ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ

Εθνικόν και Καποδιστριακόν Πανεπιστήμιον Αθηνών

—— ΙΔΡΥΘΕΝ ΤΟ 1837 ——

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Separation and Recovery of Natural & Anthropogenic Organic Polymers in soils of Attica, Greece

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ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

Διαχωρισμός και ανάκτηση φυσικών και ανθρωπογενών οργανικών πολυμερών σε εδάφη της Αττικής, Ελλάδα

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ABSTRACT

Considering the estimated maximum 300k Tn of microplastics (MPs) released yearly in the EU and the scarcity of systematic surveys for MPs, monitoring and managing their potential risks poses notable challenges to researchers and governments worldwide. Primarily owing to their small size and complex composition, the lack of standardization on methodologies for sampling & analysis, reporting units, and QA/QC procedures, present significant challenges for synthesizing research findings, interpreting data, and drawing meaningful conclusions. Furthermore, up to now the focus of research has been mostly on developing methods to estimate the amount and type of MPs in the marine environment. Standardized protocols for the recovery and characterization of MPs in soils are still under investigation, although it has been established that they are characterized by elevated adsorption capacity as the result of their large specific surface area; thus, they adsorb typical soil contaminants such as metals and metalloids, promoting their dispersion in the environment.

In this study, soil samples were selected from the systematic soil dataset of the Attica region in Greece, taken at depths of 0-20 cm and 20-40 cm. Spiking experiments were conducted using a composite mixture of soil collected at natural forest sites and blank samples spiked with cryomilled polymers. Various techniques and reagents were tested to enhance recovery and facilitate characterization. Different reagents were compared regarding their effectiveness on the degradation of organic particles, including Strand solution (0.67 mol/L NaClO, 0.45 mol/L KOH), KOH (2.6%), H_2O_2 (30%), and one using porcine pancreatic enzymes. Mineral particle removal involved density separation with supersaturated NaCl and $ZnCl_2$ solutions, followed by filtration to retain MPs. Modifications were made in the choice of reagents for repeated experiments. A multi-method approach for identifying and characterizing MPs included analysis with an optical microscope, FTIR, and Raman spectroscopy for evaluating the MP recoveries based on different treatments. All pre-treatment methods successfully reduced certain organic matter aggregates, and both chlorine solutions were effective in extracting lighter plastic particles like polyethylene (PE) and polystyrene (PS). Notably, despite their higher density, PVC, PET, and tire particles were still recovered from both density solutions through observations using an optical microscope. However, the KOH and Strand solutions were unable to decompose organic aggregates. It is crucial to continue the experimental process by utilizing FTIR analysis for either natural or anthropogenic organic polymer counting, in order to apply the recovery methods to soil samples with unknown microplastic contamination

Keywords: Microplastics (MPs), Contamination, Characterization methods, Density separation

ΠΕΡΙΛΗΨΗ

Λαμβάνοντας υπόψη την εκτιμώμενη μέγιστη ποσότητα των 300 χιλιάδων τόνων μικροπλαστικών (MPs) που απελευθερώνονται ετησίως στην ΕΕ και τη σπανιότητα των συστηματικών ερευνών για τα MPs, η παρακολούθηση και η διαχείριση των πιθανών κινδύνων τους εμπεριέχουν σημαντικές προκλήσεις για ερευνητές και κυβερνήσεις σε όλο τον κόσμο. Κυρίως λόγω του μικρού μεγέθους και της σύνθεσής τους, της έλλειψης τυποποίησης στις μεθόδους για την δειγματοληψία και ανάλυση, τις μονάδες αναφοράς και τις διαδικασίες ποιοτικού ελέγχου των μετρήσεων, η γεωχημική έρευνα στο πεδίο αυτό χαρακτηρίζεται από σημαντικές προκλήσεις για τη σύνθεση των ερευνητικών ευρημάτων, την ερμηνεία των δεδομένων και την εξαγωγή σημαντικών συμπερασμάτων. Επιπλέον, μέχρι σήμερα η κύρια εστίαση της έρευνας αφορά στην ανάπτυξη μεθόδων για την εκτίμηση της ποσότητας και του τύπου των MPs στο θαλάσσιο περιβάλλον. Τα τυποποιημένα πρωτόκολλα για την ανάκτηση και χαρακτηρισμό των MPs στα εδάφη βρίσκονται ακόμα σε πρωταρχικό στάδιο, αν και έχει βρεθεί ότι τα μικροπλαστικά σωματίδια στο έδαφος χαρακτηρίζονται από αυξημένη ικανότητα απορρόφησης λόγω της μεγάλης ειδικής επιφάνειάς τους· έτσι, απορροφούν τυπικούς ρύπους του εδάφους όπως μέταλλα και μεταλλοειδή, διευκολύνοντας τη διασπορά τους στο περιβάλλον.

Σε αυτή τη μελέτη, επιλέχθηκαν δείγματα εδάφους από το συστηματικό σύνολο δεδομένων εδάφους στην περιοχή της Αττικής, που λήφθηκαν από βάθη 0-20 cm και 20-40 cm. Διεξάχθηκαν πειράματα ελεγχόμενου εμπλουτισμού (spiking experiment) όπου χρησιμοποιήθηκε σύνθετο μείγμα εδάφους που συλλέχθηκε σε δασικές περιοχές με τη προσθήκη μετρημένων σύνθετων πολυμερών με ελεγχόμενη άλεση για τη μείωση μεγέθους τους. Δοκιμάστηκαν διάφορες τεχνικές επεξεργασίας και διαχωρισμού για να βελτιωθεί η ανάκτηση και να διευκολυνθεί ο χαρακτηρισμός των μικροπλαστικών σε ένα ελεγχόμενο πείραμα. Συγκρίθηκαν διαφορετικά διαλύματα για την χώνευση των οργανικών σωματιδίων, ως προς την αποτελεσματικότητα αποσύνθεσης συσσωματωμάτων, συμπεριλαμβανομένων των διαλυμάτων: Strand (0,67 mol/l NaClO, 0,45 mol/l KOH), KOH (2,6%), H2O2 (30%), και χώνευσης μέσω παγκρεατικών ενζύμων. Η αφαίρεση ορυκτών σωματιδίων περιλάμβανε το διαχωρισμό πυκνότητας με υπέρκορα διαλύματα NaCl και ZnCl$_2$ και του φιλτραρίσματος για την ανάκτηση υπολειπόμενων πλαστικών και μη σωματιδίων. Τα πειράματα επαναλήφθηκαν σε κάποιες περιπτώσεις και όπου κρίθηκε απαραίτητο γίναν τροποποιήσεις. Για τον εντοπισμό και τον χαρακτηρισμό των MPs χρησιμοποιήθηκαν αναλυτικές μέθοδοι, όπως η οπτική παρατήρηση μέσω μικροσκοπίου, FTIR και Raman φασματοσκοπία για την αξιολόγηση των ανακτημένων μικροπλαστικών και υπολειπόμενων οργανικών σωματιδίων. Όλες οι μέθοδοι επεξεργασίας μείωσαν επιτυχώς ορισμένα συσσωματώματα οργανικής ύλης και αμφότερα τα διαλύματα χλωρίου ήταν αποτελεσματικά στον διαχωρισμό ελαφρύτερων πλαστικών σωματιδίων όπως το πολυαιθυλένιο (PE) και το πολυστυρένιο (PS) από οργανικά και ανόργανα σωματίδια. Ειδικότερα, παρά την υψηλότερη πυκνότητά τους, τα σωματίδια PVC, PET και ελαστικών αυτοκινήτων εξακολουθούσαν να ανακτώνται και από τα δύο διαλύματα πυκνότητας μέσω παρατηρήσεων με τη χρήση οπτικού μικροσκοπίου.

Ωστόσο, τα διαλύματα ΚΟΗ και Strand δεν αποσύνθεσαν οργανικά συσσωματώματα. Για μια ολιστική μελέτη είναι σημαντικό να συνεχιστεί η πειραματική διαδικασία με τη χρήση FTIR ανάλυσης για την καταμέτρηση είτε φυσικών είτε ανθρωπογενών οργανικών πολυμερών, προκειμένου να εφαρμοστούν οι διάφοροι μέθοδοι ανάκτησης σε δείγματα εδάφους με αγνώστου μόλυνση από μικροπλαστικά.

Λέξεις κλειδιά: Μικροπλαστικά, Ρύπανση, Μέθοδοι χαρακτηρισμού, Διαχωρισμός πυκνότητας

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CHAPTER 1. Introduction

1.1   Purpose and objectives of research

To date, laboratory protocols for the recovery of microplastics from soils have not been established, while scientific interest in the study and removal of these particles is increasing. It is essential to focus on methods to recover them from different environments. The aim of this thesis is to study and compare methods for the recovery and analysis of microplastics from soil samples, to establish and use a protocol for researching soils contaminated with microplastics. The thesis includes information and data on laboratory methods for polymer identification and microplastic removal processes. Additionally, details are provided regarding the origin of soil samples used for method comparison, laying the groundwork for future research into microplastics in Greek soils, specifically in the Attica basin.

This thesis is part of the broader research project of PRIORITY, which is related to development, implentation, and consolidation strategies to tackle the global challenges of micro-and nanoplastics in the environment. It is part of the European COST ACTION CA20101 program, specifically the Short-Term Scientific Mission (STSM) titled "Linking microplastic pollution with geochemical data to determine overall anthropogenic impact on soils." The practical aspect of the project lasted from September 4, 2023, to September 29, 2023, and was funded by the European Union (Cost-Action), continuing with the implementation of objectives and deliverables until completion. Soil samples from the Department of Geochemistry and Environment were used for investigating microplastic recovery methods within the framework of the project "Granting of EAGME for the preparation of a handbook of guidelines for the preparation of soil geochemical distribution maps in the Greek Territory". The project, funded until the end of 2023 by national resources, is carried out by the Department of Geochemistry and Environment of DOPE, with acronym "TRACE ELEMENT DISTRIBUTION," serial number: 2020ΣΕ06100004, and project code E.A.G.M.E.: 22204.0.

This thesis is connected to the first part of the project, which involves soil sampling in a pilot area of the Greek Territory (part of Attica and Boeotia), aiming to test the Preliminary Guide (PG) for the preparation of soil geochemical distribution maps in the Greek Territory prepared by EAGME (Kontomichalou 2022), coordinated by Dr. Alexandros Liakopoulos, Director of the Department. The sampling team of the project consists of A. Liakopoulos, A. Argyraki, and A. Kontomichalou. In this context, samples were collected for microplastics research in soil samples from a part of Attica with different land uses, urban, agricultural, or forested. Specifically, fifty-three (53) samples were collected from forested areas of Attica.

In this context, the objectives of the thesis are as follows:

- Application of the sampling protocol in the field for collecting samples for microplastics investigation.

- Application of different methods for microplastic recovery in soil samples.
- Use of analytical methods for the identification of standard microplastics with known polymer compositions.

To conduct research on microplastics in any environment matrices, it is crucial to test methods for recovery and separation of microplastics from other particles that may form aggregates in the sample. Therefore, this thesis will focus on the soil sampling protocol, comparing various methods for microplastic recovery through organic matter digestion and separation of soil fractions from pure polymers. Finally, analytical polymer identification methods will be applied for comparison and future application in samples from the pilot program.

1.2   Description of the study area

1.2.1   Geography and geology

The pilot project area for implementing the sampling protocol extends to areas in Attica and Boeotia, covering approximately 2600 km^2, which are administratively within the boundaries of 121 Municipalities and Municipal Communities. The area of the pilot project corresponds to approximately 1/50 of the total area of the country (131,957 km^2). The total sampling locations (117 sites) are within 9 map sheets (MS) of the Geographic Service of the Army (GSA), specifically the sheets (Title (number)):

1. Chalkis (379)

2. Nea Psara (Eretria) (236)

3. Athens-Elefsis (017)

4. Rafina (322)

5. Kifisia (161)

6. Athens-Piraeus (019)

7. Athens-Koropi (018)

8. Plaka (301)

9. Lavrion (186)

The map of sampling locations and their placement on the MS of the GSA are shown in Figure1. Based on the total area of the pilot project, the sampling density is calculated at 1.1 samples / 25 km^2.

Figure 1. Total soil sampling locations of the pilot project for the implementation of the pilot soil sampling pilot project of the preliminary soil sampling guide of EAGME. On the background map the administrative boundaries of municipalities and municipal communities are also depicted and dotted the boundaries of the GIS map sheets

Geomorphologically, the area encompasses both plains, hills, and mountainous regions. This justifies its selection as a pilot area for the broader project of soil geochemical surveying in Greece, given its accessibility from the headquarters of EAGME and the National and Kapodistrian University of Athens (NKUA). The highest elevation within the study area is found at Mount Parnitha (1413 m). Additionally, the area includes the mountain ranges of Mount Pentelicus (altitude 1109 m), Mount Hymettus (altitude 1026 m), Mount Mavrovouni (altitude 648 m), and Mount Parnes (altitude 636 m). The plain relief of the area comprises the Schimatario-Oropos basin, the Marathon plain, the Thriasian Plain, the Athens Basin, and the plain area of Mesogeia. Geologically, the area consists of basement rocks belonging to the Alpine orogenic cycle, as well as younger sedimentary formations. Basement rock outcrops are observed in the mountain ranges and on several hills, with overlying sequences of sedimentary formations were found.

Alpine Formations:

The Alpine geological formations in the study area comprise four main tectonic units: the Attica Unit, the Lavrio Unit, the Sub-Pelagonian Unit, and the Almyropotamos Unit. These units consist of both metamorphic and non-metamorphic rocks. Starting from the Lavreotiki Peninsula in the southern tip, the geological basement of northeastern Attica

belongs to the Attico-Cycladic Metamorphic Complex, beneath the Pelagonian cover. The area's lithostratigraphy is complex, with two major tectonic units: the Lower Tectonic Unit and the Upper Tectonic Unit. The Lower Tectonic Unit consists mainly of metamorphic rocks with interbedded marble layers, while the Upper Tectonic Unit comprises HP-LT metapelites and metapsammites.

In the eastern half of the Athens Basin, Mount Pentelicus and Mount Hymettus are structured by the lower tectonic units, which include Middle Jurassic marbles and phyllites and Triassic dolomites. The Alepovouniou Unit, limited to the western foothills of Mount Hymettus, includes upper tectonic crystalline marbles and marly schists.

In the western half of the Athens Basin, the Sub-Pelagonian Unit exposes non-metamorphic rocks such as argillaceous schists, sandstones, conglomerates, and tuffs, evolving into neritic marbles and dolomitic marbles. The Athens Unit, situated on top of the basin, consists of dense, recrystallized limestone and clastic deposits of Upper Cretaceous age.

In the northwest end of Attica, the Almyropotamos Unit is characterized by slightly metamorphosed limestone and dolomites of Mesozoic age. Ophiolitic complexes are present within these units, primarily at their tectonic contacts, with occurrences reported in various locations, including the western foothills of Aigaleo and Mount Hymettus.Overall, the Alpine formations in the study area exhibit diverse lithologies and tectonic histories, contributing to the complex geological landscape of the region.

Post- Alpine Formations

The post-alpine formations are of Neogene – Quaternary age, predominantly lacustrine to lacustrine-deltaic in character, and typically occupy areas of basins formed by neotectonic activity, primarily expressed as wrench tectonics, forming tectonic troughs and tectonic horns. Mettos (1992) presents a detailed description of such sedimentary formations of northwest Attica and southeast Boeotia. According to his research, continental upper Miocene deposits cover a large area across the entire Attica peninsula in the form of lacustrine and fluvial-deltaic deposits. These deposits in the Mesogea basin area cover a large area extending to the southernmost tip of Lavreotiki. In the Mesogea basin area, they are occasionally overlain by continental Pleistocene deposits. The type of deposits results from the morphology of the basins and their feeding sources. The size and rounding of the clasts depend on the distance from the source of the material. The binding material is sandy, marly, or marly-sandy. On the southern side of Mount Pentelicus, conglomerates prevail with a large thickness, transitioning laterally and upward to brownish materials associated with the Pikermian fauna. These materials transition upward to off-white, sub-yellow marls, alternating with slabby marly limestone. Above the Neogene, the marine deposits of Rafina were deposited. According to Mettos (1992), characteristic of the Rafina deposits are the rapid alternations of terrestrial and marine sediments. The lithological types characterizing these deposits are reddish-brown sand, conglomerate with flattened pebbles, fossiliferous marly-clayey

sand, sandstone, sandy marl-clayey sand, yellowish sand-sandstone, reddish-brown sand, and marly sandstone. Within the Athens basin, in the Tatoi-Kifissos area, the Neogene deposits start with white marl and slabby marly limestone with marl layers. The upper members evolve into sandy marls and marly limestone with conglomerate layers. Subsequently, these sediments transition into non-cohesive conglomerates alternating with pebble layers with sands, as well as sandy-clayey layers of light khaki color. The types of pebbles include crystalline limestone, schist, bedrock limestone of Mount Parnitha, as well as sandstone of Paleozoic age. It is concluded that the primary source of supply is the metamorphosed substrate of Mount Pentelicus. Neogene sediments also occur farther south in the hills of the western-northern basin (Dasos Chaidariou, Peristeri, Nea Liosia, Zephyri, Nea Philadelphia, Nea Ionia, Neo Heraklion), while marine sediments are found in the broader coastal area of the southern basin (Piraeus, Faliro, Vouliagmeni). A portion of the study area is also located in the eastern part of the Thebes basin Tanagra-Malakassa. The development of the basin, within which the deposition of the sedimentary formations began, is placed in the Miocene, with representative lithological types being various terrestrial and lacustrine phase sediments, such as marly-conglomerate, marly limestones, marls, and travertine-like limestones with horse fossils (Papanikolaou et al. 1988). This basin is the result of tectonic movements with vertical displacement that took place from the Lower Miocene and continue to this day. These movements were of varying intensity, so the space within the basin does not have uniform development. Thus, depending on the location, the supply source, and the type of material offered, the sediments do not have the same lithological structure throughout the basin.

West of Tanagra, there are thick deltaic deposits consisting of alternating sands and pebbles, cohesive sandstones, and sandstone-pebbles in beds of 2-3 m thick. The deeper layers of the sediments consist of clays rich in marly material of ash-green color, while towards the top, fossiliferous marls develop alternately with slabby marly limestones and sandy marls with thin layers of brown sands. Throughout the area, deposits of Quaternary age also appear, which are either weathered or anthropogenic in origin, alluvial deposits covering a significant surface area superficially. The Quaternary deposits are mainly represented by old and young alluvial cones and lateral alluvial fans. Also included are the calcareous sandstones that appear mainly on the eastern coasts of Attica.

The influence of geology on soil geochemistry has been studied locally in the past, both for areas of Boeotia (Antibachi et al. 2012, Kelepertzis et al. 2013, Kelepertzis and Stathopoulou 2013), and Attica (Argyraki and Kelepertzis 2014, Argyraki et al. 2018, Kaitantzian et al. 2013, Massas et al. 2013). Overall, the soil geochemistry is influenced by the underlying lithology. A simplified lithological map of the study area is shown in Figure 2.
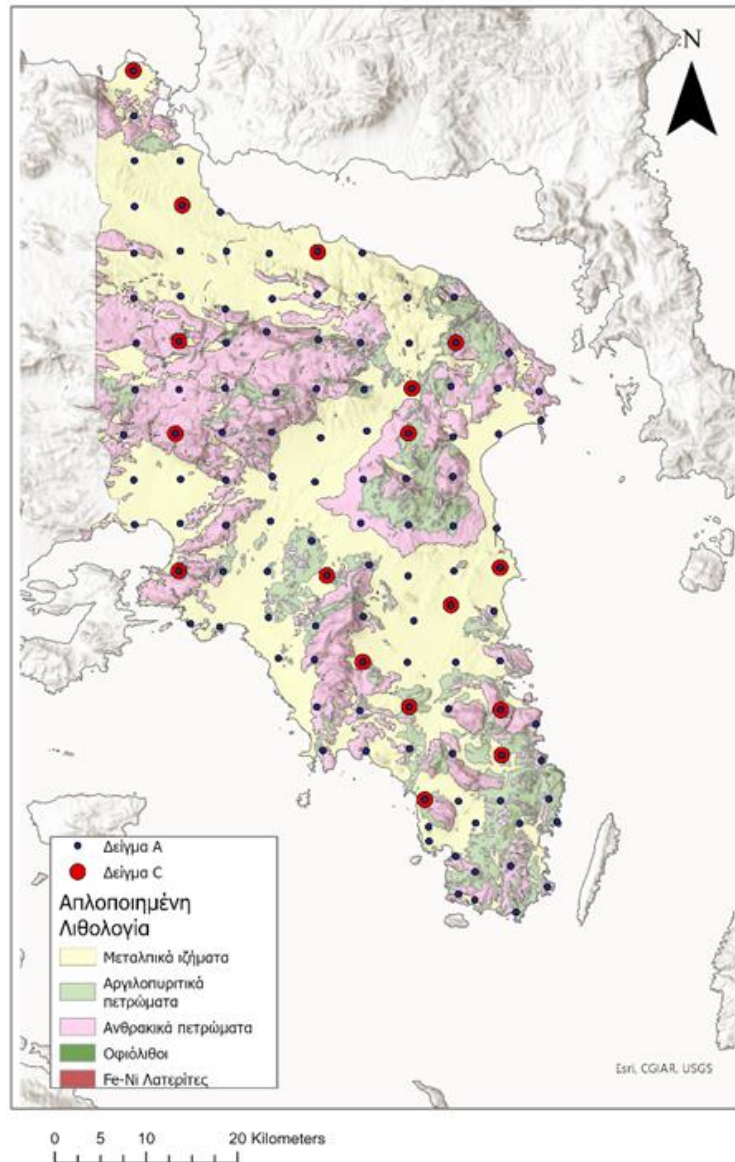
Figure 2. Simplified lithological map of the study area showing areas of post-alpine sedimentary rocks (yellow), aluminosilicate rocks (light green), carbonate rocks (pink), ophiolite rocks (dark green), and Fe-Ni laterites (brown).

CHAPTER 2. Literature review

2.1 Microplastics, an emerging pollutant in the environment

Microplastics (MPs) and nanoplastics (NPs) represent a new type of emerging pollutants. The widely used definition for MPs is particles smaller than 5 mm in their largest dimension, a definition accepted by the National Oceanic and Atmospheric Administration (NOAA) and the European Union's Marine Strategy Framework Directive (2008/56/EC). Meanwhile, plastic particles ranging in size from 0.001 μm to 0.1 μm are defined as NPs and are potentially more harmful compared to microplastics, as they are small enough to penetrate cell membranes and enter cells (Klaine et al., 2012). Microplastics are prevalent in various environments, including oceans, freshwater bodies, soil, and air, raising concerns about their effects on ecosystems and human health. It is necessary to examine all possible types of microplastics to understand the source of contamination. There are two types of microplastics: primary and secondary (Figure 3). Primary microplastics are deliberately produced to be small, such as the microbeads used in cosmetics and personal care products, while secondary microplastics are formed by the breakdown of larger plastic particles. Secondary microplastics are the most common type found in the environment (Habib et al., 2022).

The polymeric components of primary microplastics mainly include polyethylene (PE), polypropylene (PP), and polystyrene (PS), depending on the type of products manufactured by their production facility, while secondary microplastics mainly consist of polyester (PES), acrylic (PAN), and polyamide (PA), forming fibers dispersed in the environment (Padervan et al., 2020). Additionally, polymers contain additives (plastic additives, PAs) such as plasticizers, flame retardants, antioxidants, dyes, heat stabilizers, radiation stabilizers, and materials to improve or modify their mechanical properties. Compounds such as bisphenol A, phthalates, and polybrominated diphenyl ethers are used to improve plasticity during plastic synthesis. Microplastics may have toxic effects or disrupt the endocrine system of organisms by ingesting PAs (Avio et al., 2017; Tsang et al., 2017; Padervand et al., 2020). Fragments generally also contain raw materials (monomers and oligomers) that have not reacted and can leach into the environment during their use or disposal (Hong et al., 2017). In other cases, microplastics may have absorbed heavy metals such as Cu, Cr without their absorption ratio being adequately studied.

Moreover, the absorption of organic pollutants into microplastics, such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, pesticides, and organophosphorus compounds, further burdens them (Tsang et al., 2017; Llorca et al., 2020). Thus, these additives and resistant organic pollutants are environmental concerns, potentially introducing dangerous chemicals into habitats (Cole et al., 2011). One of the reasons additives are used in plastics is to delay oxidation and degradation of the polymer from exposure to UV radiation (Barnes et al., 2009).
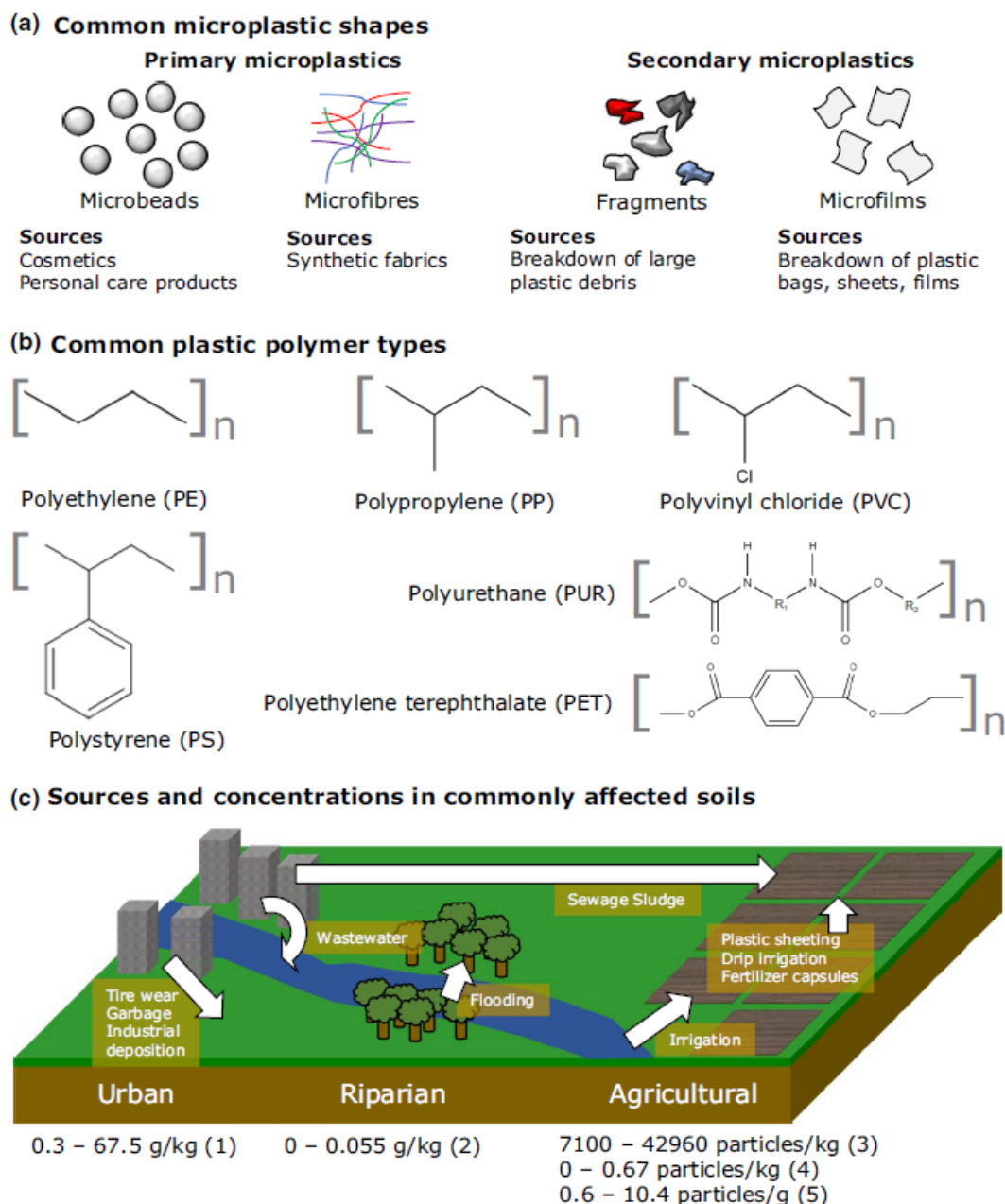
Figure 3. MPs shapes, polymer types and sources/ concentrations in soil. Source: Helmberger et al. 2020.

There are many mechanisms that can degrade or corrode plastics or even MPs, resulting in PAs being deposited in the ecosystem. Oxidative degradation is caused by slow oxidative breakdown at moderate temperatures, while thermal degradation of plastics occurs at high temperatures and is therefore a degradation mechanism that does not occur under normal environmental conditions but only in a laboratory setting. In addition, hydrolysis of MPs is the result of the reaction of the polymer with water, but it is not a very common degradation mechanism in seawater (Andrady, 2011).

Microplastics in Soil

Research initially focused on the occurrence and potential harmful outcomes of microplastics in aquatic environments. However, soil ecosystems have been recognized

as a major sink of microplastics, but the impacts of microplastics on soil ecosystems (e.g., above and below ground) remain largely unknown. Agricultural and urban soils are thought to be the most important reservoir for microplastics (Hurley and Nizzetto, 2018). Soil nature influences the migration of microplastics, and microplastics change the properties of soil, such as soil structure and function as well as microbial diversity. Specifically, microplastics in the soil on a large-scale decrease infiltration from rain and irrigation water, posing negative effects on the water holding capacity of the soil and possibly causing anoxia (Liu et al., 2014a, Liu et al., 2014b, Liu et al., 2014c). These pollutants also act as obstacles to evaporation, which can cause an increase in the levels of soil moisture (Qin et al., 2015). By affecting the properties of soil, it directly affects plants and animals, which are directly related with food quality and safety, ultimately threatening human health.

On a larger scale, the contamination starts with plastic residues that have been found in the soil because of mulching operations are converted into microplastics by the passage of time and environmental actions. The resulting microplastics disperse in the soil and combine with other pollutants such as heavy metals, pesticides, and persistent organic pollutants (POPs), causing combined toxic effects on soil flora and fauna. These MPs may be ultimately transferred to rivers, oceans, and other water bodies by agricultural water runoff causing water pollution in lakes (Sighicelli et al., 2018), rivers (Anderson et al., 2017), and the sea (Zhang et al., 2017).

Distribution of microplastics in soil

The vertical and horizontal distribution of microplastics in soil can be influenced by several factors, including soil biota, soil features such as soil macropores (pores > 75 μm), soil aggregation and soil cracking, and agronomic practices such as plowing and harvesting (Rillig et al., 2017a, 2017b). The general literature on microparticle migration in soil by bioturbation (Gabet et al., 2003) suggests that plant processes (e.g., root growth and uprooting) and inputs from various animals (e.g., larvae, earthworms, vertebrates, etc.) can serve as preferential paths for microplastics movement. Microplastics can be swallowed and excreted by earthworms (Cao et al., 2017), vertically transported from shallow to deep soils by the burrows of anecic earthworms, and laterally spread across wide areas by the movement of geophagous earthworms and mosquitoes.

Moreover, in an abiotic way, microplastics can be transferred to the soil from the surface and to deeper levels but also in an abiotic way, microplastics can be transferred to the soil from the surface and to deeper levels. Based on different abiotic factors and corresponding mechanisms, the pathways leading to the transformation and change of MPs in the soil can be divided into three main types: i) weathering and chemical degradation, ii) thermal degradation, and iii) photodegradation. Especially, this transformation and change pathways are usually not independent but are mostly performed simultaneously.

Soils in agricultural areas are polluted with macro, meso, and microplastics. Plastic fragments are emitted into the environment from plastic materials used on farms (e.g., irrigation systems, plastic mulches, plastic nets). Although those plastic materials are useful and serve several needs in the agricultural system, if they become residues, and those residues are not collected, then they fragment due to environmental conditions and become macro, meso, and microplastics. Scientists working on the prevention of plastic pollution need to first know how much macroplastic is present in various areas of the planet. This is where the assistance of citizen scientists becomes crucial.

In the H2020 MINAGRIS Project (Micro and Nanoplastics in Agricultural Soils, www.minagris.eu) and in other microplastic-related projects, the involvement of different stakeholders is very relevant, not only for discussions, but also as citizen scientists. The MINAGRIS project is a European project where several agricultural areas from different case studies in Europe are assessed. So, scientists working for the MINAGRIS project are assessing and monitoring the concentration of macro, meso, and microplastics in those agricultural sites. However, no information is being collected in urban areas or in conservation areas. This is where citizen scientists can play a big role. For example, within the MINAGRIS project consortium, the Soil Plastic app was developed. With this app, users can report the presence of macroplastics (>5mm plastic debris) in the terrestrial environment. The person who is observes the plastic material can also take one or several photos and save them to the apps' dataset. The photos taken can be used in the future by experts involved in the project.

2.2 Overview of recovery methods

In the last seven years, more studies have reflected on the importance to assess/monitor the concentration of MPs in soils. It is known that MPs produce an impact on soil life (at least in high concentrations), therefore it is important to assess the concentration of MPs in soils. For that, several methods have been developed. Among the steps for assessing the MPs concentration, is the extraction of plastic debris, that means the separation of the MPs from the bulk soil, which can be done by density separation. In this process, substances that separate the plastic debris from the soil are used together with deep centrifugations. After the separation occurs, the organic matter that remains with the microplastic particles, needs to be removed. For that, also several methods have been developed, for example, enzyme digestion or acid and alkaline digestion. The identification of MPs is done mostly via chemical imaging using infrared spectroscopy, it takes place by comparing the wavelength produced by the particles, with the wavelengths in FTIR (Fournier-transform infrared spectrometer) libraries. These libraries have typically been compiled by companies or universities using mainly pristine plastics. In situations where high-end equipment such as the FTIR is not affordable, a simple technique developed by Zhang et al. (2018) allows for the identification of light density microplastics using a high-resolution optical microscope and a heating method.

Although the pervasive presence of microplastics has been demonstrated soil systems, pretreatment and analytical methods are variable among different research groups. To date, there is no established standard method for the sampling, extraction, purification, and identification/quantification of microplastic particles in soil samples (Möller et al. 2020). Qualitative and quantitative monitoring of microplastics is necessary to identify the polymer variety in environmental samples, to assess environmental pollution and contamination of living organisms by microplastics, and to understand the environmental fate of microplastics (i.e. their degradation pathways). Moreover, standardized analytical methods for separating, identifying, and quantifying the most abundant types of plastics found in the environment are necessary for data comparison.

One of the simplest methods used to extract microplastic particles from soil samples is manual sorting, which is often applied for particles >1 mm (Piehl et al. 2018), so other methods started to be researched for the similar environments that microplastics belong. The prerequisite for the establishment of standardized methods for the recovery of microplastics from soil samples should include the separation of mineral phases and the reduction of organic matter as well as the recovery should not affect the morphology and/or structure of the polymer composites. Moreover, extraction and separation are two essential steps before any microplastics analysis in the soil and sediment. For effective extraction and separation of microplastics in soil and sediment, density separation (DS) and organic removal (OR) are the most used techniques. In addition to density separation, an organic removal step is needed prior to microplastics analysis in soil and sediment samples as the presence of organic matter on the surface of microplastics may alter FTIR and Raman results.

CHAPTER 3. Methodology

3.1 Sampling method

Research on the dispersion of microplastics focuses on sampling in sewage, sea, surface water, sediments, and the bottom of lakes and seas. Until today, there is no specific sampling protocol for soils, but several studies have been conducted on sampling methodology or similar techniques used for soil geochemical research. Sampling of soil presents significant variations depending on the area of interest and the depth of collection, as the first five cm show higher concentrations of microplastics than the first ten cm due to anthropogenic activity. It is essential that the sample that was sent to the laboratory for analysis is representative of the point of interest.

Required equipment

Soil sampling for microplastic analysis entails some limitations in the use of certain materials to avoid sample contamination. During excavation, metal shovels, hammers, and trowels were used, while the storage containers were glass jars with lids covered with aluminum foil, with a maximum capacity of 500g. The amounts of soil samples varied according to the capacity of the glass containers but covered the necessary quantity for the subsequent analyses. According to Möller et al. (2021), for studying soil MPs contamination in any given area, a soil weight sample of at least 250 g should be collected as a representative soil sample weight per site. Plastic bags and a plastic shovel were used for soil geochemical sampling to avoid contaminations. To take necessary protective measures, the team wore protective masks during the soil collection process. The sampling team was also equipped with handheld GPS devices capable of entering the marked map of sampling points on the planned 5 x 5 km grid (E.A.G.M.E. team) or with a mobile phone with an integrated GPS and a marked map of sampling points in kml file format, accessible via the Google Earth app for smartphones. They also had printed maps with marked sampling points and folders with pre-printed sampling sheets to fill out in the field (Figure 4).

Figure 4. On the left: Sampling tools set for soil sampling. On the right: Print screen of a "smart" phone with the Google Earth application and the planned sampling locations, printed sampling form for field notes input, and a map of the sampling area with marked positions

Outdoor Sampling Procedure

The purpose of sampling to map the geochemical or microplastic landscape is to collect enough material outdoors that is representative of the population being studied (e.g., soil). Outdoor samples should be as free of pollutants as possible and collected in well-marked containers. Sampling locations should be encoded and marked on a map with precision. Ideally, the sampling follows a predefined protocol that clearly and thoroughly describes the sampling process. This procedure should be communicated to the sampling personnel who should receive appropriate training. Thus, the same methodology can be followed for microplastic research sampling, as it falls within the framework of environmental research for mapping pollution dispersion in soil environments.

The sampling locations visited by the team of the University of Athens (NKUA) are presented in the Figure 5. Within the framework of the pilot program, many of the selected sampling locations for soil geochemical sampling were identified with those chosen for microplastic detection. A total of 143 locations were collected, of which 88 were identified for microplastic sampling.
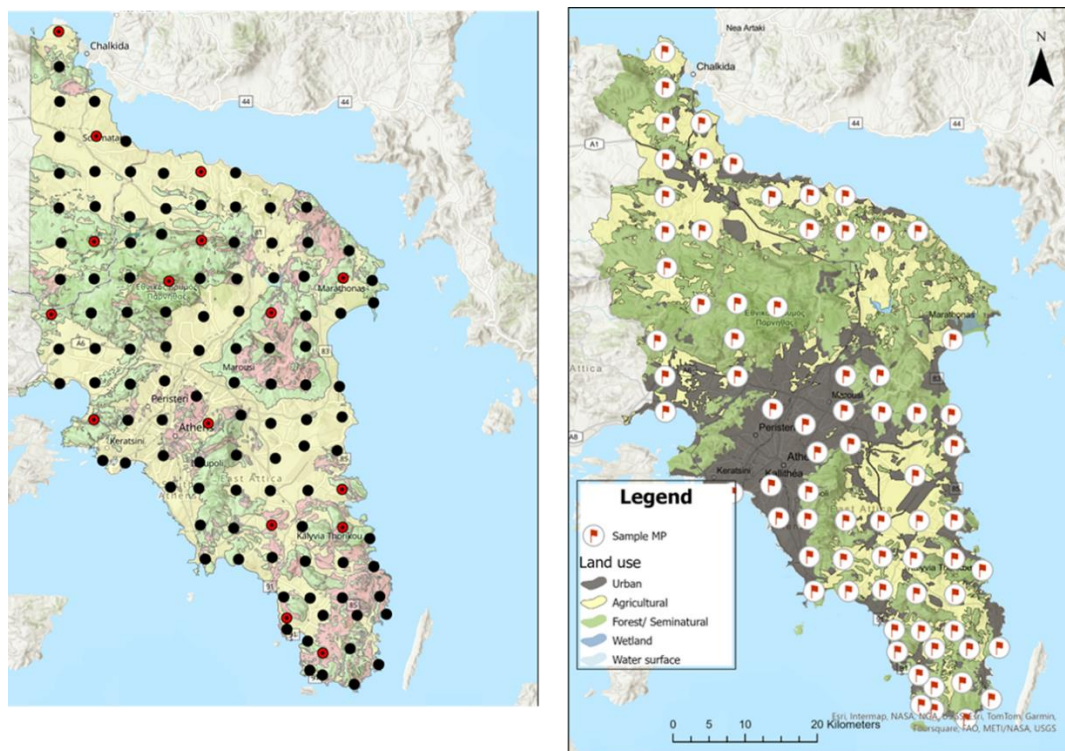
Figure 5. The selection of sampling locations for microplastic sampling (map on the right) was based on the establishment of the first Soil Geochemical Atlas of Greece, in Attica region (map on the left). The data were provided by the Hellenic Survey for Geology & Mineral Exploration.

The microplastic research samples come from different land uses, namely urban areas, geographic regions, forests/semi-natural areas (Figure 5). The sampling procedure for taking regular samples at each location is described in detail as follows:

- The surface of the site is cleared of vegetation, dead organic material, rocks, debris, and anything within the first 1-2 centimeters of the site. A photo is taken with the site code (close-up and general plan of the area).
- The tools are "immersed" into the soil of the area, and the excavation process begins using an axe, shovel, and carving tools as needed for the site.
- The soil material is gathered on cardboard to mix and homogenize it, removing manually any clusters of rocks with a diameter >2 cm and roots found within the soil.
- Once the soil collection is complete, the material is quadrupled, and the sample collection is carried out using a metal scoop, carefully collected, and placed directly into a glass container.

Observations are recorded for the area, the site, and the Sampling Form is completed, while accurate photos are taken to depict the recorded information. In cases where a "C" depth sample needs to be taken at the site, and if possible, it is carefully taken without mixing material from the surface, and the above procedure is followed. After the sample/samples collection at the site is complete, the site is restored to its original state. Upon leaving the site and returning to the vehicle, the tools are cleaned with a wet cloth to remove material from the sampling site and avoid contamination of the next sample. Lastly, all containers are collected, recorded, and placed in a transport box to be transported to the laboratory.

Sample Labeling

Sample labeling was done with clear and stable writing on the glass containers, indicating the identification details of each sample, i.e., the sample code. For surface samples 0-20 cm, the code "A" is referenced, while for depth samples, the code "C" is used, and the numbering of the samples was based on the correlation of coordinates of each point in the WGS system with the encoding of the system (CRS) ETRS89- LAEA Europe. This process begins with the study of the pilot program's locations and ends with their sampling. At the end of each sampling, a sampling recording form is completed with all sample details. The sampling recording form includes the following information, which is crucial for repeating the sampling under the same conditions:

- The type and precise code of the sampling location and depth
- Coordinates of the sampling point
- Description of the sampling location and land use
- Name of the sampling supervisor.

3.2 Laboratory Sample Processing

One of the purposes of this study is to compare methods for separating microplastics from soil samples, which includes the removal of organic matter and mineral particles. For this reason, a comparison of certain extraction methods for organic particles, such as lignin

and cellulose, and density-based separations for mineral particles was conducted, as suggested by Mattson et al. (2022). For the spiking experiment, specific microplastics were chosen, after a cryo- milling process. Twenty particles from each polymer category were peaked for each microplastic recovery technique. Specifically, "PET" and "PVC" particles affected by deep-sea conditions at 2400 m depth were selected, with a brown shade of color, as well as "PE" polyethylene and "PS" polystyrene particles affected by corrosive conditions of surface seawater. Additionally, an extra selection of particles was added, black car tire particles that can be found in urban and forest environments during soil sampling for MPs, to simulate real pollution conditions.

The process of selecting methodologies in the experimental procedure for the spiking experiment is documented in Table 1. The experiments were conducted at the laboratories of the National and Kapodistrian University of Athens, Laboratory of Economic Geology and Geochemistry, in Greece and at the Kristineberg Center for Marine Research and Innovation, in Sweden.
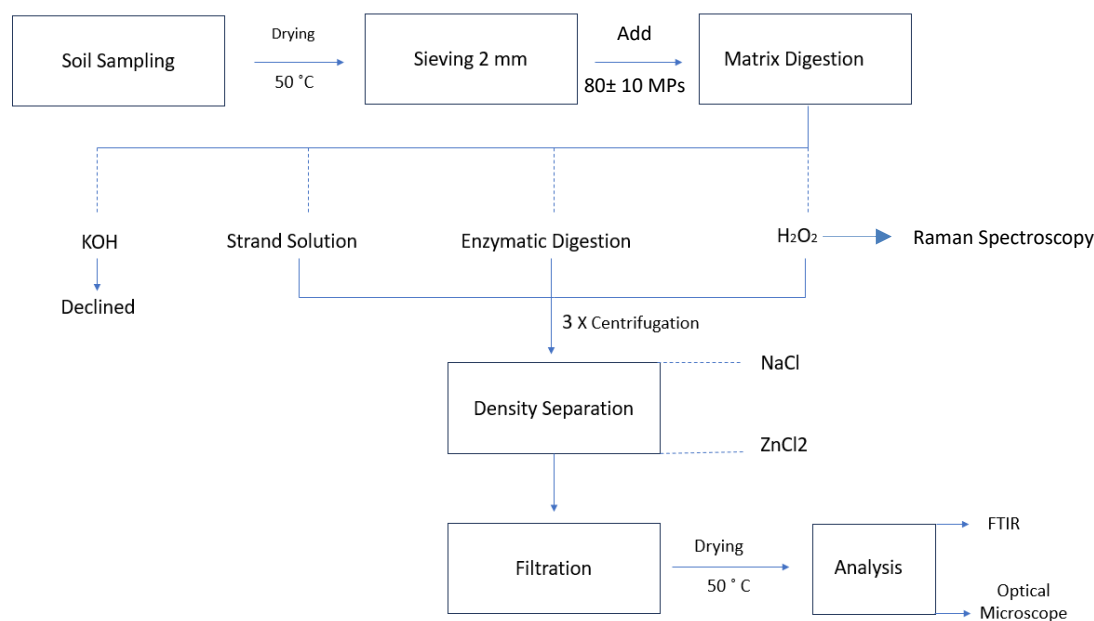

Table 1. Experimental path of the MPs spiked soil experiments.

The coding of the samples (Table 2) was based on the selection of the methods applied to the MPs spiked composite soil samples of 50 g each produced after mixing soil from various sampling locations within the forest/seminatural areas mentioned in the previous paragraph. Of the total of eight spiked samples, two are blanks (Bl1, Bl2). The letter A or B stands for the density separation method (solution) and the numbers 1-3 stand for the pretreatment method (reagent).

Sample Name	Pre-treatment Method	Density Separation
A1	H_2O_2	$ZnCl_2$
A2	Enzymes	$ZnCl_2$
A3	Strand	$ZnCl_2$
B1	H_2O_2	NaCl
B2	Enzymes	NaCl
B3	Strand	NaCl
Bl1	H_2O_2	NaCl
Bl2	H_2O_2	$ZnCl_2$

3.3 Pretreatment   Table 2. Sample coding for the MPs separation experiments.   **of samples**

To begin the sample processing, it is necessary to do pretreatment of all samples to facilitate the separation of microplastics from soil fractions. It is essential to remove any residual moisture to ensure that the samples are completely dry during weighing and processing and to prevent the growth of microorganisms in the samples. The samples are placed in aluminum containers and heated to 45°C in a thermally controlled oven for at least 48 hours to remove all moisture. To eliminate debris and large pieces of organic matter (tree roots and wood pieces), conglomerates containing these materials, as well as potential microplastics, must first be disintegrated. Using a porcelain mortar and pestle, all sample conglomerates were disintegrated and crushed into uniform smaller-sized granules to facilitate subsequent sieving (Figure 6).

Material that passes through the 2 mm sieve is retained for mixing. All soil samples must be thoroughly mixed to ensure that the sample is as representative as possible of the sampling medium. The most common method is referred to as quartering. The splitting process should be carried out as follows: The material obtained from sieving should be divided into quarters, and each quarter should be mixed separately. Two quarters are then mixed to form halves. The two halves are mixed to form a homogeneous core. Approximately 50g of soil is then weighed from the homogeneous core to produce a composite sample. To form composite samples from all sampling points for all land uses, it was calculated that each composite sample should consist of 500 g, so that all samples are equally mixed. After the sieving process, the remaining sample is stored in the corresponding container, and the portion to be used is placed in a glass container with a maximum capacity of 500g. To avoid contamination during pre-processing, metal sieves,

porcelain mortars, and glass containers were used. After each sieving process, the items were cleaned with acetone and compressed air to remove soil particles.

Figure 6. Using a porcelain mortar and pestle for sample disaggregation.

Sieving is another frequently used method for isolating microplastics from water and sediment matrices. The sieves are usually made of metal, such as stainless steel or copper (Figure 7).

Figure 7. Stainless steel sieve, containing a soil sample.

For microplastics analysis, duplicate samples of equal weight and homogeneity had to be created from each sampling point. Processing and analyzing duplicate samples for each point are important for studying repeatability, as will be discussed in a subsequent deliverable regarding the final assessment of the samples. The duplicate samples were labeled in glass beakers as "1A, 1B" for clear distinction. The depth was marked as "E" for surface samples and "C" for depth samples.

3.4 Digestion of organic matter

3.4.1 Inorganic digestion

Microplastics are particles with size of less than 2 mm and they can be mixed and form aggregates with minerals and natural organic matter in the soil, making their separation method more difficult and increasing the time in their final recovery methodology (Liang et al. 2021). Aggregates require an analytical method that usually consists of two main axes for the removal of organic and inorganic material. H removal of organic matter by the digestion process requires the use of reagents that will not affect the morphology of the synthetic polymers, while reducing the mass of natural polymers derived from plants or animal organisms.

Several reagents are commonly employed for organic matter reduction, each with distinct mechanisms and applications. For this reason, inorganic chemical reagents were used for digestion and an enzymatic digestion method. Potassium Hydroxide (KOH) is frequently utilized for its ability to hydrolyze ester bonds and solubilize organic compounds, particularly humic and fulvic acids. Strand solution, comprising strong acids like hydrochloric acid (HCl) and nitric acid (HNO_3), offers efficient dissolution of mineral components while partially affecting organic matter. Enzymatic digestion harnesses the specificity of enzymes such as cellulase and protease to selectively break down cellulose, proteins, and lipids. Additionally, Hydrogen Peroxide (H_2O_2) digestion with temperature reduction provides oxidative breakdown of organic matter, enhancing solubilization for subsequent analysis. Understanding the characteristics and efficiencies of these reagents is essential for designing effective soil analysis protocols tailored to specific research or agricultural needs.

Potassium hydroxide (KOH) treatment

Among the various activators, such as KOH, H_2O_2, HCl, the KOH reagent stands out for its promising attributes. KOH reagent is often used to separate microplastics from marine tissues of marine organisms (such as mussels' tissue) (Kühn et al., 2017). It boasts a moderate activation temperature and produces a highly developed microporous structure with an ultrahigh specific surface area. The chemical reaction between potassium hydroxide (KOH) and cellulose involves saponification (Papoutsis et al., 2020). This process involves KOH hydrolyzing the ester linkages within the cellulose molecule, breaking them down into their constituent components. Despite the form or environmental exposure, most polymers exhibit resistance to potassium hydroxide (KOH) solution. However, exceptions include cellulose acetate from cigarette filters, certain biodegradable plastics, and a single polyethylene sheet. However, KOH digestion may not completely solubilize all organic matter components, especially in soils with high levels of recalcitrant organic compounds or complex organic structures.

Strand Solution treatment

The treatment with inorganic chemicals is based on a protocol developed by Strand and Tairova (2016) but slightly modified as it consisted of a mixture of 0.67 mol/L NaClO, 0.45 mol/L KOH, and 0.022 mol/L $Na_4P_2O_7$, but in this study we used only KOH and NaClO. The basic solution can increase the possibility that its effect on the organic material may not be as efficient as other reagents.

Hydrogen peroxide (H_2O_2) treatment

Currently, the widely known way to remove natural organic material from soil is oxidative digestion using hydrogen peroxide (H_2O_2). The use of hydrogen peroxide aims to efficiently decompose aggregates and oxidise organic compounds in soil, including materials such as wood and roots, thus ensuring a cleaner and more accurate analytical control of the microplastic content of soil samples. For the digestion process it has been chosen to use hydrogen peroxide (H_2O_2 30%) 100ml per 50g of sample in a beaker.

Due to the violent exothermic reaction (Munno et al., 2018) that takes place during the oxidation of organic matter, the temperature of the soil sample can reach up to 75 ˚C. To avoid melting or etching of the surface of the microplastics, digestion is carried out within, an ice bath to ensure that the reaction temperature is kept below 40 °C (Maw et al. 2022) (Figure 8). Reagent is added in two to three batches, with continuous stirring from a stirring magnet for two hours until bubble production stops. The transient addition of reagent is made to prevent spilling of the produced bubbles and to maintain a constant temperature, which was monitored with a thermometer throughout the mixing of the reagent with the sample.

All inorganic reagents followed the same procedure for the pretreatment of the soil samples:

- Stirring at 160 rpm, for 2 hours
- 24 hours settling time.
- Room Temperature
- Temperature control
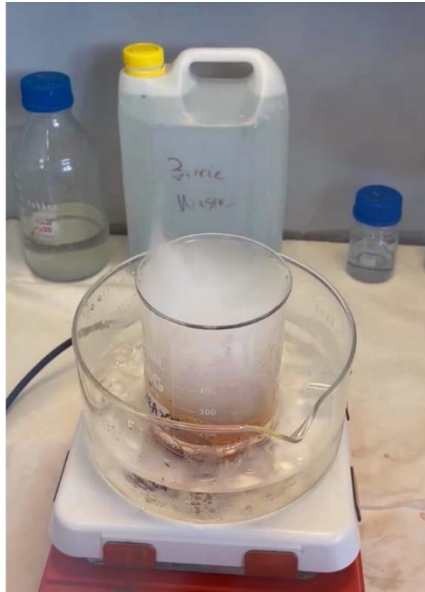- Water bath if the reaction is exothermic.

Figure 8. Organic matter digestion with H_2O_2, in controlled temperature ice bath.

3.4.2 Enzymatic Digestion

Enzymatic digestion offers high specificity and efficiency in targeting specific organic compounds present in soil samples. Enzymes such as cellulase, protease, and lipase can selectively break down cellulose, proteins, and lipids, respectively. However, the efficiency of enzymatic digestion may vary depending on the substrate specificity of the enzymes used and the complexity of the organic matter matrix. The most important enzymes in soil agroecosystems are those involved in breaking down cellulose and other plant cell components and in the transformations of C, N, P and S elements (Sherene et al. 2017).

For instance, lignin and cellulose are mainly composed of plant cell walls that vary substantially in their contents depending on the species, variety, and climate. Traditionally, cellulose was thought to be degraded by three main types of enzyme activity: endoglucanases, exoglucanases, including cellodextrinases and cellobiohydrolases for the non-reducing end acting cellobiohydrolases and β-glucosidases. For the enzymatic pretreatment, 0,08g porcine pancreatic enzymes (Creon 25 000, Abbott Laboratories GmbH, Germany, Mylan) and 10 mL of Tris hydrochloride solution (Trizma, pH 8.0, 1 M, 0.2 μm fltered, Sigma-Aldrich, T3038, USA) per gram of soil were used. The processing of the samples was carried out in the same way as with the inorganic reagents except that different reaction times and temperatures that were used. The procedure that was followed for mixing the spiked samples with the enzymes:

- Stirring at 150 rpm, overnight
- Temperature set at 37.5 °C
- To maintain pH 8, Tri's solution was added, when the solutions pH value was different.

3.5 Centrifugation

To perform a density-based separation process, centrifugation of the samples is necessary as a rinsing step. Until today, there have been few experimental descriptions of centrifugation as a method for microplastic separation to extract microplastics from sediments, and it was observed that buoyancy was minimally affected by the presence of sediments (Phuong et al. 2018). As the use of centrifugation is essential post-sedimentation, as an additional rinsing step, the samples were washed with the addition of deionized water, followed by vigorous agitation and centrifugation for 30 minutes at 1000 revolutions per minute. This washing process was repeated three times (Mattsson et al. 2022). The supernatant liquid was removed using a 50 ml disposable syringe between repetitions, and fresh deionized water was added. The supernatant liquid was stored in a beaker until all centrifugation stages were completed. In cases where the supernatant liquid contained soil particles (silt), centrifugation was repeated at a higher speed, for example, 1300 rpm.

3.6 Density Separation

For the recovery of microplastics from mineral and organic matter particles, it is necessary to apply a separation method in which the morphology of the microplastics is not affected. As it will separate the soil and any inorganic substances that didn't decompose in the digestion process. Density separation is a key procedure for the analysis of microplastics in soil and sediments. Due to the complexity of the sample fractions and the difference in density of each particle, different types of particles can float (or remain undisturbed) when mixed with different solutions at different densities.

The density of most plastics (0.8- 2.3 g cm^{-3}) is much lower than the density of sediment or soil (1.52- 2.7 g cm^{-3}) and this difference can be a useful information for separating microplastics from minerals. The density of microplastics can be affected by the concentration of additives, polymer's type and even by any adsorbed substances and organisms (Quinn et al., 2017). In most studies, high concentration solution of NaCl (1.2 g cm^{-3}) is used for microplastic separation. It is considered a reliable method and environmentally friendly technique recommended by MSFD Technical Subgroup of Marine Litter and National Oceanic and Atmospheric Administration (NoAA). However due to the low density of NaCl, high density polymers such as polyvinyl chloride and polyethylene terephthalate may not be separated by this method (J. Frias et al., 2018). In contrast, low-density polymers such as polypropylene or polyamide can be easily separated (Imhof et al., 2012).

Also, as a density separation solution we used zinc chloride ZnCl$_2$ $(\rho \approx 1.58$ g/cm$^3)$ as it has been suitable for the separation of most polymer types, including high density polymers (PET, PVC). Zinc chloride (ZnCl$_2$) is also considered as one of the best density separation salts, according to researchers' flotation of Polyamide, Polystyrene, Polyvinyl chloride, Polyethylene terephthalate, Polyethylene, and Polypropylene is possible with

solution of density 1.37 g mL^{-1} with maximum recovery and minimum interference (Maes et al., 2017)

To compare the efficacy of two different widely used density separation solutions, NaCl (density 1.2 g/cm^3) and ZnCl$_2$ (1.8 g/cm^3), the pre-treated samples were divided into two groups further processed with ZnCl$_2$ or NaCl (Figure 9). Both saline solutions followed the same procedure, i.e:

- 5 minutes of mixing the sample with the saline solution, in a glass beaker
- 24 hours of settling time
- Room temperature
- Repeat if separation has not been achieved

In the case of the ZnCl$_2$ solution it was necessary to repeat the process, due to organic particles that did not float (Figure 10). Therefore, the supernatant solution was filtered through a 300 μm metal mesh and remixed with the saline solution in a glass beaker. After the second-density separation, the solution was filtered through two metal meshes, with sizes 300 μm and 100 μm, respectively. For the NaCl solution all pretreated samples extracted with NaCl, only one separation was performed since fewer mineral and organic particles were extracted compared to the ZnCl$_2$ solution. After filtration, all the filters were rinsed with Milli-Q water to remove salt crystals.

Figure 9. Left: Three beakers with soil samples and ZnCl$_2$ solution, after 24 hours. Right: Three beakers with soil samples and NaCl solution, after 24 hours

Figure 10. Second separation process for the $ZnCl_2$ solution

To achieve the final collection of microplastics from the solutions, a filtration method was applied, in which solid particles are separated from a liquid by filtraring through a metal mesh. In this study, the particles were collected from two metal meshes, 300 μm 100 μm each, in a separation tower (Figure 11). The collected liquid for the centrifugation step, was also filtered through the meshes. Upon completion of particle collection, rinsing was performed using a water flask with Milli-Q water to remove the sodium chloride crystals. Finally, the meshes were transferred to glass petri dishes and placed in a thermostatically controlled oven for a 24 hour period at 50 ˚C to dry. All samples are wrapped in foil and stored in the room without any other objects to avoid any contamination.

Figure 11. Filtration with two metal meshes inside the separation tower.

Prevention of contamination of the samples

Synthetic polymers are ubiquitous in everyday life due to their multifaced nature (Yuvedha et al., 2019). Even the processes of sampling, preparation and analysis of

samples containing microplastics are affected by the presence of synthetic polymers in the environment. Therefore, numerous sources of contamination, such as sampling equipment, clothing and airborne particles can contaminate analysis and quantification procedures. Due to their ability to be suspended in the atmosphere, microplastics, and in particular microfibres, may alterate the analyses results, which can lead to an overestimation of microplastic concentrations in samples.Therefore, particular emphasis should be placed on sample contamination prevention. Samples should be protected as far as possible, both immediately after sampling and during their treatment. To protect the sample from airborne microorganisms and contaminants, after sampling, the collected samples should be stored in glass containers with aluminium lids (Tagg et al., 2015). Strict measures should be taken in the laboratory and during the analysis of the samples. Potential sources of contamination such as plastic laboratory containers were avoided and replaced with other materials (Galloway, 2015). All glass containers were thoroughly filled with tap water and distilled water after each experiment. The estimation of the concentration of microplastics in the atmosphere may be carried out by using an open container or paper filter exposed to the laboratory atmosphere. Specifically, two samples were selected as blanks, which were open glass bottles placed in the laboratory space and then they were processed according to the protocol chosen for two of the pretreatment (hydrogen peroxide) and density separation methods (both NaCl and $ZnCl_2$). In this way, microfibres and microplastics can be detected from the laboratory area or the robe worn by the analyst.

3.7 Analytical methods for MP counting and polymer identification

The samples after separation from the soil and organic material are ready for analysis. In this study the analytical methods used were optical microscopy, Fourier-transform infrared spectroscopy and Raman spectroscopy. Through the analytical methods discussed below, the removal of organic matter, the recovery of microplastics and the efficiency of the method will be examined. Due to the R&D (research and development) character of this thesis and the time constraints of the STSM conducted in the Kristineberg laboratories, not all samples were examined by using FTIR and microscope mapping. At the same time, the Raman instrument was used in the laboratories of the NKUA (core facility) for a corresponding spiked soil sample containing PE particles. The sample was analyzed by Raman, which was treated with H_2O_2 and separated using NaCl solution of the same concentration as used in the rest of the experiment.

3.7.1 Optical Microscopy

Optical analysis is one of the most widely used methods for analyzing the physical characteristics of microplastics. The main operation process of optical analysis involves observing samples with the naked eye or with a microscope, and then microplastics can be sorted and approximately counted based on their color, shape, and size.

The advantage of optical analysis is its simple operation, and it is considered a non-destructive method in terms of sample mass (von Moos et al., 2012). However, optical

analysis cannot provide information regarding the chemical composition of microplastics (Lavers et al., 2016). Additionally, optical analysis can be time-consuming for identifying complex polymers, and its accuracy and effectiveness are relatively low because experimental results can be easily influenced by various factors such as sample mixtures, color, shape, microplastic structure, as well as subjective judgments of the research team (Dekiff et al., 2014; Lavers et al., 2016). The error rate for microplastic identification still exceeds 20% under a conventional bright-field microscope (Hidalgo-Ruz et al., 2012), and the error rate exceeds 70% when microplastics are transparent (Song et al., 2015). Therefore, optical microscopy is often combined with electron microscopy to reduce the error rate in microplastic detection. When particle sizes are smaller than 100 μm or contain other particle mixtures, optical analysis is no longer applicable (Hidalgo-Ruz et al., 2012; Song et al., 2015). In cases where additives such as plasticizers, biocides, flame retardants, stabilizers, antioxidants, and organic pigments (stabilizers) are intentionally added to plastics during the manufacturing process and are usually not chemically linked to the polymers, chemical additives are recognized as one of the main factors contributing to the toxicity of complex plastics (Gallo et al., 2018; Isaac and Kandasubramanian, 2021). Therefore, optical analysis is usually used only as an auxiliary method for microplastic analysis and not as an independent method of microplastic analysis.

In this study, microplastic identification and analysis began with optical analysis through polarized light microscopy. During the laboratory part of this research, polarizing microscopes were used based on bright-field (BF) and dark-field (DF) microscopy, with typical lenses used being 10X, 20X, and 50X.

Bright-field Microscopy (BF): A modified arrangement of a simple optical microscope. The basic feature is the way the sample is imaged, based on the phenomenon of light diffraction to create contrast. It appears with a bright background, and features on the surface are visible as dark. Characteristics in the sample will only be visible if they absorb light or the intensity of the light can scatter it, significantly enough to spread it in large angles, so that light is not collected in the objective lens. It is noted that polymers are generally transparent, meaning they have very low contrast, except in cases where they have intense color.

Dark-field Microscopy (DF): This type of microscope is like bright-field microscopy with the only difference being that there is an opaque disc covering the center of the lens resulting in the sample being illuminated only by oblique rays that do not enter the lens but only if they undergo diffraction through the sample. Thus, the image observed is a bright object on a dark background.

For the application of microscopy on samples, metal forceps were used, and the transfer of filters to glass slides or coverslips was carried out, and observation was done by using gloves and a metal spatula in case any microplastic was covered by organic matter. Subsequently, pictures were taken using a scale and brightness processing by switching from BF to DF.

3.7.2 Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared (FTIR) spectroscopy is the most widely used technique for the detection of microplastics due to several reasons like directness, reliability, non-destructive approach (Ojeda et al., 2009) and it also produces individual band patterns by specific infrared spectra for different plastic types (Hidalgo-Ruz et al., 2012). It is based on the absorption of infrared radiation by the molecules of the substance. Infrared absorption spectra can be due to a variety of energy transitions. These transitions result from transitions from one vibrational and rotational energy state to another. For a molecule to absorb infrared radiation, its energy must be just enough to cause a vibrational or rotational transition. In FTIR, the infrared radiation passes through the sample to create absorption or transmittance spectra. The spectrum is created when infrared frequency is absorbed, matching the vibration frequency of a bond, is known as the molecular "fingerprint" of the sample (Kalogerakis et al., 2017). The advantages of this technique vary in microplastic research, such as being a non-destructive method, as samples are not heated or dissolved in a solvent. It is considered environmentally friendly, and very small sample quantities are required for spectrum acquisition (Chen et al., 2020). Additionally, it avoids false positive inclusion of non-plastic particles and reduces false negative errors due to plastic particles that do not have distinct color or texture. Moreover, it can provide information about particles, such as the extent of oxidation that may have occurred. FTIR has a limitation in that it can only detect microplastics up to 20 μm or 15 μm and leads to underestimation, or if the particles are thick (>50-100 μm) leading to total absorption. This can be overcome by using micro-FTIR, which has the ability to characterize the sample below 10 μm or by excluding bigger particles (Wang & Wang, 2018).In some cases, a sample could contain a semi-synthetic (fiber), then the band value would fall in 1105 cm^{-1} which is the characteristic band for semi-synthetic fibers. Usually, the samples collected from the environment have a different composition, they are often weathered and tend to aggregate with other natural organic particles, which makes it difficult for the matching algorithm to match with the spectral library, which is commercially available, so there is a need to enhance the polymer spectra library (Cai et al., 2019).

In this study, the FTIR spectroscopy was utilized to analyze all samples. Typically, in microplastic monitoring programs, FTIR is predominantly used in two ways: either scanning all suspected particles as a composite sample or analyzing a subset from the sample, as a single particle, to verify the results of optical identification via microscopy. In this research, the former approach was adopted for the samples. Initially, the samples were examined using polarized microscopy and then transferred to a filter of smaller pore size to be consolidated in the FTIR sample holder.

3.7.3 Raman Spectroscopy

For microplastic identification Raman spectroscopy is also used, it is a vibrational spectroscopy technique that delivers information in the form of vibrational spectra, on basis of inelastic scattering of light. The spectrum obtained is like a fingerprint of chemical structure, which allows identification of particles present in sample (Araujo et

al., 2018). It shows better spatial resolution for samples, highly sensitive to non-polar functional groups, spectral bands are narrowed down and low interference due to water are few benefits of Raman spectroscopy over FTIR techniques (Elert et al., 2017; Ivleva et al., 2017; Kappler ¨ et al., 2016). Raman micro spectroscopy can detect microplastics and deliver its chemical and structural characteristics, which is not possible with other spectroscopic techniques (Crawford and Quinn, 2017). In Raman spectroscopy auto-fluorescence background from organic matter present at on microplastics is a great hindrance for identification of the microplastics, as it overshadows the raman peaks. In the present study Raman spectra were obtained by using the Renishaw InVia confocal Raman microscope hosted in the Core Facility of the School of Science in National and Kapodistrian University (Figure 12).
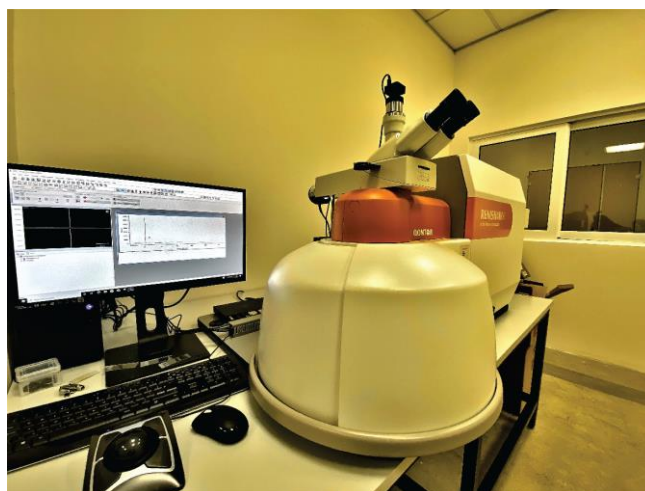

Figure 12. The Renishaw InVia confocal Raman microscope hosted in the Core Facility of the School of Science in National and Kapodistrian University.

The difference between FTIR and Raman spectroscopy lies in the fact that the spectrum generated by the former technique depends on the change in the permanent dipole moment of the chemical bond, while Raman depends on the change in the polarity of the chemical bond (Käppler et al., 2016). At the operational level, while FTIR spectroscopy utilizes the frequency of infrared radiation, Raman spectroscopy applies a monochromatic laser, and this energy is absorbed by the sample before creating a spectrum (Bretas Alvim et al., 2020). Käppler et al. (2016) propose the use of both FTIR and Raman spectroscopy to obtain more comprehensive and reliable results for the analyzed particles. Despite the high sensitivity of Raman analysis for detecting small particles (<20 μm), the method may be hindered by additional substances present in plastics, resulting in significant modifications to the polymer spectrum compared to the spectrum of the polymers alone, making their identification difficult (Lenz et al., 2015; Qiu et al., 2016; Araujo et al., 2018). Interferences affecting the process include the presence of unknown frequencies and fluorescence (Sun et al., 2019). Additional substances on the surface of microplastics may overlap the characteristic spectrum of the base polymer, potentially leading to overestimation of microplastics (Sun et al., 2019; Bretas Alvim et al., 2020). Another

issue regarding Raman spectroscopy is fluorescence. Dyes and pigments often emit intense fluorescence in the presence of light, making the identification of polymer spectra difficult (Massonnet et al., 2012). Additionally, the performance of Raman spectroscopy depends on the equipment, the wavelength of the applied laser, and the operator. In the present work the most frequently polymers considered as microplastics are PP and PE, which are usually identified by measuring pristine polymers from existing surveys or identified by means of software (Figure 13).
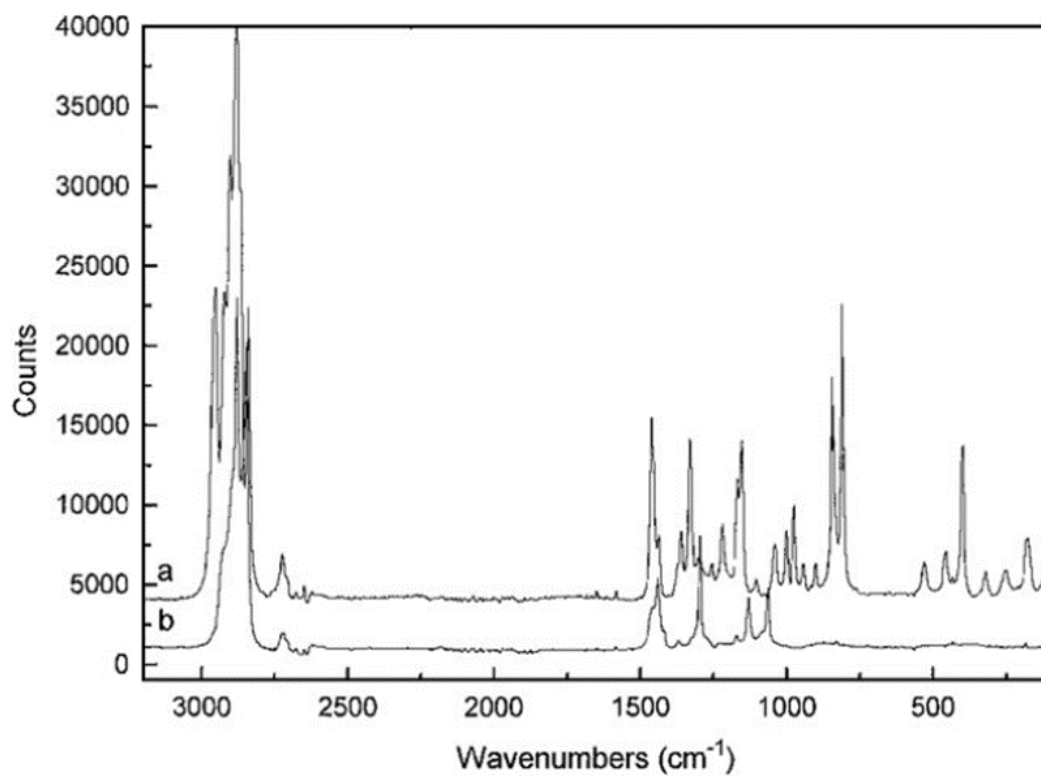
Figure 13. Raman spectrum of pure (a) polypropylene and (b) low density polyethylene (Ch. Tselios et al., 1999).

CHAPTER 4. Results and discussion

In summary, each digestion method offers unique advantages and considerations regarding the efficiency of organic matter extraction from soil samples. Understanding the mechanisms and limitations of these methods is crucial for selecting the most appropriate approach based on the specific objectives of the analysis is crucial for selecting the most appropriate approach based on the specific objectives of the analysis.

4.1 Pilot experiment results obtained by Raman spectroscopy

Prior to the experiments on recovery of spiked microplastics, a pilot experiment was performed for the effect of hydrogen peroxide (H_2O_2) on two types of polymers, polypropylene (PP) and high-density polyethylene (HDPE). The choice of hydrogen peroxide was based on its wide use in similar studies of microplastic recovery from sediment samples and soil samples for organic matter reduction. The test experimental procedure used the same conditions as those of the methodology of Chapter 3 on plastic particles with a size range of 500 µm-1.5 mm.

To examine the oxidizing activity of the reagent, Raman spectra of prestige PP and PE particles were obtained:

A.   Before treatment with H_2O_2
B.   After 2-hour treatment with H_2O_2 (30%).

- Raman spectra of polyethylene (HDPE) before treatment (Figure 14) shows main vibrations at 1062, 1129, 1170, 1295, 1416, 1440, 2850, and 2883 cm^{-1}. The bands at 1062 and 1130 cm^{-1} have been assigned to the asymmetric C–C and symmetric C–C stretching vibrations of the C–C bonds. The band at 1170 cm^{-1} represent the bending mode (rocking CH2 vibration) of the CH2 bonds, and the bands at 1295 cm^{-1} and 1416 cm^{-1} are twisting CH_2 and wagging CH_2 vibrations of the CH_2 groups, respectively. The bands at 2850 cm^{-1} and 2883 cm^{-1} belong to the symmetric CH_2 and asymmetric CH_2 stretching vibrations of the CH_2 groups. Thus, we can identify that the polymer is HDPE, and not LDPE, because the intensity of the symmetric CH_2 stretching mode at 2850 cm^{-1} compared to the asymmetric CH_2 stretching mode at 2883 cm^{-1} should be lower for an HDPE than to a LDPE.
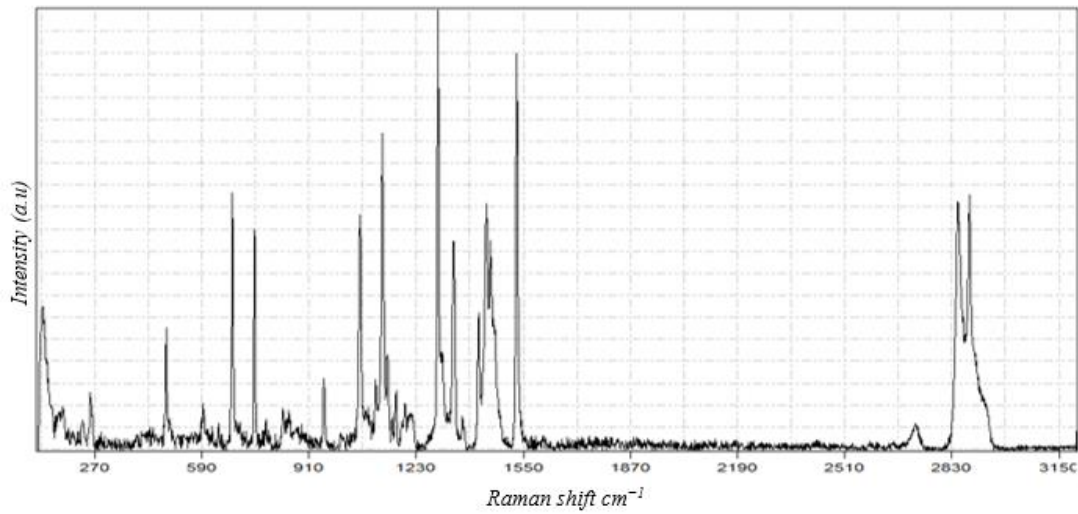
Figure 14. Raman spectra of polyethylene (HDPE) before treatment.

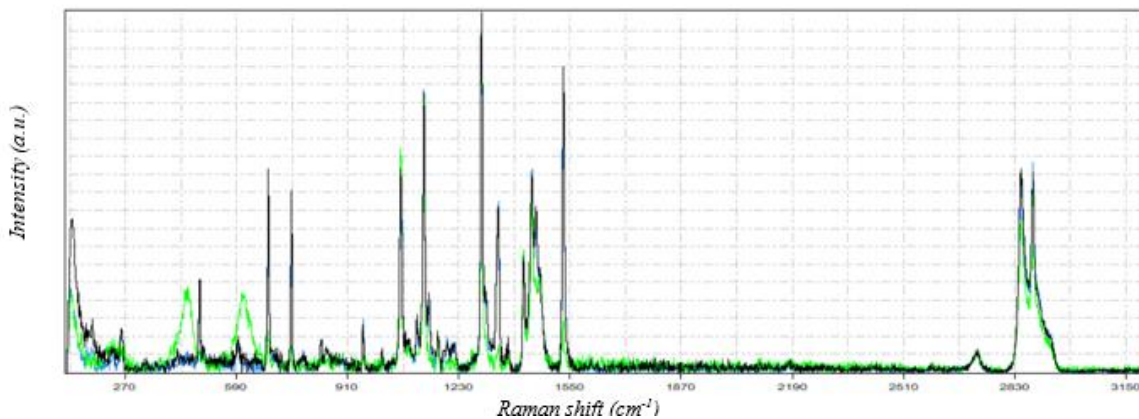- Raman spectra after treatment are presented in Figure 15.

Figure 15. Raman spectra of HDPE. Black: untreated, blue: treated once with H_2O_2 for 2 hours, green: treated twice

There is a minor variation in the treatment of the polymer when it has reacted once with H_2O_2 and a greater variation in the double treatment. The peaks 2850 cm^{-1} and 2883 cm^{-1} in both cases have the asymmetric CH_2 stretching mode reduced in intensity.

- **Illustrated Raman spectra of PP polymer**
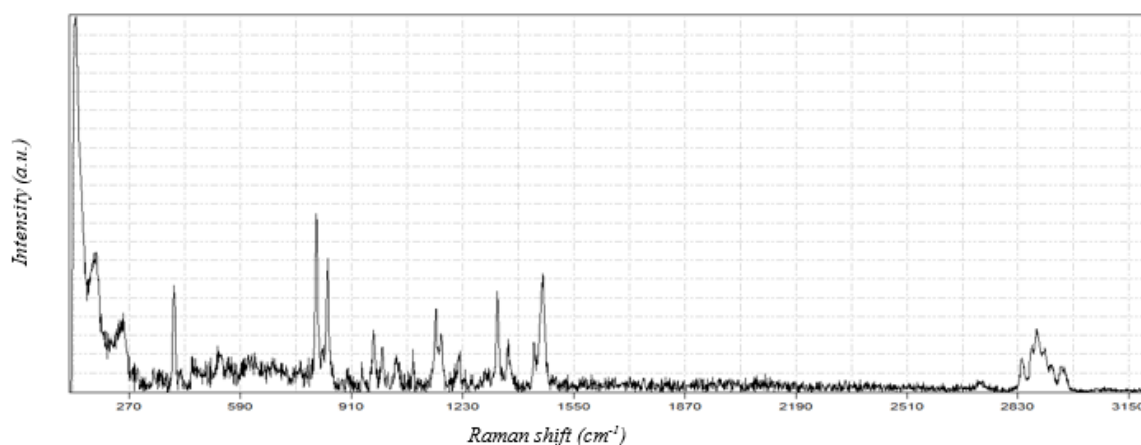- **A. Before treatment with H_2O_2 (30%)**

Figure 16. Raman spectra of polypropylene (PP)

.

Polypropylene is usually isotactic. The most intense vibrations in isotactic polypropylene (PP) in this case are at 398, 809, 841, 973, 1330, 1360, 1458, and 2883 cm^{-1}. The band at 398 cm^{-1} has been assigned to CH_2 wagging and CH bending, vibration at 809 cm^{-1} to CH_2 rocking and stretching of C–C and C–CH_3, at 841 cm^{-1} to rocking of CH_2 and CH_3 and stretching of C–C and C–CH_3, and at 973 cm^{-1} to CH_3 rocking and C–C stretching. The remaining bands have been assigned as follows: 1330 cm^{-1} to CH bending and CH_2 twisting, 1360 cm^{-1} to symmetric bending of CH_3 and CH bending, 1458 cm^{-1} to asymmetric bending of CH_3 and CH_2 bending and 2883 cm^{-1} to symmetric stretching of CH_3 group.

We observe that the peaks after the double treatment with H_2O_2 decrease in intensity in most vibrations (Figure 17). The vibration at 809 cm^{-1} decreasing to a much greater extent, so it is related to the motion of CH_2 rocking and stretching of C-C and C-CH3.
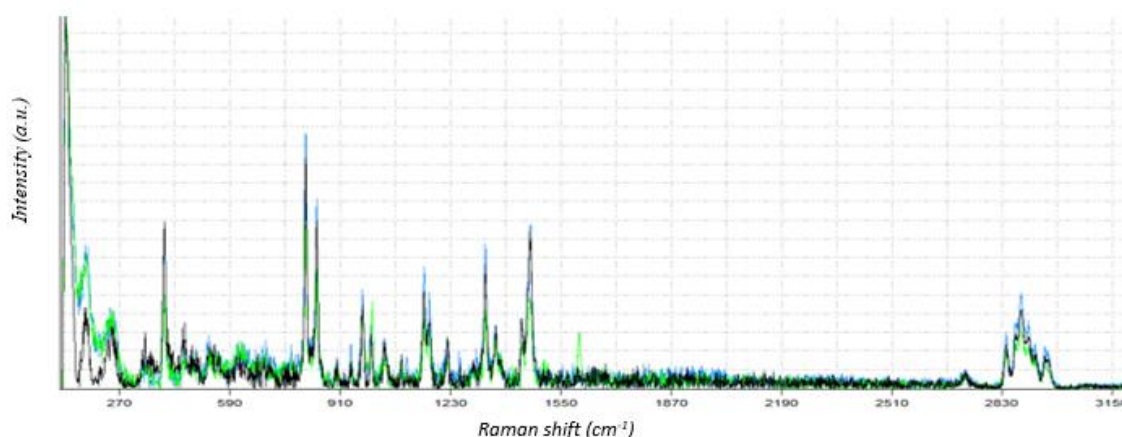

Figure 17. Raman Spectra of polypropylene. Black: untreated, blue: treated once with H2O2 for 2 hours /50- 55 Co, green: treated twice

According to the results, no substantial differences were observed between the spectra of microplastics before and after treatment with hydrogen peroxide. Based on observations, there has been no erosion of the surfaces of the microplastics by the hydrogen peroxide reactor. Using H_2O_2 as a pretreatment method for separating microplastics from soils can lead us to several conclusions related to environmental applications. The analysis of Raman vibrations contributes by understanding the chemical reactions, potential by products and degradation pathways of particularly in scenarios where oxidative processes may occur, such as exposure to sunlight or reactive oxygen species in the environment.
Raman shift (cm^{-1})

Including the use of Raman spectroscopy we can see whether the treatment can contribute to the assessment of retrieving microplastics from complexed soil. By understanding how the polymer's structure and properties are affected, researchers can evaluate the effectiveness of H_2O_2 treatment, potential byproducts or degradation products, and the overall environmental implications of using such chemical treatments in waste management or recycling processes.

4.2 Optical microscopy and FTIR spectroscopy results

It is important that before any qualitative or quantitative analysis of microplastic samples, a visual observation of the samples would be done by using an optical microscope. In this study, an optical microscope was used for mapping the total surface area of the sample filter (metallic mesh 100 μm). The samples that we analyzed using optical microscopy are "A1", "A2", "B1", "B2" Also, the sample "A2" was analyzed using FTIR spectroscopy.

A.   "A1"

For sample "A1" the hydrogen peroxide digestion method and density separation using zinc chloride to remove organic and mineral particles were performed. Figure 18 shows organic particles remained after density separation in the 100 μm filter, as well as possible plastic particles from the spiked tire wire particles. It is also observed that elongated particles, can be identified as pieces of twigs, have lost the phloem that normally surrounds them.
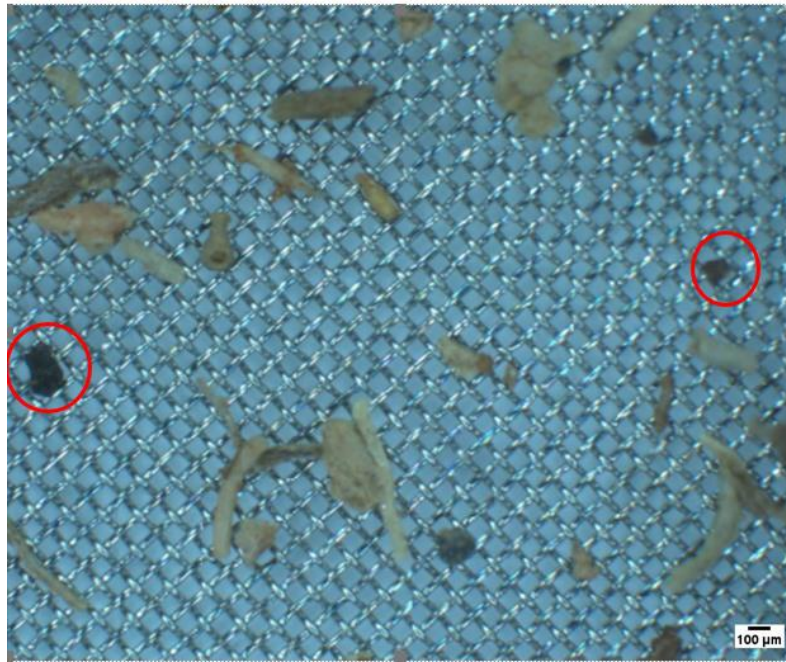
Figure 18. Plastic compounds on the metal mesh.

At the same metal mesh we can observe all the remained particles, both microplastics and organic matter. In the Figure microplastics have been circled based on their visual comparison with microplastics, before the spiking process.

Figure 19. Possible microplastics on a metal mesh

The diameters and lengths of some of the organic and plastic particles have been measured in the picture using the software "Image J", in order to estimate the size variation of larger and smaller particles that have been recovered. The Figure shows two microplastics with diameters of 93 μm and 100 μm respectively, of pale yellow-white shade

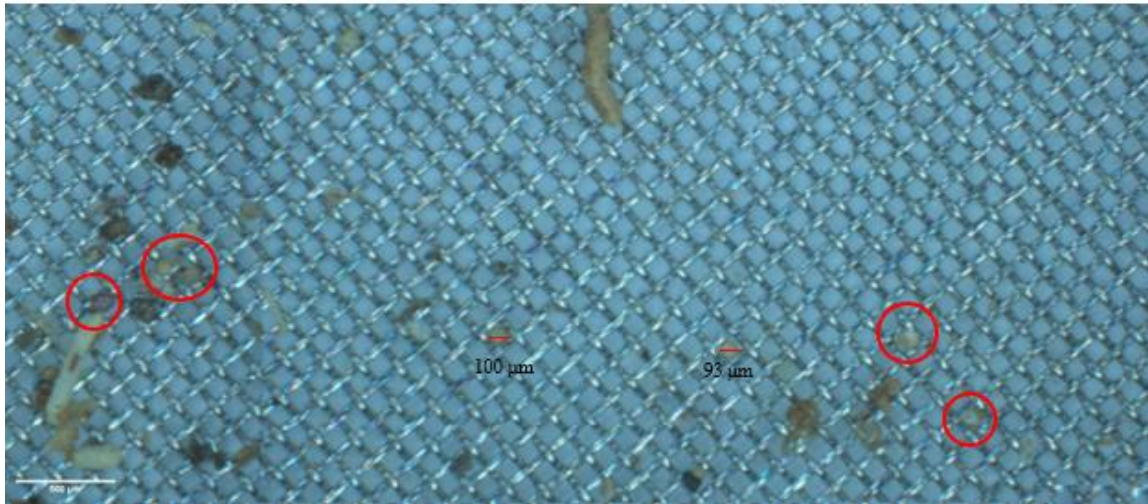Figure 20. Possible microplastics on a metal mesh and description of their diameters

Observed organics that have not had an external phloem removed but without necessarily being in large aggregates with other organics or microplastics. Pieces of twigs 3.2 mm long were also measured, indicating that due to their elongated structure they can pass through sieves of size 2 mm.

Figure 21. Aggregates with organic matter

B.   "B1"

In sample "B1", the particles were treated with hydrogen peroxide and density separation was performed using sodium chloride. In the images below, twigs and other organics have been observed in sizes up to 3 mm in length (Figure 22), while microplastics were measured in the range of 100 μm to 400 μm (Figure 23).

Figure 22. Organic matter particles, with marked length
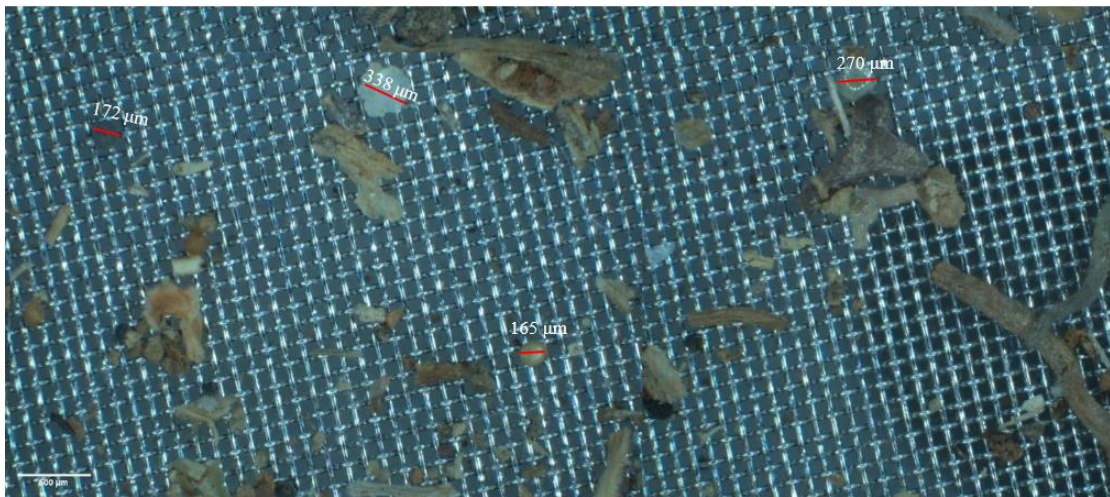
Figure 23. Possible microplastic particles, with marked diameter (100-400 μm)

C.   "B2"

Sample "B2" was digested by using enzymes and density separation was performed using sodium chloride. In the following images plastic particles with a minimum size of 100-150 μm have been observed (Figure 24), as based on optical observation we could detect spiked particles of wire particles and PET or PVC particles of "deep sea condiotions", with the characteristic brown color.

Figure 24. Possible microplastic from deep sea conditions

Figure 25. Possible plastic particles

The Figure 26 shows a pink-coloured plastic particle of unknown origin, since no such particles were mixed with the soil samples. The origin of this microplastic is presumed to be from the external sampling environment, with particle size 80 μm X 230 μm.
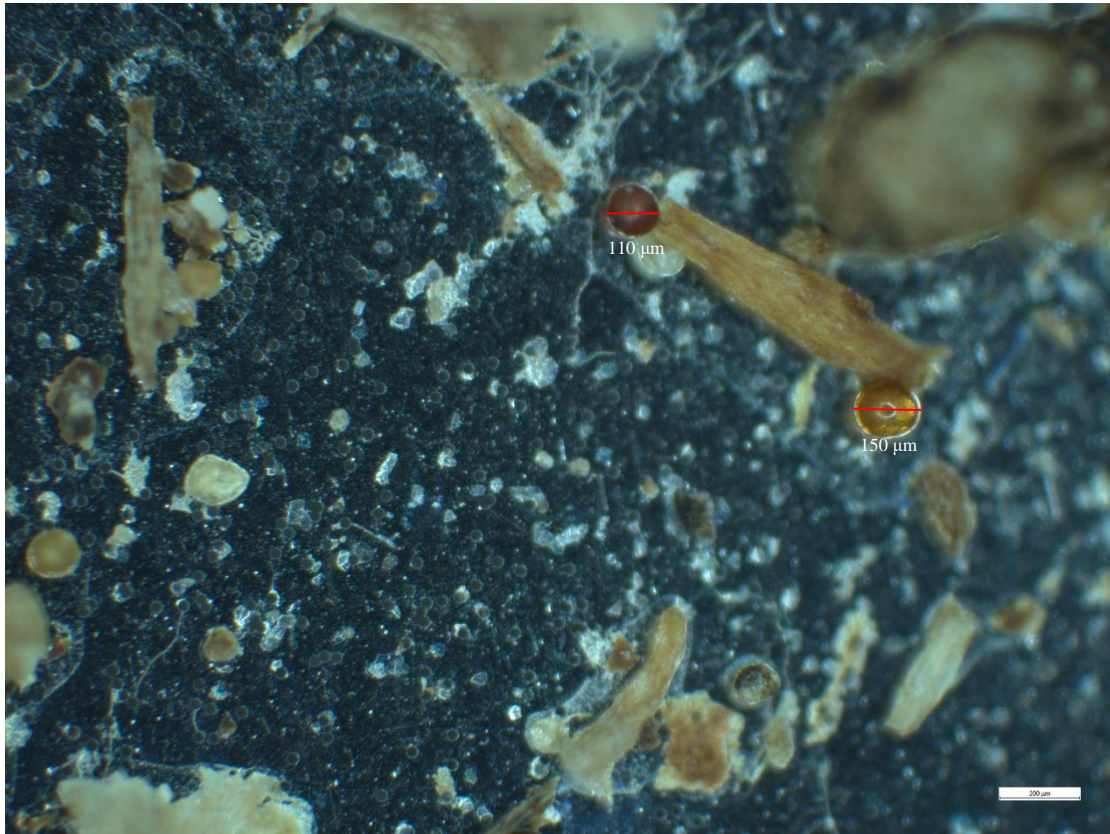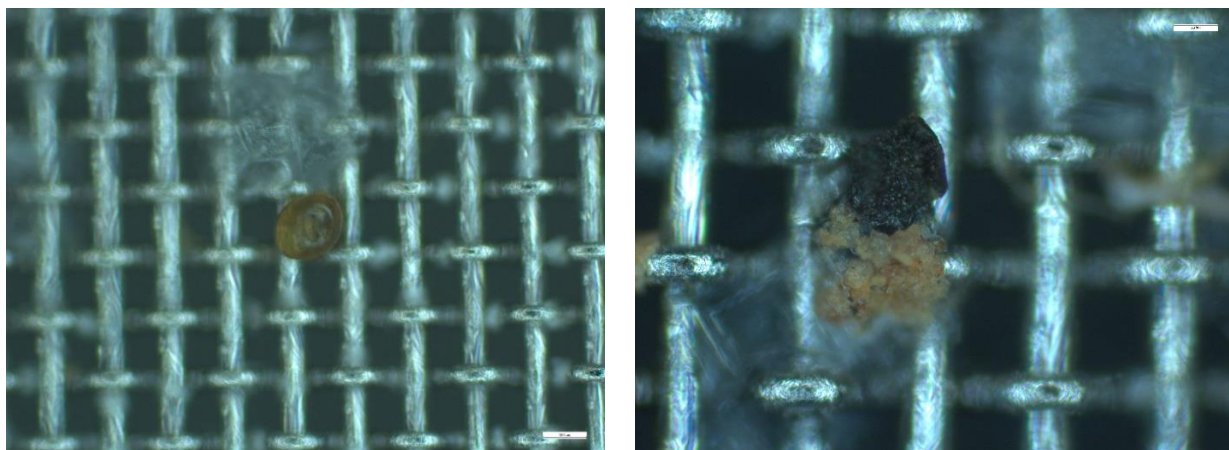
Figure 26. A pink microplastic of unknown origin

D. Rejected reagent: KOH

In the first phase of the experiments all reagents were tested with soil without the presence of microplastics to study their influence on the digestion of organic particles. The KOH reagent, as very often mentioned in the literature, has a alkaline pH value, so cellulose and lignin that is contained in organic particles in soil environments, as well as shells or organic residues are more easily to be decomposed in the presence of a different solvent or oxidising agent, than with KOH. The Figure shows that large aggregates of organic material remain, which may complicate the analysis and recovery of microplastics that may be trapped in the matrix of the organic debris or fill the gaps of the metallic meshes.
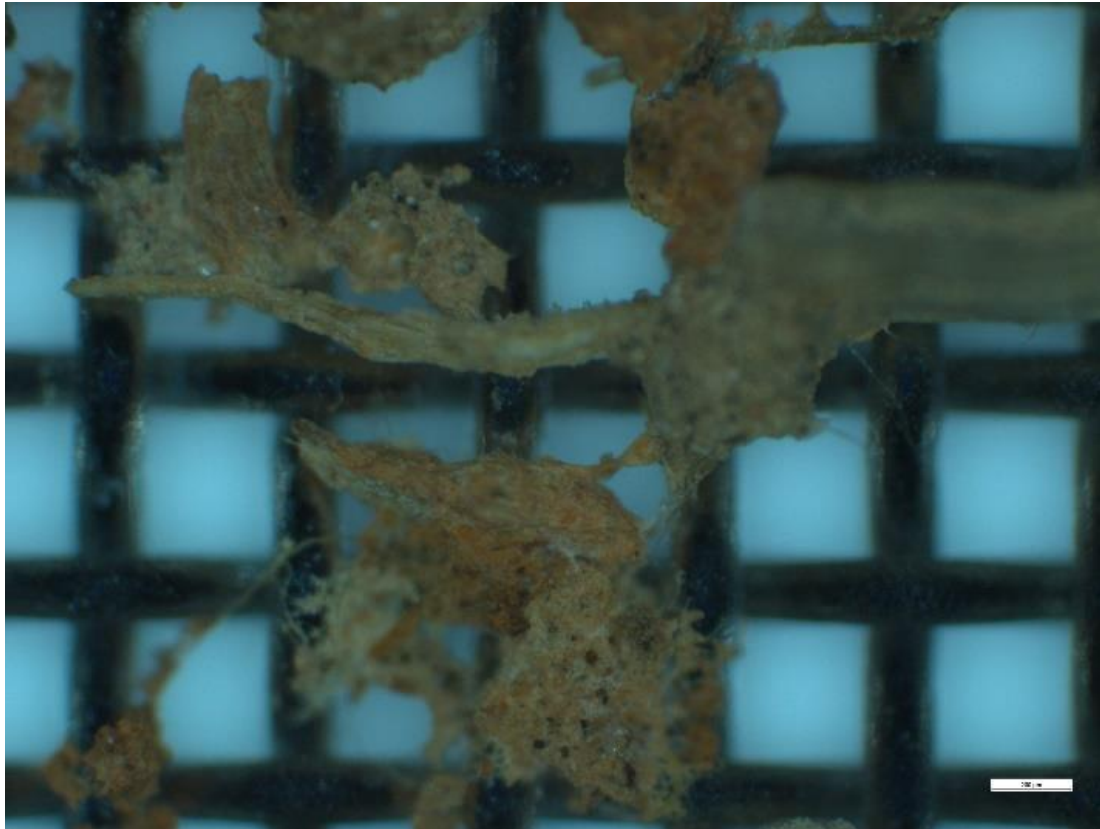
Figure 27. Aggregates with organic compounds

E. "A2"

Sample "A2", soil sample conducted with the use of enzymatic digestion and zinc chloride density separation solution, was studied using FTIR spectroscopy, due to time and laboratory constraints, it was the only sample that had a total analysis and count of polymer types, due to the FTIR's capability of mapping the filter area. The measurement using an instrument was performed point-by-point on all particles present in the filter, except for the wire tire particles which, due to their black color, could not be identified by the instrument. Therefore, a grid was generated based on selected coordinates from the metal filter (Figure 28).
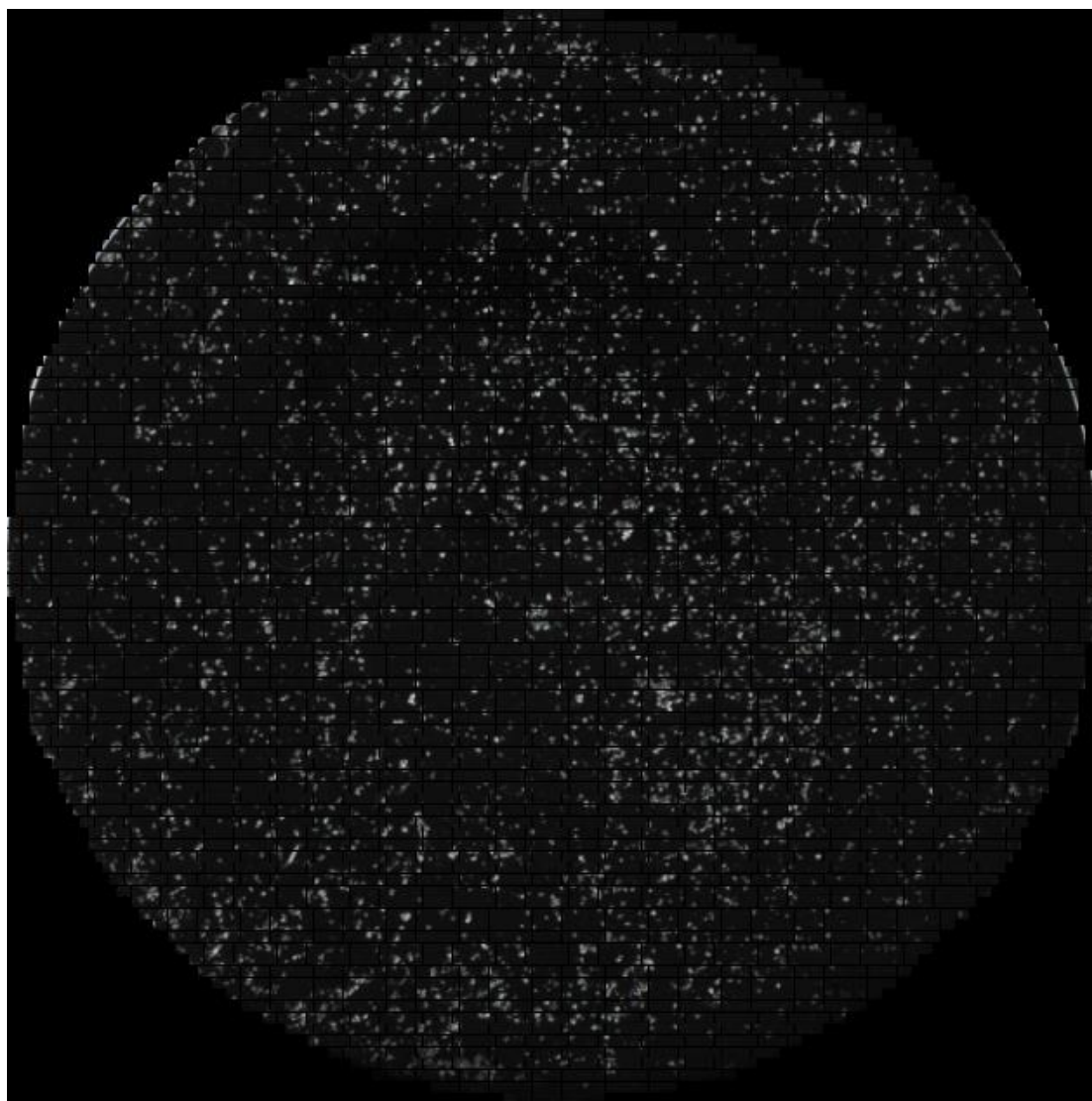
Figure 28. Performing a whole scan of the metal mesh using the optical microscope Zeiss Axio Imager M2, KMRS

During the analysis of all the co-ordinates, spectra and images were obtained. We can combine the co-ordinates with possible microplastics as shown in Figure 29.
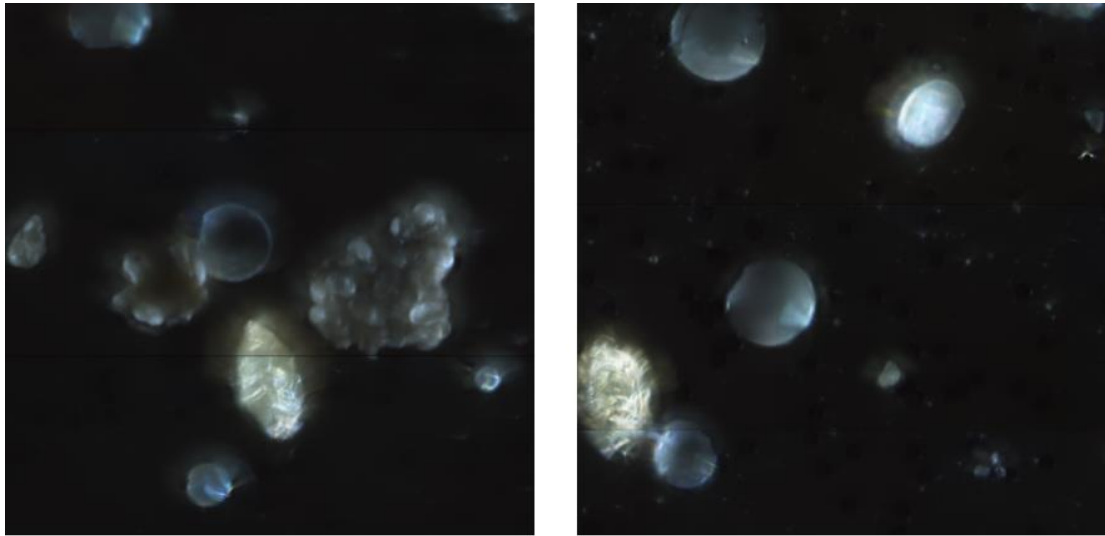
Figure 29. Coordinate display, where image and absorption spectrum has been captured from FTIR's software.

Table 2 shows the results of the identification of the infrared absorption spectra of sample A2 for the spiked polymers:

- Polyethylene (PE) & Polystyrene (PS).
- Polyvinylchloride (PVC) & Polyethylene terephthalate (PET).
- Black particles from car tires cannot be identified.

Also, other microplastics were found of various types of polymers, such as polyacetal, polypropylene, nylon, polyacrylamide and fibers.

Polymer	Microplastics total	Spiked
PE (LDPE)	59	Spiked
PE (HDPE)	5	Not spiked
PS	1	Spiked
PVC	32	Spiked
PET	4	Spiked
PP	15	Not spiked
Fiber	299	Not spiked

Table 3. Table of content for type of polymers in the sample A2 ($ZnCl_2$ separation solution)

During the experimental procedure, we spiked 20 ± 5 particles of each polymer type, measured larger than 100 μm and smaller than 1 mm, totaling 80 particles. Plastic particles measurement was conducted using the GEPARD program, where the infrared absorption spectrum for each type is identified through FTIR, followed by automatic categorization based on size and occurrence.

All particles present in the filter (natural organic and microplastics) were compared regarding shape (Figure 30). Also, microplastics were compared in terms of size (smaller than 50-100 μm and larger than 100 μm) (Figure 31). Out of the 6234 analyzed particles, the dominant shape was irregular and then spherical. This may be due to the presence of many natural organics such as twigs, irregular cellulose and microplastics without necessarily having a spherical shape.
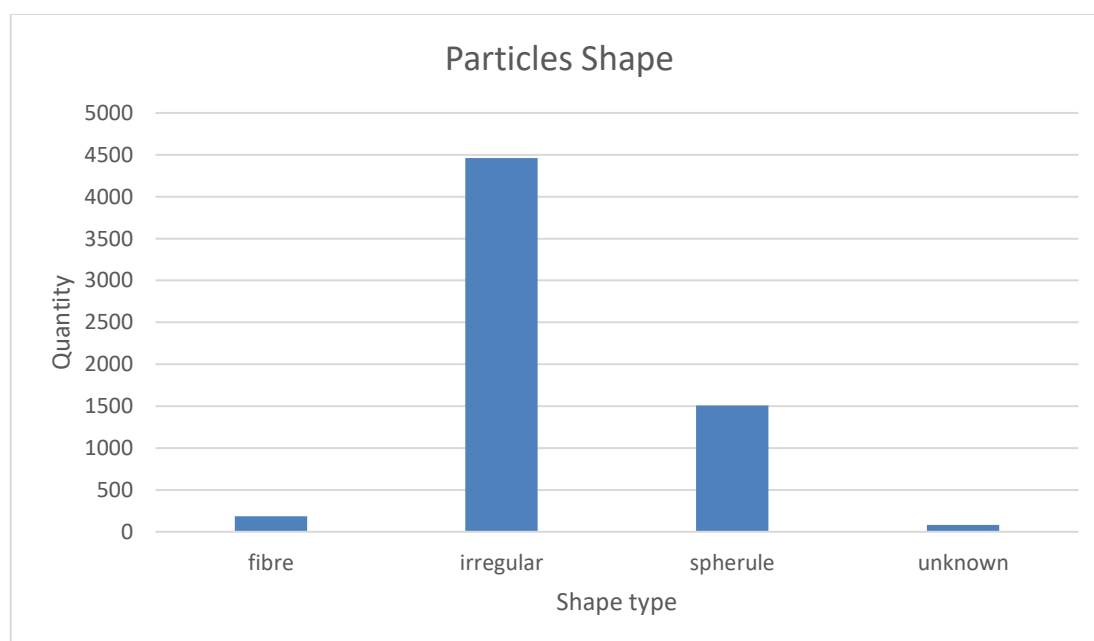

Figure 30. Shape type- quantity diagram

The spiked microplastics in the sample were 20 ± 5 of the following type of polymer:

- As shown in Figure 31 the number of polyethylene particles exceeded 20, which constituted the total spiked particles. In fact, 20 PE particles were found to be below 100 μm, and 44 particles were above 100 μm, which are more than the primary spiked particles.
- Only one polystyrene particle was detected, which is less than the 19 spiked particles.
- For PVC, 30 particles were between 50-100 μm, and 2 particles were over 100 μm, suggesting the existence of microplastics from an unknown source.
- Only 3 PET particles of more than 100 μm were found, which is less than the 20 spiked particles.
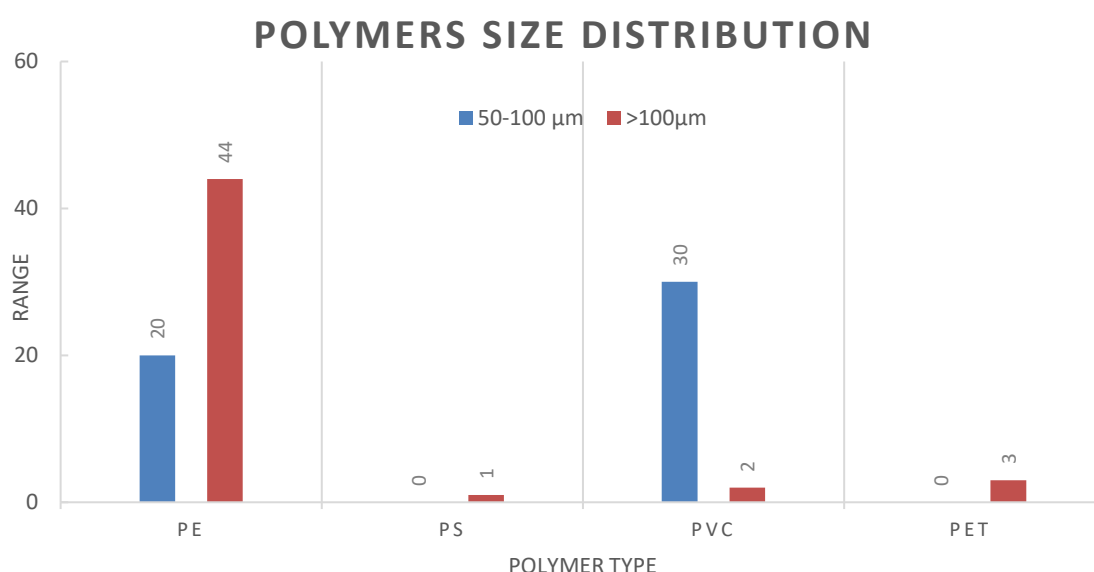
Figure 31. Polymer type-range diagram.

To compare the recovered particles of the samples separated either treated with zinc chloride or with sodium chloride, an optical count was done by using the optical microscope mapping properties and the operator's visual perception, while in sample "A2" a count was performed based on the spectra representing the plastic particles in the filter after density separation (Table 4).

Identification Method	Sample Name	Total identified particles
Optical Microscopy	A1	125 ± 5
Optical Microscopy	A2	120 ± 5
FTIR Measurement	A2	104
Optical Microscopy	B1	45 ± 5
Optical Microscopy	B2	170 ± 5

Based on the table, we observe that the samples treated with zinc chloride (marked "A") have similar values of the recovered particles. However, there is a deviation of between 5-20 particles. In specific, the optical measurement of "A2" comes out to 120± 5 particles, while the FTIR spectroscopy measurement is at 104 particles, which indicates that:

A. There is a size deviation of 16 particles, between optical and FTIR analyses.

B. There is an initial deviation from the initial 60 particles that enriched the soil samples

In a similar way, a difference is observed between the initial spiked, and the final particle count for the samples with sodium chloride solutions (marked as "B"). On the basis of the above deviations, there is a scientific concern regarding the visual identification of microplastic particles because of the risk of bias between operators and the risk of both false positive and false negative.

4.3 Analyses of blank samples

The two blank samples were used, listed as "BL1" for NaCl solution, for density separation and "BL2" for $ZnCl_2$ solution, for density separation. The samples were analyzed using cellulose filters used for filtration, by optical observation with an optical microscope. A total of 4 microfibers were found in the blanks, of which pictures "A, B, C" in Figure 27 belong to BL1 and "D" to BL2. No microplastics were found in the samples of this type. All particles visually identified as anthropogenic in the blank samples, which maybe be apart from operators clothes and lab coat.
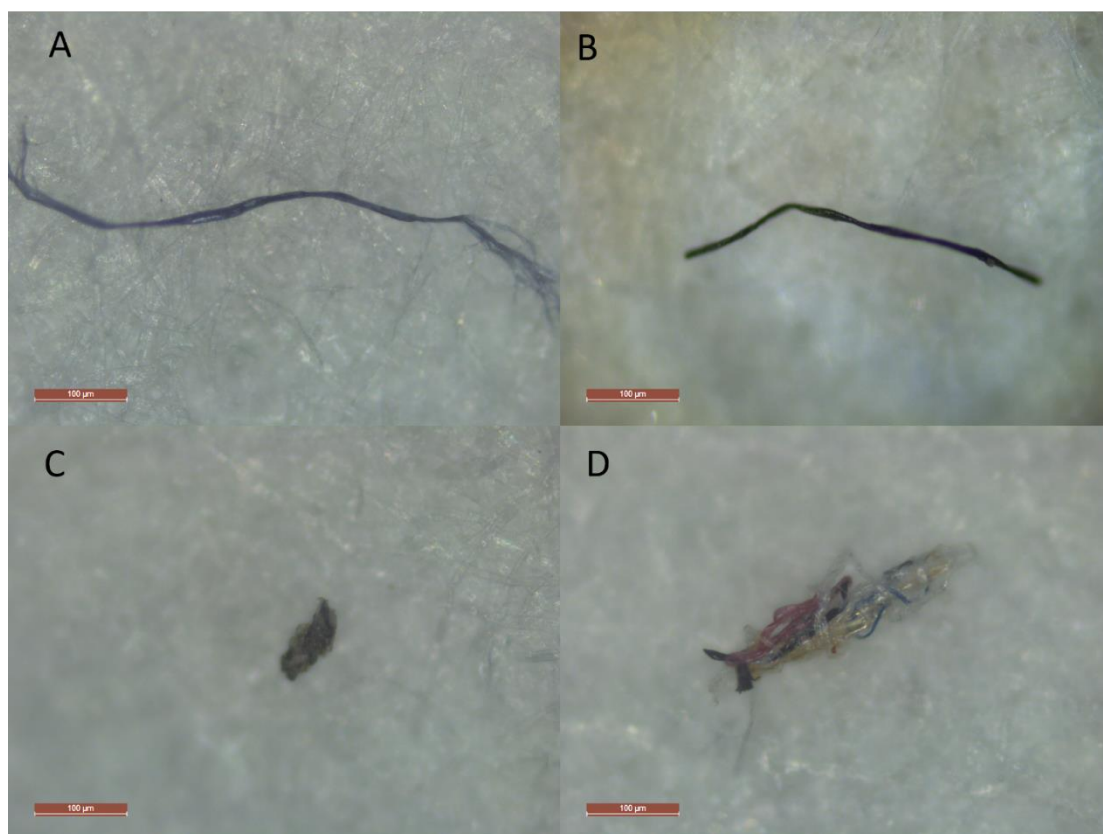
Figure 32. Examples of microfibres in Blank samples

CHAPTER 5. Conclusions and recommendations

This thesis contributed to part of the experimental stage of the STSM of Priority entitled "Linking microplastic pollution with geochemical data to determine overall anhropogenic impact on soils", funded by Cost Action 20101. This chapter presents conlclusions of various methods employed for the extraction and analysis of organic matter and microplastics from soil samples.

1 Sampling protocol

The outdoor sampling protocol for mapping microplastic landscapes involves rigorous procedures to ensure the collection of representative samples devoid of pollutants. Sampling locations are carefully chosen and marked on maps with precision, following a predefined protocol communicated to trained personnel. The University of Athens (NKUA) team conducted sampling across various land uses, including urban, agricultural, and semi-natural areas, totaling 88 locations for microplastic sampling. The sampling procedure entails clearing the site surface, excavation, homogenization of soil, and collection using glass containers. Detailed observations, site documentation, and photo records are maintained. Samples are marked clearly with identification details, and a sampling recording form is completed for each, including location, coordinates, description, and supervisor's name. Especially for microplastics sampling it was important to have glass and metal or stainless-steel equipment in order to avoid any anthropogenic contaminants. The protocol ensures consistency and replicability in subsequent samplings, crucial for environmental research and pollution mapping efforts.

2 Treatment methods

The treatment methods employed for the digestion of organic matter in soil samples involved both inorganic and enzymatic digestion techniques and the density separation with chlorine solution. Inorganic digestion was conducted using reagents such as potassium hydroxide (KOH), Strand solution and hydrogen peroxide (H_2O_2). KOH wasn't used in the final experiment, as well as the Strand solution wasn't analyzed, due to time limitation at the STSM program. Hydrogen peroxide provided oxidative breakdown of organic matter, with digestion conducted in an ice bath to prevent excessive temperature rise. Enzymatic selectively broke down cellulose, proteins, and lipids aggregates. Following digestion, centrifugation was employed for density-based separation, washing the samples with deionized water to remove sediment and reactants. Finally, density separation using solutions like NaCl and $ZnCl_2$ facilitated the recovery of microplastics from soil particles, with filtration and drying processes ensuring the purity of the collected samples. Safety measures were also taken to protect samples from contamination during handling and analysis, including the use of glass containers and avoidance of plastic laboratory materials.

3 Raman Spectroscopy Results

Tests conducted using hydrogen peroxide (H_2O_2) on polypropylene (PP) and high-density polyethylene (HDPE) showed minor variations in the spectra of treated polymers. Also, no substantial differences were observed in the spectra of microplastics before and after treatment with H_2O_2. Raman spectroscopy aids in understanding chemical reactions and degradation pathways, offering insights into the effectiveness of H_2O_2 treatment for microplastic retrieval from soil matrices.

4 Optical Microscopy and FTIR Spectroscopy Results

Optical microscopy revealed the presence residual organic and spiked plastic particles in soil samples, with sizes ranging from 100 μm to 3 mm. FTIR spectroscopy was necessary for the identification of spiked polymer types, including polyethylene (PE), polystyrene (PS), polyvinylchloride (PVC), and polyethylene terephthalate (PET). Also, not spiked microplastics were found in the analyzed sample, such as polypropylene, high-density polyethylene and microfibers. Comparison between samples treated with different digestion methods (e.g., H2O2 vs. enzyme digestion) highlighted variations in the recovered particle counts. However, more measurements should be made based on measurements by a mapping software from an instrument like the Fourier-transform infrared spectroscopyor Raman spectroscopy, because optical measurement involves random or systematic errors by the analyst.

5 Analyses of Blank Samples

Blank samples showed the presence of microfibers but not any microplastics, indicating potential contamination from operators' clothing or lab equipment.

Overall, the study underscores the importance of selecting appropriate digestion methods and analytical techniques for accurate identification and quantification of organic and microplastic particles in soil samples. Each digestion method offers unique advantages and considerations regarding the efficiency of organic matter extraction from soil samples. Understanding the mechanisms and limitations of these methods is crucial for selecting the most appropriate approach based on the specific objectives of the analysis is crucial for selecting the most appropriate approach based on the specific objectives of the analysis.

Based on the above, the following proposals are made to improve the stage of experimental and characterization procedure during the extension of the STSM project and beyond the boundaries of this thesis:

- The use of reagents such as hydrogen peroxide with or without the presence of catalysts (Fenton), without raising temperatures above 70 °C, to avoid decomposition of polymers structure

- We shall continue the comparison of solutions for density separation, in order to understand the viscosity and flotation of large and small, low or high density microplastics.
- The counting and identification of microplastics shall be performed by a combination of methods such as Raman or FTIR with SEM-EDS or Pyrolysis-GC-MS and shall not rely on the optical capability of the analyst.

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