take 2–3 weeks to successfully remove SOM [83,84]. Enzymatic digestion procedures have mostly been reported for aquatic samples, but there are studies proposing a combination of enzymatic digestion with short-term H_2O_2 reactions, including Fenton's reagent, with promising results [82]. Oxidation with H_2O_2 can also be time-consuming and can last a week at room temperature [85]. In order to shorten the reaction time, oxidation at higher temperatures is recommended or the use of Fenton's reagent as a catalyst, which was reported to achieve high SOM removal rates even at room temperatures [64]. According to the available literature, 30% H_2O_2 is most commonly used. Amounts of reagent vary between 40 and 200 mL depending on the amount of sample, incubation temperatures vary between 50 and 70 °C, and oxidation usually lasts for 72 h [58,69,86–91]. The reaction time can be shortened by stirring and was reported to last for 12 h [87] or even 2 h [91]. We decided on the use of 50 mL of 30% H_2O_2 and an incubation time of 72 h at 40 °C–45 °C so we did not risk the loss of non-thermostable MPs. Since our samples were SOM-rich, the formation of dense foam was observed.

4.4. Validation

Spiking of quartz sand with MPs. The use of sucrose solution for density separation has potential for application in other MP extraction setups and is the most innovative step of the protocol proposed here due to the low cost and absence of negative environmental impact. Therefore, this step was validated separately. Two types of pristine MPs were used, PE and PCV, as they are the lightest and the heaviest among the commonly found polymers in the environment, respectively [3,33]. Based on the recovery test, MP separation by centrifugation using sucrose density gradient is applicable for the extraction of all examined sizes of PE (Figure 2). The separation of PVC > 500 µm is also justifiable since the recovery between 85.00 and 95.00% is high, especially considering that the density separation of high-density polymers such as PVC is challenging. A recovery of 64.04 ± 1.73% for 125–500 µm PVC can also be considered satisfactory when all factors are considered, and specifically considering that the density separation of heavy polymers is only possible with high-density solutions such as NaI, $ZnCl_2$, and ZnBr, which are expensive and are potentially hazardous for human health and/or the environment [33]. The proposed method is not justifiable for the extraction of PVC < 125 µm, even considering costs and environmental friendliness, since the recovery of 28.13 ± 14.13% cannot provide any valid results.

Spiking of soil columns with MP. Since every segment of each column weighed approximately 400 g and the whole amount was supposed to be processed, it was quite challenging. The upside of wet-sieving pretreatment is that large amounts can be treated at once, but the centrifugation step requires a longer time. Our research sites are alluvial forests in Serbia, and after wet sieving, there was a lot of organic matter left in samples, and its removal by H_2O_2 oxidation would have been highly time- and labor-consuming. Instead, we decided to prepare a sucrose solution with a density of 1.2 g/cm^3, since most of the SOM is of lower density, and proceed with SOM removal using density separation. After centrifugation, PVC (1.4 g/cm^3) particles and soil sediments that remained after wet sieving were settled at the bottom of the tube, and floating organic matter was removed. The second centrifugation step was performed as previously described using 1.73 g/cm^3 sucrose solution; MPs were separated from the sediment in this step. There were small amounts of SOM residues in the samples, which we were able to separate manually since this fraction was >500 µm. The average recovery of 92.86 ± 2.23% for 1000–3000 µm sized MPs and 86.67 ± 2.89% for MPs between 500 and 1000 µm is considerably high, especially for high-density polymers such as PVC. These results are in line with the spiking of quartz sand with MP validation results. The average recovery from soil columns is slightly but insignificantly higher. This can be due to the fact that columns were spiked with a considerably larger amount of MPs, and the loss of single particles would have less effect on the overall recovery. Also, multi-stage density separations with solutions of various densities were proven to increase extraction efficiency [60,61,63–68]. This should be tested using different sucrose solutions in the future, and it might have contributed to the high recovery of the two-step centrifugation extraction, which was carried out in this experiment. This also showed that the variability of density can have other applications, e.g., adjusting the density of the sucrose solution could enable skipping the organic matter digestion step by multiple centrifugation cycles when extracting heavy plastic polymers, such as PVC, in our case. Admittedly, this could be performed with various combinations of different separation solutions, but adjusting the density of the sucrose solution is feasible, cost-free, and completely safe, and could be adapted to different research needs. Another upside of sucrose solution is that it can be easily dissolved and washed off with water, so it does not interfere with spectroscopic identification methods, which can be the case with oil density separation [41].

5. Conclusions

MPs are among the most important environmental issues due to their prevalence and impact on the environment. The effects of MPs in the soil are numerous and long-term and are not yet fully understood or defined. To approach this problem in the right way at all levels, locally, regionally, and globally, it is necessary to take several key steps in terms of understanding the "life cycle" of MPs in soil ecosystems. The availability and comparability of results, information, and experiences are a prerequisite for successfully overcoming existing obstacles, establishing facts, and increasing the scope of general knowledge on this topic. We proposed an environmentally safe and cheap protocol based on density separation using a sucrose solution. Protocol pretreatment allows for large amounts of samples; however, in that case, the centrifugation step can be more or less time-consuming depending on the laboratory's technical setup. The representativeness of the sample increases with its amount when it comes to MP pollution, so a compromise should be made between labor and research needs without compromising the validity of the results. Density separation using sucrose solution validation showed that its use in the extraction of PE is applicable for all examined MP sizes (>32 μm) with high recovery rates. It is also applicable for the extraction of PVC > 500 μm with high recovery, which was confirmed with MP extraction validation through the spiking of both quartz sand and soil columns. We conclude that the extraction of PVC > 125 μm is justifiable considering that sucrose solution is practically cost-free and completely environmentally and health safe. The validation of results indicates that sucrose solution density separation has excellent potential for MP extraction from soil samples. The validation of MP extraction using sucrose solution against other common density separation solutions for different MP polymers and sizes should be carried out in the future. The possibility of using various sucrose solutions in multi-step density separation to increase efficiency is to be examined.

The use of sucrose solution as a density separation medium can have significant benefits, especially for developing countries and laboratories with limited resources. Due to its low cost and non-toxic nature, sucrose offers an accessible alternative to more expensive and hazardous media, enabling broader participation in microplastics research in general. Moreover, the equipment required for the protocols involving sucrose solution is generally considered standard laboratory apparatus, which reduces the need for costly investments in specialized instruments. This accessibility facilitates capacity building in regions where advanced laboratory infrastructure is lacking, promoting environmental monitoring and data collection on microplastic pollution at a local and regional scale. Ultimately, implementing sucrose-based methods can help bridge the gap between resource-rich and resource-limited settings, contributing to more equitable scientific research and environmental management globally.

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Abbreviations

The following abbreviations are used in this manuscript:

MP	Microplastic
PP	Polypropylene
PE	Polyethylene
PS	Polystyrene
PA	Polyamide (Nylon)
PC	Polycarbonate
ABS	Acrylonitrile Butadiene Styrene
PMMA	Polymethyl Methacrylate (Acrylic)
PET	Polyethylene Terephthalate
POM	Polyoxymethylene (Acetal)
PVC	Polyvinyl Chloride
HDPE	High-Density Polyethylene
AMF	Arbuscular Mycorrhizal Fungi
SOM	Soil Organic Matter
RSD	Relative Standard Deviation

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