UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Microplastics in the indoor environment

João Vasco Almeida Silva Valente

Dissertação para obtenção do grau de Mestre em
Biotecnologia
(2º Ciclo de estudos)

Orientador: Prof. Manuel Ramiro Dias Pastorinho
Co-Orientador: Dr. Ana Catarina Sousa e
Prof. Luís Taborda-Barata

Covilhã, janeiro 2020

Acknowledgements

Em primeiro lugar, queria agradecer aos meus orientadores, o Professor Ramiro "Miro" Pastorinho e a Professora Ana Catarina "Cathy" Sousa pela orientação nesta dissertação, pois foram pessoas fundamentais para todo este projeto. Todos os ensinamentos,conhecimento, profissionalismo, dedicação e empenho que me transmitiram ao longo deste ano fizeram-me crescer muito tanto como investigador como pessoa. Também tenho que agradecer toda a paciência que tiveram comigo e todo o "à vontade" que colocaram desde o iníciocriou uma relação não só de professor-aluno, como também de grande amizade.

Agradecer àDr. Sara Fateixa pela colaboração, trabalho e disponibilidade na análise das amostras através da espectroscopia de Raman e também na elaboração desta dissertação. E à Dr. Ana Borges pela ajuda na análise nas amostras no microscópio de fluorescência.

A todos os meus amigos que me acompanharam ao longo deste percurso académico, são eles os caloiros de Biotecnologia 2014/2015 e desde já agradeço do fundo do coração todos os momentos que partilhámos, pois tornaram este percurso inesquecível e ajudaram-me a crescer e a tornar melhor.

Também quero agradecer a todos os meus amigos, aosconterrâneos que me acompanharamdesde sempre, tantos nos bons momentos como nos menos bons e tornaram a minha infância e juventude memorável. E também à malta do secundário, com especial carinho para a Rita Carapito e para o Vasco Lopes porque sempre mostraram empenhados e disponíveis para contribuir para o meu crescimento enquanto pessoa.

Não podia deixar de agradecer também ao Cláudio "Rôlas" Gonçalves, ao João "Jeffrey" Martins e ao Rafael "Cão-gato" Barros por terem sido, no verdadeiro sentido da palavra, grandes compinchas, vocês foram, são e vão continuar a ser dos amigos que quero levar para sempre porque personificam o verdadeiro sentido da amizade!

Á Filipa da Silva e ao Rui, um gigante obrigado por me terem recebido tão bem, em vossa casa, e tornarem a minha estadia em Aveiro espetacular. Fizeram me sentir verdadeiramente como se estivesse "em casa" e por isso estou muito grato!

Ao Path um grande obrigado por me terem acolhido tão bem, especialmente ao Emanuel Capela e à Ana Rufino, por terem sido tão incríveis comigo, sem sequer me conhecerem, fizeram tudo para sentir à vontade, para me ajudare para tornarem a minha passagem por Aveiro tão boa!

Também quero agradecer a todas as pessoas doSteelab por tornarem este laboratório tão único e no qual gostei muito de trabalhar, sobretudo ao Rafael "Cão-gato" Barros, por me ter acompanhado sempre que precisei e tornado todo o trabalho ainda mais motivador e empolgante.

Por último, os mais importantes, à minha família, avós, tios, primos e especialmente pai, mãe e irmã o meu obrigado por todo o carinho e amor que me deram, por todos os valores que me transmitiram, por acreditarem sempre em mim, por me terem dado o sempre o melhor e por me incentivarem a ser, a cada dia que passa, melhor!!

Este trabalho foi suportado por verbas do Centro de Investigação em Ciências da Saúde (CICS-UBI) financiado por Fundos Nacionais pela Fundação para a Ciência e a Tecnologia (FCT) (UID/Multi/00709/2019) e pelo projeto ICON "InterdisciplinaryChallengesonNeurodegeneration (ICON)" (Ref. CENTRO-01-0145-FEDER-000013). Apoio financeiro adicional foi fornecido pelo CICECO-Aveiro InstituteofMaterials, FCT Ref. UIDB/50011/2020 & UIDP/50011/2020 e pelo projeto tesseRESPIRA fundado pela Labex DRIIHM (PIA), Observatório Internacional Hommes-Millieux, ferramenta do CNRS/INEE-Centro Nacional de Pesquisa Científica/Instituto de Ecologia e Meio Ambiente.

Resumo

Os microplásticos (MPs) definidos como sendo partículas com tamanho entre 100 nm e 5 mm, são considerados contaminantes emergentes com efeitos deletérios no ambiente e na saúde humana.O polietileno (PE), policloreto de vinilo (PVC) e a poliamida (PA, mais conhecida por nylon), são três dos polímeros mais comuns sendo utilizados,por exemplo, em materiais de construção, embalagens, produtos de higiene e cuidado pessoal e vestuário. Devido à sua ubiquidade no meio ambiente, incluindo o ambiente doméstico, os seres humanos estão constantemente expostos.No entanto, ainda existem poucos estudos relativamente à contaminação do ambiente doméstico por microplásticos e seus efeitos na saúde.

Com este trabalho pretende-se desenvolver uma estratégia integrada para estudar os microplásticos presentes no ambiente doméstico e os seus possíveis efeitos deletérios. Para tal, os níveis de MPs em amostras de ar e de pó doméstico foram avaliados e a citotoxicidade de três MPs distintos (polietileno, PVC e poliamida) foi avaliada em três linhas celulares, nomeadamente em células epiteliais intestinais (Caco-2), em hepatócitos (HepG2) e em neurónios dopaminérgicos (N27). Adicionalmente, e dadas as limitações das técnicas de quantificação de MPs atualmente existentes, foi desenvolvida uma nova estratégia de isolamento e purificação de MPs de amostras complexas recorrendo a sistemas aquosos bifásicos constituídos por líquidos iónicos.

Os resultados obtidos da análise de MPs,em amostras de ar e de pó recorrendo a separação por densidade com cloreto de sódio (NaCl) e digestão com peróxido de hidrogénio (H_2O_2) e posterior visualização microscópica após coloração com vermelho do nilo, demostraram que a maior quantidade de MPs corresponde às amostrasrecolhidas na cozinha.

O novo método desenvolvido para o tratamento das amostras, utilizando líquidos iónicos, permitiu extrair os MPs da matriz de pó.Contudo a separação dos diferentes MPs não foi possível e, por isso, esta técnica ainda precisa de ser otimizada. Ainda assim, a espetroscopia de Raman mostrou ser uma técnica eficiente para identificar MPs em amostras de pó doméstico.

Os ensaios de citotoxicidade demostraram que a linha celular de neurónios dopaminérgicos foi a mais sensível à exposição a microplásticos, sendo a poliamida o microplástico testado menos tóxico e o PE o mais tóxico. Para as concentrações testadas (0,01; 0,1; 1; 10; 100; 1000; 2000; 4000 $mg.L^{-1}$) a toxicidade dos três MPs para as células epiteliais intestinais e para hepatócitos foi reduzida oque demostra a baixa toxicidade destes MPs quando testados na sua versão pura e isoladamente, isto é, a sua forma mais nativa sem a adição de plastificantes, corantes, retardantes de chama ou estabilizadores.

Palavras-chave: microplásticos, líquidos iónicos, pó doméstico, citotoxicidade, microscopia fluorescência, espectroscopia de Raman.

Abstract

Microplastics are plastic particles with sizes between 100 nm and 5 mm, being consideredan emerging class of contaminants with deleterious effects on the environment and human health.Polyethylene (PE), Polyvinyl Chloride (PVC) and polyamide (PA-commonlyknown as nylon) are three of the most common polymers used in buildings and construction, packaging,personal careproducts and clothing. Due to MPs ubiquity in the environment, especiallyindoor, humans are continuously exposed. However, only a few studies regarding the contamination of the indoor environment by microplastics and their effects on health were performed to date.

This work intends to develop an integrated strategy to study the microplastics present in the indoor environment and their potentialdeleterious effects.For this, the levels of MPs in air-born and house dust samples were estimated and the cytotoxicity of three different MPs (polyethylene, PVC and polyamide) was evaluated in three cell lines, namely intestinal epithelial cells (Caco-2), hepatocytes (HepG2) and dopaminergic neurons (N27).In addition, and givingthe limitations of currently existing quantification techniques, a new strategy for isolating and purifying MPs from complex samples was developed using two-phase aqueous systems made up of ionic liquids.

The results obtained from the analysis of MPs in air and dust samples using sodium chloride (NaCl) density separation and digestion with hydrogen peroxide (H_2O_2), and subsequent microscopic visualization after Nile Red staining showed that the largest amount of MPs corresponds to samples collected in kitchens.

The new procedure developed for sample preparation using ILs generally allowed to extract the MPs from the dust matrix.However, the separation in a single step of the different MPs was not achieved and requires further optimization. Nevertheless, Raman spectroscopy proved to be efficient to identify MPs in house dust samples.

The cytotoxicity tests showed that the dopaminergic neuron cell line was the most sensitive to microplastics' exposure, with polyamide being the least toxic microplastic tested and PE the most toxic. For the tested concentrations (0.01; 0.1; 1; 10; 100; 1000; 2000; 4000 $mg.L^{-1}$) the toxicity of the three MPs for intestinal epithelial cells and for hepatocytes was reduced, which shows the low toxicity of these MPs when tested in its pure and isolated, that is its native form without the addition of plasticizers, colourants, flame retardants or stabilizers.

Key-words: microplastics, ionic liquids, house dust, cytotoxicity,fluorescence microscopy, Raman spectroscopy.

Index

List of Figures

List of Tables

List of acronyms

ABS - Aqueous Biphasic System
BBzP – Butyl benzyl phthalate
CCK-8 – Cell counting kit-8
DDT - Dichlorodiphenyltrichloroethane
DEHP – Di(2-ethylhexyl) phthalate
EVA – Ethylene-vinyl-acetate
FTIR - Fourier-transform infrared spectroscopy
HCH - Hexachlorocyclohexane
IL – Ionic Liquid
MSFD – Marine Strategy Framework Directive
MPs – Microplastics
NOAA – National Oceanic and Atmospheric Administration
PA – Polyamide
PAH - Polycyclic Aromatic Hydrocarbon
PAN - Polyacrylonitrile
PCBs - Polychlorinated biphenyls
PE - Polyethylene
PET – Polyethylene terephthalate
PP - Polypropylene
PS – Polystyrene
PUR – Polyurethane
PVC – Polyvinyl Chloride
REACH – Registration, Evaluation, Authorisation and Restriction of Chemicals
SBR – Styrene-Butadiene Rubber
TDCIPP -Tris(1,3-dichloro-2-propyl) phosphate
WWTP – Wastewater Treatment Plant

Chapter 1

1. Introduction

1.1. Microplasticsas emerging contaminants

In 2018, the plastics production reached almost 360 million tonnes worldwide, of which 62 million tonnes were produced in Europe alone[1].Such figures are a consequence of the high demand for plastics in modern society,which includesbuilding and construction materials, automotive materials, packaging and a wide range of consumer products,such as hygienic products, textiles, toys and stationery[1]. Many plastics exhibit a high degree of resistance to chemical and thermal degradation and therefore theyare prone to be accumulated in the environment [2].Besides, several plastics are designed for single-use,being immediately discarded after their short usage,ending up in the environment [3]. Although recycling strategies are improving and the amount of plastic waste sent to recycling has doubled since 2006, about 25% of post-consumer waste plasticwas still sent to landfills in 2018[1]. This creates an important problem as it represents a large introduction of plasticsinto the environment. Recently, plastic pollution has received increasing attention fromthe scientific community as well as the general public and policymakers. Of particular relevance are single-use plastics and microplastics. Microplastics are generally described as plastic debris smaller than 5mm in size [4] and are considered as emerging contaminants. They result from numerous sources including the degradation of larger plastic particles.

The toxicological features of plastics and microplastics are associated with their physical properties (e.g. size, particularly relevant in the case of micro and nano plastics) and also with the chemical compounds that are added during the manufacturing process and/or adsorbed during its life cycle. During the manufacturing process, several additives are added to plastics. These additives may leach from the plastics, being releasedinto the environment and their present toxicity towards humans and wildlife. In addition, microplastics, when released in the environment, can adsorb pollutants because their chemical structure was affectedby radiation or other processes and the polymers' chains become available to bind environmental contaminantssuch as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and trace metals[5,6].

Microplastics can be found in air, water and soils, leading to continuous exposures. Such exposures are diverse and may change as the size and shape of microplastics suffer modifications along their life cycle [7-9]. Besides size and shape,variations in colour and composition are another concerning issue, since theycan influence the fate and effects of microplastics [6]. Because microplastics comprise particles in the micro-scale, they can be

ingested by animals at any trophic level of afood chain and the smallest (<150 μm) particles have thecapability of entering cells [10]. This "migration" will contribute to increasing the microplastics' concentration within organisms, resulting in bioaccumulation and then biomagnification in organisms at higher levels of the food chain[6]. The accumulation of microplastics along the food chain will inevitably contribute to human exposure to these emerging contaminants.

1.2. Classification of microplastics

The classification of microplastics is still a topic under debate[4]. In 2003, for the first time, the term "microlitter" was used to describe the marine litter composedof plastic with sizes between 63-500 μm [11]. One year later the term "microplastic" was popularized as a reference to plastic fragments with diameters below ≈20μm [12]. Then, in 2008, the National Oceanic and Atmospheric Administration (NOAA) brought together experts in order to discuss and approve a working definition that describes microplastics as all plastic particles <5 mm in diameter and this definition became the most used one[13]. However, there is no international agreement uponwhich size a particle of plastic should be considered microplastic [14]. This fact is related tothe lack of standardized methods to analyse microplastic particles [6,15-17].

The **categorization of microplastics** can provide some clues to identify thepossible sourcessince plastic particles stay pretty much the same along their path in theenvironment[6]. However, attention should be paid because if sampling is far away from the source some processes that change the particle´s appearance may occur. These processes may be biological, physical or chemical and encompass abrasion, fragmentation and weathering [6,18]. Thus, some characteristics beyond size can be used to create a system in which microplastics are classified based on behaviour and some similarities in terms of characteristics. Usually, they are subdivided according to origin (product type), chemical composition (polymer type), morphology (shape) and colour [4,6].

In terms of **origin, microplastics** can be primary or secondary. **Primary microplastics** are directly released into the environment as small particles and their main sources include laundering of synthetic clothes (35% of primary microplastics); abrasion of tyres through driving (28%); intentionally added microplastics in personal care products, for example, microbeads in facial scrubs (2%)[19]. **Secondary microplastics** are generated in the environment from the degradation of larger plastic objects, such as plastic bags, plastic bottles or fishing nets [6,19].

In **terms of composition**, (micro)plastics are composed of high molecular weight molecules (polymers) which consist on the repetition of individual units of relativelylow molecular mass molecules (monomers)[20]. These structures can suffer some modifications and are classified as **thermoplastics**, if shape changes with heat, and **thermosets** when the chemical composition

is altered when heated. In addition, there are also **elastomers** that have high elasticity and viscosity which allows high deformations before they break up.

Polymers are usually classified as**natural** or **synthetic**. There's no doubt that DNA, proteins, wool, silk and cellulose are natural polymers and are not plastics, while petroleum-based products(polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS), polyvinylchloride (PVC), etc…) are associated with plastics. The plastics most produced and commonly found in the environment are PP, PE, PS, PET, Polyamide(nylon) and polyvinylchloride (PVC) [21,22]. PVC together with Polyurethane (PU) and Polyacrylonitrile (PAN) are considered to be the most toxic ones [23].Recently,**bio-based plastics** are being produced as an alternative to fossil feedstock and include conventional polymers such as bio-PET and bio-PE but also biodegradable polymers like polylactic acid and polyhydroxyalkanoates. In addition, polymers can be made with inorganic monomers (e.g. silicone) which are elastomers and sometimes are excluded from plastics definition since they do notmeet the International Organization for Standardization (ISO) definition for plastics: "material which contains as an essential ingredient a high molecular weight polymer and which, at some stage in its processing into finished products, can be shaped by flow" [4,24].The polymers that are made with more than one type of monomers are classified **copolymers**, such as acrylonitrile-butadiene-stryrene, ethylene-vinyl acetate (EVA) and styrene-butadiene rubber (SBR), being acrylonitrile-butadiene-stryrene and ethylene-vinyl acetatethermoplastics and styrene-butadiene rubber an elastomer [4]. The **composite polymers** have two components, a polymer matrix plus a polymeric or nonpolymeric reinforcement. They include both thermoset composites (glass-fibre-reinforced polyester or graphite-reinforced epoxy) and thermoplastics (polyester textiles mixed with cotton or wool [4].

Polymers may also be classified according to their composition, however, this characterization is sometimes difficult. For example, rayon and cellophane are natural processed polymers that undergo high modifications during manufacture and thus could be considered as synthetic polymers and be included in the plastics definition because theysuffer a huge human intervention. On the other hand, dyed natural fibers used for textiles are only slightly modified and the principal structure of the natural polymer keeps practically unchanged when the low molecular weight compounds are added to the peripheral chains, and therefore they should not be strictly considered as plastics[4,25].

Furthermore, **additives**should also be taken into consideration.These compounds are added to polymers during the manufacturing process in order to improve or add some characteristics that are crucial to the function of the plastic. Additivesinclude,for example,plasticizers, stabilizers, flame retardants, flow modifiers, processing aids, impact modifiers, antioxidants, pigments, biocides and fragrances. Some polymers such as PVC that have a high percentage of additives in their constitution, often exceeding 50% of the total weight [6] and thus they originate a toxic mixture with significative hazardous levels[3].However, REACH only classify a substance as a polymer if it has an additive content of under 50% [4,26].Additionally, plastics

could be a source of dangerous and hazardous unreacted monomers, possibly carcinogenic and mutagenic [23], and environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), metals, pesticides and pharmaceuticals[3,5]. Also, additives have low molecular weight and because they lack chemical binding to the main structure of the polymerthey are susceptible to leaching. Furthermore, new surfaces are easily exposed facilitating the migration of additives through fragmentation processes [27].

In **terms of morphology**, the microplastics are commonly classified as **fibres, fragments, spheres or beads, pellets, films and foams**. The **fibers** are long and with low thickness, normally are flexible and can be individually isolated or grouped in a bundle. PET and nylon polymers constitutes two fibers normally used in textiles; **fragments** have a rigid structure and irregular shape and can be made of any polymer since they result from the fragmentation processes; **spheres** have a round surface and usually are made of PE incorporating most of the hygieneproducts; **pellets** look like spheres but are a bit longer (cylindrical shape) and are used in the manufacturing of every plastic item, so they can be made of any polymer; **films** are flat, thin and malleable, resulting from the rupture of PE, PET and PVC sheets and **foams** are cloud-like, soft and compressible particles made of polyurethane (PUR) that are usually used in building insulation, mattresses and pillows [1,4,6].

Colouris used as an additional descriptor that can provide information about the potential sources since it does not change very much and thus it can be a useful tool to identify contaminations during sample collection and preparation. In addition,weathering processes can be identified through discolouration [4]. Also, colour plays a key role inorganisms because some colour plastic particles may be mistaken as food [28] and eventually ingested, which contribute to increase the bioaccumulation and biomagnification of microplastics along the food chain.

1.3. Methods

In order to study the presence and distribution of microplastics in the environment, it is crucial to collect samples directly where they occur. However, the widespread environmental dissemination of these materials implicates that they will be present in numerous types of matrices including, for example,water, sediments, air and biota[29-32]. Thisleads tothe collection of a huge varietyof microplastics rendering data analysis and comparison between studies more difficult[15,33-35]. Furthermore, no standardized methods for their collection have been established[36].

Due to the ubiquitous distribution of (micro)plastics in the environment,**sample treatment and analysis**becomesvery complex due to the easiness on the occurrence of contaminations. Utmost careis required fromthe researchers in order to prevent background contamination[22,37].Sources of contamination may include abrasion of synthetic clothing,

uncleaned laboratory equipment, plastic tools, improperly sealed samples or even contaminated ambient air [38-40].Thus, it is important to implementa quality assurance system to obtain more robust and trustworthy results,whilepreventing error-prone methodologies[22].

Despite the fact that standard operating procedures to fully eradicate microplastics contamination are still missing [41,42],somebehaviours have shown to beuseful. These include wearingcotton lab coats and clothing [41,43-45]; Rinsingall materials and tools with Mili-Q waterin order to freethem from any particle contamination; Replacing all the recipientsto be used (Petri dishes, beakers, flasks, etc),that normally are made of some type of plastic, by glass; Substituteplastics tools by stainless steel items and ban the use ofmicropipette plastic tips [46]that should be replaced byglass pipettes or microsyringes without plastics parts.

Furthermore,all the work with environmental samples should be performed in a laminar flow clean bench which can reduce the contamination up to 96.5% [22].

Sample preparation includes purification and isolationsteps that are required to remove (mostly natural) particles that could tamperwith the results (biofilms, sand, chitin and wood lignin), and to conserve microplastics and prevent the generation of secondary microplastics[46-48].Density separationand digestion are two common processesusedto separate microplastics present insediment and water samples.

1.3.1. Density separation

Sodium chloride (NaCl)is the most common solution (density=1.2 $g.cm^{-3}$)used to separate plastics from the sample´s matrix [49]. Thisisusually performedby mixing the samples with the salt solution andrecovering the supernatant, which contains microplastics, for further filtration [21]. Despite the fact that for some polymers that have higher densities than NaCl, such as PVC, PET and Polyester (above 1.2 $g.cm^{-3}$) [39],therecovery rates being under 90% and presenting larger standard errors[50], the NaCl salt solution keeps being used because it is widely available,cheap, eco-friendly[51] and it is recommended by NOAA (National Oceanic and Atmospheric Administration)[41] and the MSFD (Marine Strategy Framework Directive) Technical Subgroup [42].

1.3.2. Digestion

Generally, samples have an organic content that needs to be removed in order to isolate microplastics and thus prevent their overestimation, without damaging the original structure and chemistry of the polymers.To reduce the organic matter content,samples are submitted to oxidative digestion. Hydrogen peroxide (H_2O_2, 30%)is typically usedto treat microplastic

samples [47,49]. H_2O_2also eliminates lipids, chitin, or wood lignin which fluoresce upon being stained with fluorescent dyes as for example Nile red [46].

According to NOAA, both sediment and water samples should be treated with H_2O_2 (30%) combined with Fe (II) sulfatesolution (0.05 M) (Fenton's reagent) heated at 75ºC [41].There are also acidic, alkaline and enzymatic digestions but thesetend to modify the polymers' integrity[33], depositing residues in the plastics' surfacewhichrender the spectroscopic characterizationdifficult[31,52,53]. Furthermore, theirefficiency is highly dependent onthe sample´s organic material [54].For example, polymers like nylon, polystyrene (PS), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyethylene terephthalate (PET), polypropylene (PP) and polyvinyl chloride (PVC)are damaged, discoloured and haveresidues addedby acidic digestions with nitric acid (HNO_3) [31,52,55-57]. This also occurs with KOH and NaOH in alkaline digestions [31,52,57-60]. Digestion with the enzymeproteinase K with $CaCl_2$was responsiblefor calcium deposition in thesurface of the particles[61] but at the same time,it had no effect on polymers [54].

1.4. Contamination Sources

Microplastics are ubiquitous particles and thus present in terrestrial, aquatic and air environments [62]. It is estimated that around 75-90% of microplastics are originated from land-based sources and 10-25% from the ocean [40,63].

The main entries of **primary microplastics** to terrestrial ecosystems and soils are aerialdeposition, sedimented microplastics, landfills and sludge application to agricultural soils[8,64,65]. Primary microplastics can also enter the environment from their widespread usage in personal care products such as shower gels, hand, facial and body cleansers, shampoos, and toothpaste [6,40]. These products have in their composition polyethylene microbeads that act as abrasive scrubbers [66]. In addition, primary microplastics also have origin in industrial abrasives for sandblasting constituted of acrylic, polystyrene and polyester beads [62,67,68]; raw materials/residues used for plastic manufacturing accidentally lost, inadequately handled or runed-off from processing facilities [63,69]; drilling fluids and medical applications like dental tooth polish [40] and drug vectors [2].

Upon usage, microplastics enter the urban water cycle and thus wastewater treatment plants (WWTPs). Sewage influentsgenerally containcosmetic and industry microbeads, fibres from clothes'washing and tyre debris from road runoff. At WWTPs different technologiesare used to prevent MPs introduction into the environment[8]. Some of these technologies efficiently remove up to 95% of microplastics [70-72]. However, becausehigh volumes of effluents are discharged, the amount of MPs that are not removed in WWTPs and thus enters into the environment is considerable. During wastetreatment, sludges with a significant amount of MPs are generated[73-75]. These sludges are normally used as fertilisers in agricultural soils[40]where

they last for several years [76]. Additionally, it is estimated that MPs applied to land through sludge applications may exceed 400,000 tonnes, which is higher than the mass reported to be present in oceanic surface waters worldwide [77].

It should be noted that in developing countries, agricultural and municipal plastic wastes are directly released to open fields and landfills which contributes to MPs runoff [40] and thus increasingthe possibility to reach and contaminate other (eco)systems. Furthermore, landfills easily lead to wind-blown particles [78] already detected in the atmospheric fallout [32,79], although suspended MPs in the air are difficult to determine because of the complexdynamics of the atmosphere[80].

Secondary microplastics are a consequence of the degradation of larger plastic objects. There are some organisms capable of generating secondary microplastics whether by grinding small plastics particles in their gizzard, like earthworms [64], scrapping or chewing off plastic debris like collembola or mites [62] or even by abrasion caused by digging mammals. Also, it is important to highlight the ability of earthworms to transport MPs through the soil from the surface to the bottom layers. This may result in increased MPs durability, in increased ability to reach groundwater which could potentiate negative effects in other aquatic environments and also in the fragmentation to nano-sized particles during the process leading to further environmental risks [64].

Nevertheless, secondary microplastics have the most diverse sourcesasthey result from the breakdown of large plastic objects [81,82].Some human activities promote microplastics' releaseinto the environment. These include for example inadequate industrial disposal of products after plastics' manufacture and their atmospheric transport [3] as well as abrasion in landfills and recycling facilities [40]. Also, general littering is produced from everyday objects, packaging, bottles, houseware items, wrappers, electronics and synthetic textiles[1,83-85]. Some of these secondary generated MPs will enter WWTPs, especially fibres from clothing [9,86] as previously explained. The application of sludges as fertilizers furthercontributes to the widespread occurrence of microplastics[65]. Besides, plastic mulches, polytunnels, polymer seed coatingsto control germination [87,88] and bale twines and wraps, containers and netting are important sources of microplastics to the terrestrial environment [8,89]. Furthermore, construction materials, artificial turf and household dust [72,90,91] are usually responsible for airborne microplastics [72]. Recently, it was found that cars' tires abrasion [92,93]and road surface markings paint[8] also leads to the formation of these particles. However, the atmospheric environment could include several particles from the most diverse sources due to resuspension [32]. In addition, weathering phenomena like storms and sewer overflows facilitate the dispersion of these tiny particles [8].

Recently, microplastics were detected in human food items. Several studies reported the occurrence of these particles in bivalves [38,45,94,95], fish [28,96-98], table salt [99,100] and drinks, including beer and tap water[101,102].

1.5. Toxicity

In spite of microplastics being considered inert for a long time, it is currently accepted that MPs have the potential to harm organisms [103-107].

The high surface area of MPs and their omnipresentnature are two fundamental characteristics responsible for cytotoxicity, oxidative stress, translocation to other tissues and chronic inflammation. On the other hand, MPs can be a constituent of particulate matter, being able to release some chemicals from their original matrices, to adsorb organic pollutants [108,109] or be part of vectors for hazardous microorganisms [110]. Consequently,it has been proposed that the incidence of immune and/or neurodegenerative diseases increases due to the contact with MPs [111].

The ability of MPs to adsorb organic contaminants is one of the main drivers of its toxicity. These contaminants include for example polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichlorodiphenyl trichloroethanes (DTTs), hexachlorocyclohexane (HCHs), dioxins and furans[112-115].

MPs can also accumulate virus and bacteria in their surface and thus facilitate infectionsbymicroorganisms[116]. This obviously increases the concern around these particles, since some potential pathogenic bacteria can multiply on the surface of MPs and trigger further effects which might be harmful not only for humans but also for other organisms [117,118].

In order to assess the state of the art on microplastics, a search was carried out on *Scopus* on 10 October 2019using the terms “microplastic” and “toxicity”. Only articles in the fields of pharmacology, toxicology and pharmaceuticalswere considered. A total of 70 research articles were retrieved. From the references of those articles, 3 more articles were included. The results are summarized in Figure 1 and Annex 7.1.

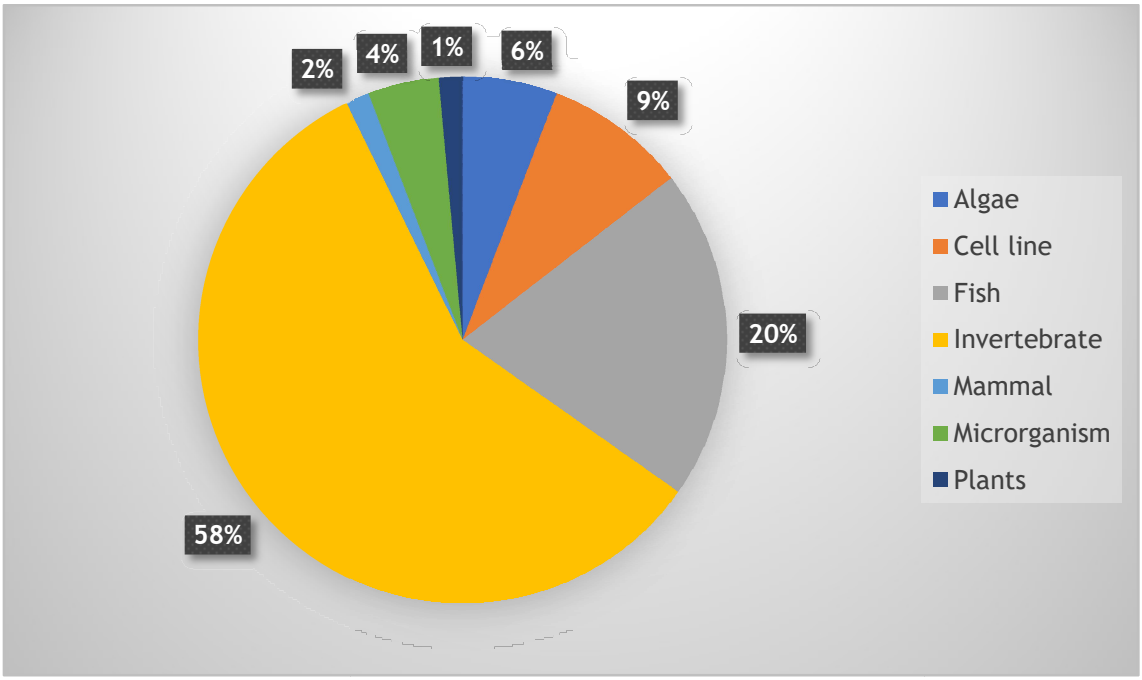


Figure 1. Graphical representation of the proportion of studies on the effects of MPs in the different classes of organisms. Scopus search performed on 10th October 2019 usingthe keywords "microplastic" and "toxicity".

A high number of studies analysedthe harmful effects in organisms, especially those living in the aquatic environment. Generally, the analysed effects include mortality, behaviour effects, genotoxicity and reproductive impairment [28,119-125]. In contrast, fewer studies focus on terrestrial organisms and even less in mammals.In mice, for example, it has been reported that polystyrene MPs have harmful effects [126], more specifically changes in composition and diversity of the gut microbiota (gut microbiota dysbiosis) which lead to several disorders, including abnormal physiological indicators and metabolic disorders [126,127]. Hence, these changes lead to microbial pathogenesis of rheumatoid arthritis [128] and bypass graft infection and bacteremia[129].

1.6. Human exposure and health impacts

Exposure to microplastics occursthrough three different pathways: ingestion, inhalation and dermal contact [130].

Ingestion commonly includes food contaminated with microplastics (bivalves, fishes, tap water, beers and salt, for example). It is estimated that just from the consumption of bivalves a European could be exposed to 11,000 microplastics per year [45]. But settling dust also represents a major intake of these particles and should be considered as it may be more significant than ingestion from food [95]. Although limited research is available, a study in mice reported that particles (fine particles <2.5 μm in diameter and coarse particles with <10 μm

in diameter) may reach the gastrointestinal system, leading to an alteration of the inflammatory response conditions and changes in the gut microbiome composition and metabolism [131].

Inhalation is derived from the release and resuspension of microplastics with low density in air and from the abrasion of large plastics[32]. Around 272 microplastics are expected to be inhaled per day bya male individual[132]. However, this value may varyasthere are many factors that can influence sampling including cleaning schedules, activities, furniture materials and season[5]. Some reports analysed workers from para-aramid, polypropylene, polyester and nylon flock facilities and no clear evidence of increased cancer risk was registered, although they hada higher prevalence of respiratory irritation, coughing, dyspnoea and reduced lung capacity, leading tointerstitial lung disease [133-136].

Dermal exposureis suspected to occur only for particles below 100 nm as they can pass through the dermal barrier [130]. Such particles include monomers and additivesof plasticsbeingthe endocrine disruptors bisphenol A and phthalates the most common ones. Human exposure to MPs also occursdue to the use of cosmetic products and toothpaste scrubbing. Some of thoseparticles, due totheirsize, can penetrate into the skin, enter in the circulatory system and end up in the liver and kidneys[137]. Some studies have been performed in order to evaluate the potential effects of dermal exposure. *In vivo*experiments with mice and rats exposed toPVC microplastics with stabilizers and plasticizers incorporated demonstrated that exposure could result in degeneration and necrosis[138].Also, cell viability and oxidative stress of PE and PS microplastics were evaluatedin human epithelial cells, being confirmed that oxidative stress is one of the mechanisms of cytotoxicity [139].

Chapter 2

2. Objective

Microplastics are widespread in the environment. However, there are limited studies focusing on microplastics in the indoor environment. This work aims to develop an integrated strategy to study microplastics in the indoor environment. For that,the levels of microplastics in indoor air and dust samples were assessed and the toxicity of the most common microplastics towards human cell lines was evaluated. Furthermore, given the limitations of the currently available techniques for MPs quantification, a new method for sample treatment and MPs isolation and purification from dust samples is proposed.

Chapter 3

3. Materials and Methods

3.1. Quantification of Microplastics from indoor samples

The quantification of microplastics in the indoor environment was performed using two different matrices: air samples, and house dust samples. For indoor air samples, two different sampling campaigns were performed in the same house.

3.1.1. Indoor air

3.1.1.1. First sampling campaign

3.1.1.1.1. Sample collection

The first air samples were collected between 21 and 28 March 2019 from a house with four adults (ages between 22 and 55 years old) located in Teixoso, Covilhã, Portugal. Sampling was performed in the kitchen andin a bedroom. One unlidded petri dish was placed on each room at 1.2 m height since it is the breathing height of an adult [30] and another petri dish was maintained sealed, in order to serve as a field blank. After seven days, petri dishes were retrieved and sealed with parafilm. Special attention was paid to possible contamination issues by microplastics and therefore no plastic materials were used during sampling.

3.1.1.1.2. Sample treatment

In the laboratory, the glass Petri dishes were washed with Milli-Q water directly to avacuum filter system equipped with a hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma). Each filter was treated with 20 mL of hydrogen peroxide (H_2O_2, 30% w/v; Fisher Scientific) and keptat room temperature for 2 days in a 250 mL glass Erlenmeyer. This eliminates biological materials, such as lipids, chitin or lignin, and prevents overestimation of MPssincethese compounds are also stained by Nile Red and consequently fluoresce under the fluorescence microscope [46].Then, the filters were thoroughly rinsed with Mili-Q water and removed from the flask. The remaining solution was vacuum filtered into a new filter membrane (10.0 µm pore size, 25 mm diameter; Sigma) while rising the flask walls. Finally, the filter was stored inside a sealed glass petri dish and leftto dry for 24h at room temperature before microscopicanalysis.

3.1.1.2. Second sampling campaign

3.1.1.2.1. Sample collection

Sample collection was performed between 26 of April and 3 of May 2019 in the same house as described in 3.1.1.1.1.In this second campaign, all the sampling preparation and treatment procedureswere performed inside a vertical laminar flow chamber. For this campaign,we selected only one room- the kitchen- as it registered the highestdeposition of microplastics. This stead, four Petri dishes were placed (two blank fields) at 1.2 m height as described in 3.2.1.1.1.

3.1.1.2.2. Sample treatment

The Petri dishes were washed with Milli-Q water directly to a vacuum filter system equipped with a hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma). Contrary to the previous treatment, a density separation procedure was performed in order to separate microplastics that float in a NaCl solution [4749]. The separation was achieved using a NaCl solution (d=1.2 $g.cm^{-3}$) prepared by dissolving 130 g of NaCl powder (VWR; purity >99%) in 500 mL of Milli-Q water. The filter membrane with the sample was placed in a beaker with 100 mL of NaCl solution,shaken by hand for 10 s and then left for sedimentation for 24 h. After that, the top phase (supernatant) was removed (with a glass 10 mL pipette) and vacuum filteredthrough a hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma). Next, a digestion (or "oxidation" as referred to by some authors) step was performed as described in 3.2.1.1.2.A vacuum filtration with a new hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma) was then performed. The filter was stored inside a sealed petri dish and leftto dry for 24h at room temperature before microscope analysis.

3.1.2. House dust

3.1.2.1. Sample Collection

The house dust sample was collected atthe same house from which air samples were obtained. The vacuum cleaner bag routinely used by the participants to vacuum the entire house was retrieved. The whole bag was taken to the laboratory, where the dust sample was sieved through a 63 µm mesh, as this is the size of particles that easily adhere tohands and thus are likely to be ingested by humans[140]. The sieved samples were stored in glass vials.

3.1.2.2. Sample Treatment

For the house dust sample, thedigestion was performed before density separation with a ratio of 16.78 mL of H_2O_2 per gram of dust.

3.1.3. Microplastics visualization with Nile Red

The fluorescent Nile Red dye was used for labelling microplastics in order to perform their identification through confocal light microscopy. The dye stock solution was prepared by dissolving 10 mg of Nile red powder (N3013; Sigma) in 10 mL of methanol (≥ 99.9%; Sigma). The stock solution was preserved in 2mL aliquots at -20°C under dark conditions. A fresh working solution of 1 $\mu g.ml^{-1}$ was prepared every day prior to the confocal microscopy analysis. For the analysis, 2-3 drops of Nile red solution were carefully added to each membrane filter using a Hamilton syringe with a stainless-steel needle. Then, filters were placed on glass microscope slides and were kept for 10 min in the dark. The imaging was performed in anAxio Observer Z1 microscope with Axio cam MRm camera andmetal halide arc lamp. The Nile Red fluorescence of the microplastics wasperformedona hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma) in green fluorescence using 552 and 636 nm as excitation and emission wavelengths as described byErni-Cassola, et al. [46]. The acquired images were taken in five random fields at 10x.

The Nile Red staining step was performedina vertical laminar flow chamber for the third sampling campaign, as described in 3.2.1.3.

3.1.4. Fluorescence quantification

The identification and quantification of fluorescent particles was performed in ImageJ software (version1.8.0) using a macro that performed the following tasks: (1) set the scale, (2) subtract the background using a rolling ball radius of 1500 pixels, (3) convert images to 8bit, (4) adjust black and white thresholds using 9 and 175 as the lower and higher values of pixel brightness, respectively, and (5) quantify particles based on area (400 $-\infty \mu m^2$). This limit was set to avoid the quantification of filter membrane´s pores (10 µm). The pores are detected by the software (Figure 2) and therefore they need to be discarded from fluorescence quantification [46]. The details of the macro used are provided inAnnex 7.2.

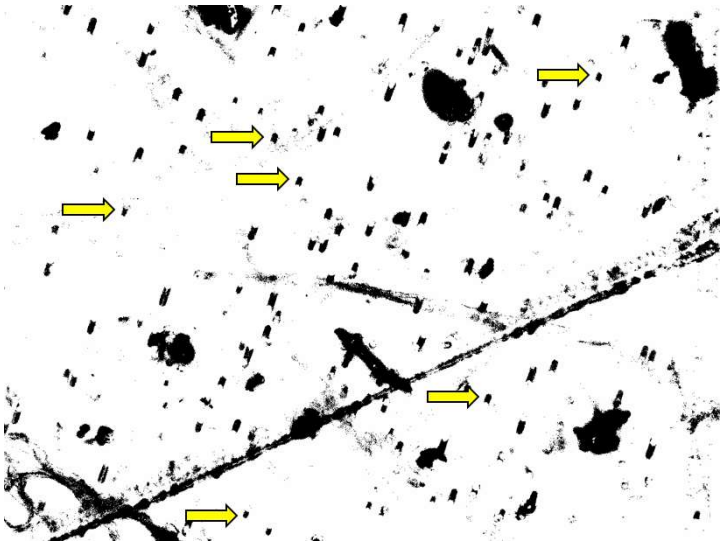


Figure 2 Examples of hydrophilic polycarbonate membrane pores.

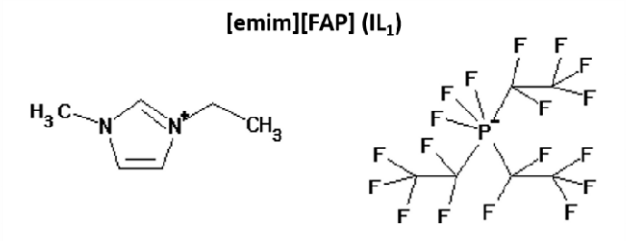
3.2. Alternative method for sample treatment and density separation

A novel method for sample treatment and density separation of microplastics from the dust matrix was developed using ionic liquids.

3.2.1. Ionic Liquids

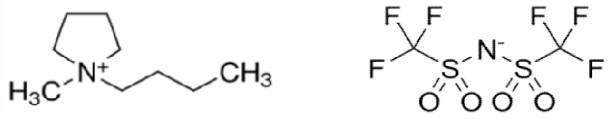
The ILs tested were 1-ethyl-3-methylimidazolium tris(pentafluoroethyl)trifluorophosphate, [C_2mim][FAP] (purity >99%) acquired from Merck and 1-butyl-3-methylpyridinium bis(trifluoromethylsulfonyl)amide, [C_4mpy][NTf_2] (purity > 99%) from Iolitec. The chemical structures of both ILs are presented in Figure 3. The purity of the ILs was checked by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and found to be in agreement with the purity given by the suppliers. Both ILs are liquid at room temperature and are non-water soluble at 25ºC. These ILs were chosen based on their densities: [C_2mim][FAP] presents a density of 1.71 $g.cm^{-3}$ and [C_4mpy][NTf_2] has a density of 1.41 $g.cm^{-3}$.

[emim][FAP] (IL_1)



1-ethyl-3-methylimidazolium tris(pentafluoroethyl)trifluorophosphate

[C4mpy][NTf2] (IL_2)



1-butyl-3-methylpyridinium bis(trifluoromethylsulfonyl)amide

Figure 3. Chemical structures of the Ionic Liquids (IL) used.

3.2.2. Screening tests

To understand the potential of ILs to separate the different microplastics it was necessary to perform a "macroscopic evaluation" of the system with the purpose of accessthe interactions between house dust, microplastics, ILs and H_2O.The screening tests started with an evaluation of the components in a "single" phase system (without mixing ILs and H_2O) and then a biphasic system was established through a combination of ILs (non-water miscible) and H_2O.

3.2.2.1. "Single" phase systems

3.2.2.1.1. House dust

At first, the behaviour of dust in the presence of ILs and H_2O was evaluated. Approximately, 50 mg of house dust (<63 µm particle size) were added to the system constituted by 500 µL of each IL or 500 µL of H_2O. Then, in order to reduce the amount of house dust sample to a minimum (important aspect for epidemiological surveys), the amount of house dust was reduced to 5 mg (<63 µm particle size) and the same systems were created, e.g., house dust with 500 µL of each IL or 500 µL of H_2O.After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for "single" phase systems of house dust tested are summarized in Table 1.

Table 1. "Single" systems of house dust combinations studied for initial screening.

Sample code	MPs	House Dust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
S-50H-W	✗	✓ 50 mg	✗	✗	✓
S-50H-1	✗	✓ 50 mg	✓	✗	✗
S-50H-2	✗	✓ 50 mg	✗	✓	✗
S-5H-1	✗	✓ 5 mg	✓	✗	✗
S-5H-2	✗	✓ 5 mg	✗	✓	✗

3.2.2.1.2. Microplastics

The behaviour of each MPs (PE, PVC, PA) was tested individually and together in thepresence of the two different ILs. Approximately, 1 mg of each microplastic were added to the system constituted by 500 µL of each IL. After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for “single” phase systems of MPs tested are summarized in Table 2.

Table 2. "Single" systems of microplastics combinations studied for initial screening.

Sample Code	MPs	HouseDust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
S-PE-1	PE	✗	✓	✗	✗
S-PVC-1	PVC	✗	✓	✗	✗
S-PA-1	PA	✗	✓	✗	✗
S-PE-2	PE	✗	✗	✓	✗
S-PVC-2	PVC	✗	✗	✓	✗
S-PA-2	PA	✗	✗	✓	✗
S-MP-1	PE, PVC, PA	✗	✓	✗	✗
S-MP-2	PE, PVC, PA	✗	✗	✓	✗

3.2.2.1.3. Microplastics and house dust

The combination of house dust (5 mg; <63 µm particle size) and MPs (PE, PVC, PA) was studied in the presence of the ILs. Each microplastic was tested individually and together with house dust. The amount of house dust and MPs used were the same as described above, as well as the ILs. After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for "single" phase systems of MPs and house dust tested are summarized in Table 3.

Table 3. "Single" phase systems combinations studied for initial screening.

Sample Code	MPs	HouseDust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
S-MP-5H-1	**PE, PVC, PA**	✔ 5 mg	✔	✘	✘
S-MP-5H-1	**PE, PVC, PA**	✔ 5 mg	✘	✔	✘

3.2.2.2. "Biphasic" systems

3.2.2.2.1. House dust

The behaviour of house dust in the presence of a combination of ILs and H_2O was evaluated. Approximately, 5 or 50 mg of house dust (<63 µm particle size) was added to the system constituted by 500 µL of each IL and 500 µL of H_2O. After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for "biphasic" phase systems of house dust tested are summarized in Table 4.

Table 4. "Biphasic" systems of house dust combinations studied for initial screening.

Sample Code	MPs	HouseDust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
B-5H-1-2	✗	✓ 5 mg	✓	✓	✗
B-5H-1-W	✗	✓ 5 mg	✓	✗	✓
B-50H-1-W	✗	✓ 50 mg	✓	✗	✓
B-5H-2-W	✗	✓ 5 mg	✗	✓	✓
B-50H-2-W	✗	✓ 50 mg	✗	✓	✓

3.2.2.2.2. Microplastics

The behaviour of each MPs (PE, PVC, PA) in the presence of the two different ILs and H_2O was tested individually and in the mixture. Approximately, 1 mg of each MPs were added to the system constituted by 500 µL of each IL and 500 µL of H_2O. After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for “biphasic” phase systems of MPs tested are summarized in Table 5.

Table 5. "Biphasic" systems of microplastics combinations studied for initial screening.

Sample Code	MPs	HouseDust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
B-PE-1-W	PE	✗	✓	✗	✓
B-PVC-1-W	PVC	✗	✓	✗	✓
B-PA-1-W	PA	✗	✓	✗	✓
B-PE-2-W	PE	✗	✗	✓	✓
B-PVC-2-W	PVC	✗	✗	✓	✓
B-PA-2-W	PA	✗	✗	✓	✓
B-MP-1-W	PE, PVC, PA	✗	✓	✗	✓
B-MP-2-W	PE, PVC, PA	✗	✗	✓	✓

3.2.2.2.3. MPs and house dust

The combination of house dust (5 mg; <63 µm particle size) and MPs (PE, PVC, PA) was studied in the presence of the two different ILs and H_2O. Each MPs was tested individually and in mixture with house dust. The amount of house dust and MPs used were the same as described above as well as ILs and H_2O. After mixing, all systems were centrifuged at 4000 rpm for 5 min.

The different combinations tested for "biphasic" phase systems of MPs tested are summarized in Table 6.

Table 6. "Biphasic" systems combinations studied for initial screening.

Sample Code	MPs	HouseDust	IL_1 ([C_2mim][FAP])	IL_2 ([C_4mpy][NTf_2])	H_2O
B-PE-5H-1-W	**PE**	✔ 5 mg	✔	✘	✔
B-PVC-5H-1-W	**PVC**	✔ 5 mg	✔	✘	✔
B-PA-5H-1-W	**PA**	✔ 5 mg	✔	✘	✔
B-PE-5H-2-W	**PE**	✔ 5 mg	✘	✔	✔
B-PVC-5H-2-W	**PVC**	✔ 5 mg	✘	✔	✔
B-PA-5H-2-W	**PA**	✔ 5 mg	✘	✔	✔
B-MP-5H-1-W	**PE, PVC, PA**	✔ 5 mg	✔	✘	✔
B-MP-5H-2-W	**PE, PVC, PA**	✔ 5 mg	✘	✔	✔

The different steps for IL treatment and MPs density separation are summarized in Figure 4.

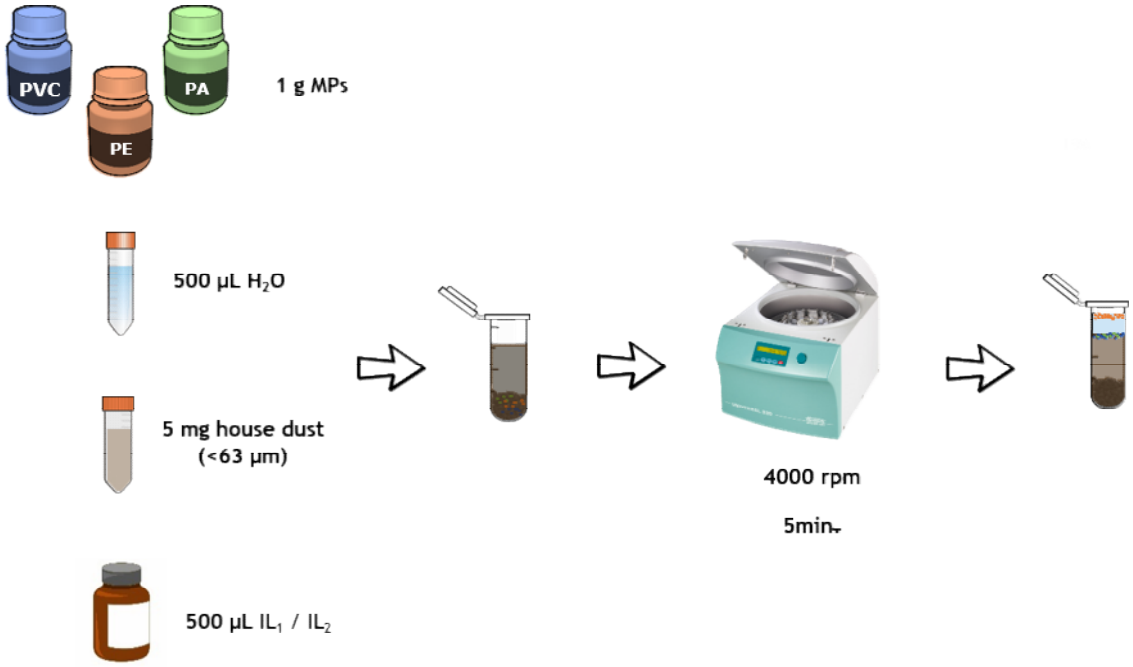


Figure 4. Schematic representation of the steps involved in ionic Liquid treatment and density separation procedures.

3.2.3. Optimization tests

After the establishment of the ideal conditions, a two-step method was performedin order to separateand extract microplastics from house dust samples. In this two-step approach, the ionic liquid [C_4mpy][NTf_2] was used in the first step and H_2O in the second one.Two different approaches were used: the first one using 1.5 mL polypropylene tubesand the second one using decantation ampoules.

3.2.3.1. Trial 1

After the initial experiments described in 3.2.2. a two-step procedure to separate and extract MPs present in house dust samples was carried out. Firstly, 5 mg of house dust was added to500 µL of [C_4mpy][NTf_2] (IL_2) in a 2 mL Eppendorf and centrifuged at 4000 rpm for 5 min to allow the separation of MPs from the house dust matrix. Secondly, the recovered MPs in the surface of the IL were added to 500 µL of H_2O in order to fractionate the MPs according to their densities.In addition, the same procedure was performed with 3 mgof PE, PVC and PA to confirm the behaviour of these MPs along withthe various steps.Figure 5 describes the overall process.

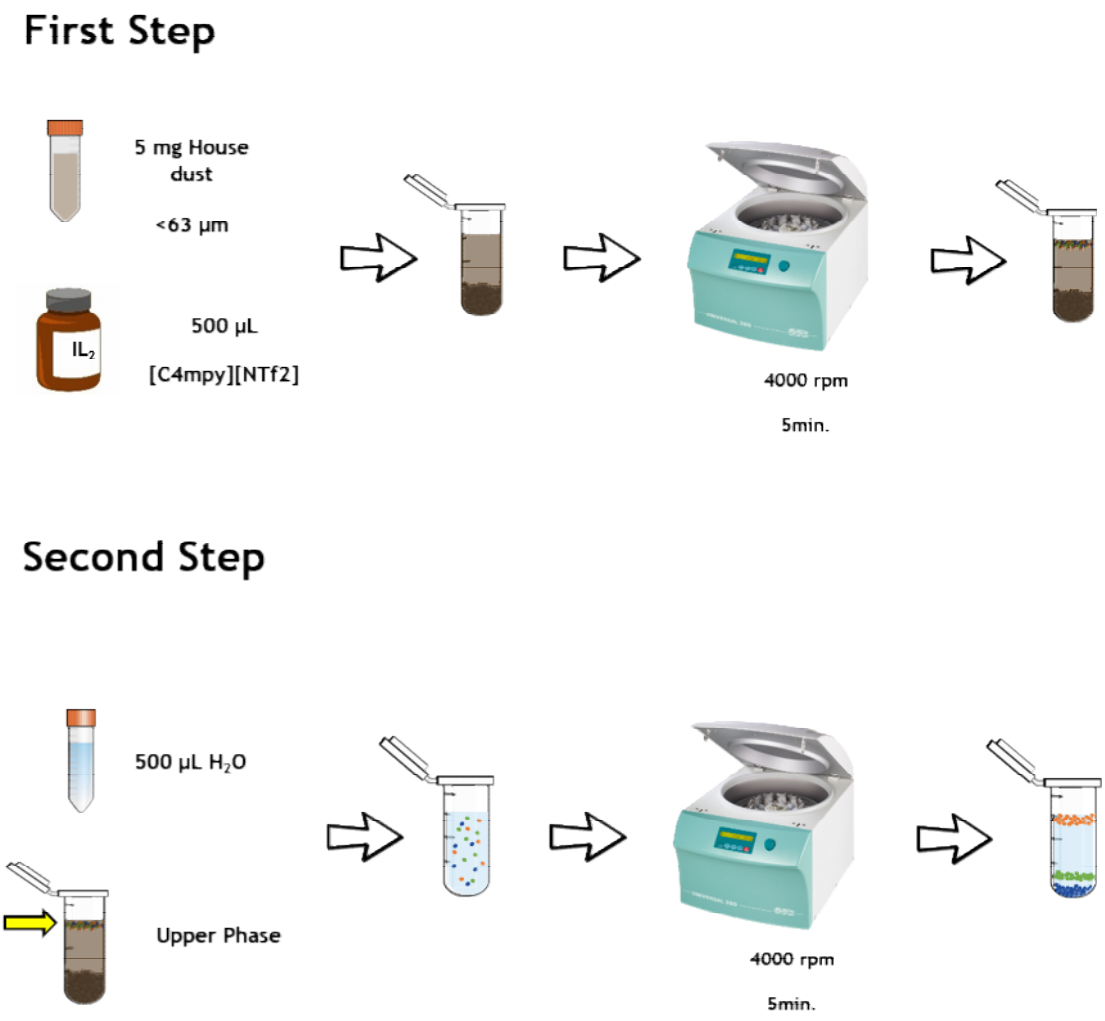


Figure 5. Optimization of the separation and isolation of microplastics with 1.5 polypropylene tubes using IL treatment and density separation.

3.2.3.2. Trial 2 (Ampoules)

In order to improve the recovery of the topphase at the start of the second step and the extraction of each microplastic, the procedure described in 3.2.3.1. was carried out with10 mL decantation ampoules, as illustrated in Figure 6.

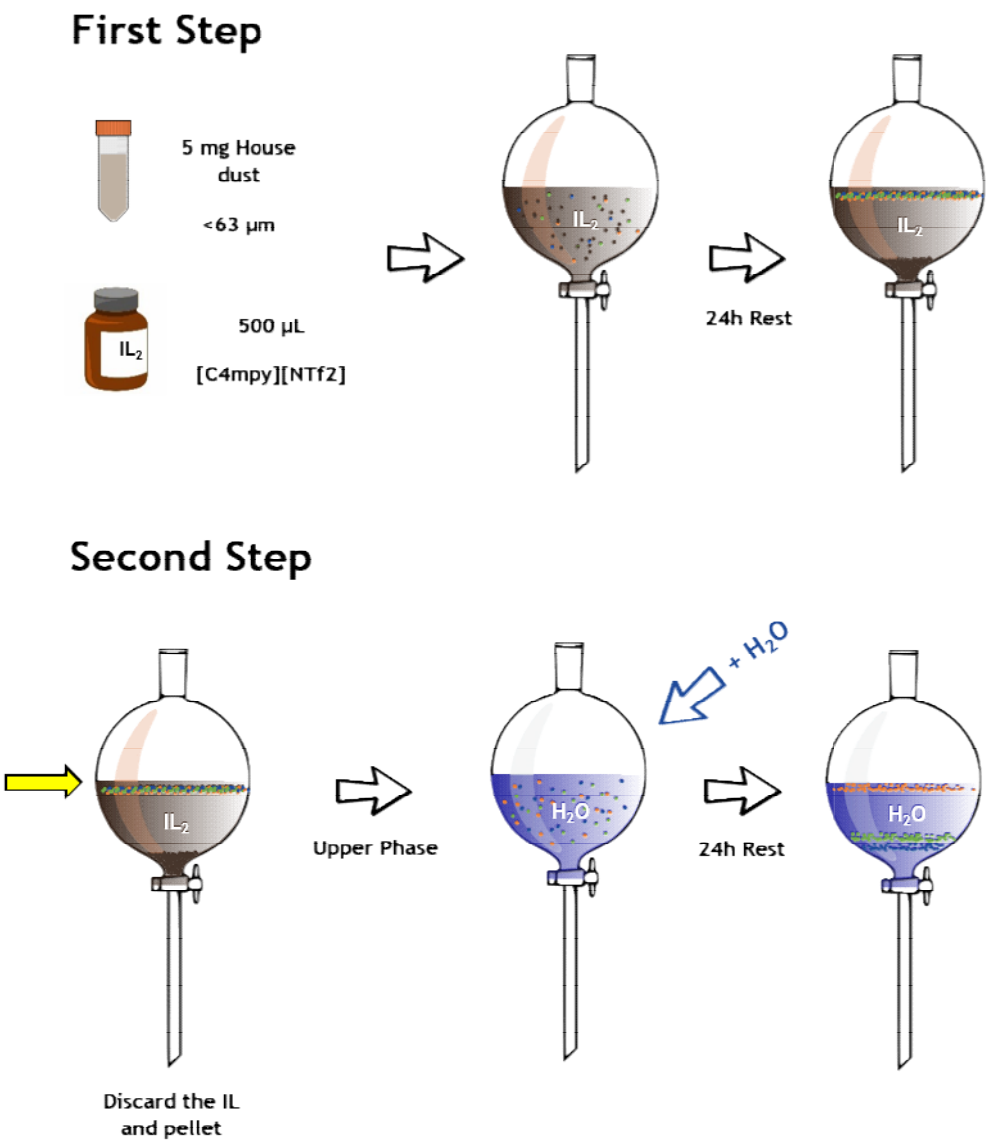


Figure 6. Optimization of the separation and isolation of microplastics with ampoules using IL treatment and density separation.

3.2.4. Micro-Raman Spectroscopy

To confirm the identity of the MPs (PE, PVC, PA) in the different phases, Micro-Raman spectroscopy was performed.This technique was chosen as it allows to differentiate polymers according to their spectral bands and by their size in the optic images. In addition, Raman spectroscopy shows better resolution (down to 1 mm), wider spectral coverage, higher sensitivity to non-polar groups, lower interference and narrower spectral bands than Fourier-transform infrared spectroscopy (FTIR)[141].

The samples for Raman spectroscopy were collected during the steps described in 3.2.3. (tubes and ampoules) and vacuum filtered through hydrophilic polycarbonate membranes

(10.0 µm pore size, 25 mm diameter; Sigma). The filters were stored inside a sealed petri dish until Raman analysis.

Also, controls of PE, PVC, PA and house dust were prepared by mixing into 1 mL of Milli-Q water and then vacuum filtered through the same hydrophilic polycarbonate membranes (10.0 µm pore size, 25 mm diameter; Sigma). Moreover, the ionic liquid ($[C_4mpy][NTf_2]$; IL_2) and hydrophilic polycarbonate membraneswere also analysed.

The samples from the step described in 3.2.3.1. (Trial 1 - 1.5mL tubes) and 3.2.3.2. (Trial 2 - 10 mL ampoules) were analysed by Raman spectroscopy using a combined Raman-AFM confocal microscope WITec alpha300 RAS+ (WITec, Ulm, Germany) with an Nd:YAG laser operating at 532 nm with the power set between 4.0 mW and 7.5mW. Samples were observed using a 100x and 50x objectives and each Raman spectrum was acquired with an integration time of 2s, 10 acquisitions each. The Raman maps were created by acquiringa Raman spectrum in each pixel, in a total of 22500 spectra, using different areas (between 22500 μm^2 and 122500 μm^2) by integrating over specific Raman bands using WITec software for data evaluation and processing. The different polymers were identified in the Raman images by integrating the area of a specific Raman band for each polymer, namely 1130 and 2860 cm^{-1} for PE, 2940 cm^{-1} for PVC and 1641 and 2917 cm^{-1} for PA. The Raman spectra of the different polymers were used as the basis set in the analysis using the software tool of WITec Project, providing the colour-coded combined Raman images.

3.3. Evaluation of microplastics cytotoxicity

3.3.1. Preparation of Microplastic solutions

All solutions were prepared inside a vertical laminar flow chamber to prevent contamination. Only the weighing of microplastics was performed outside the laminar flow chamber. Based on previous published data the following range of concentrations was selected: 0.01; 0.1; 1; 10; 100; 1000 $\mu g.ml^{-1}$[142]. A stock solution of each MP was prepared in NaCl at a concentration of 100 000 $mg.L^{-1}$. This stock solution was prepared by mixing 100 mg of each microplastic (PE-Polyethylene (PE powder; Sigma), PVC-PolyvinylChloride (PVC powder; Sigma) and PA-Polyamide (Nylon-12 powder; Sigma)) in 1 mL of NaCl solution (9 mg. mL^{-1}; 2.25 g NaCl dissolved in 250 mL of Milli-Q water) and was preserved at 4ºC under dark conditions. Thesecond stock of MPs of 10 000 mg.L-1 was prepared in cell culture medium by adding 200 µL of the first stock to 1.8mL of culture medium. Working solutions (final volume 2 mL) were prepared by successive dilutions of 200 µL in 1.8 mL of culture medium (0.01; 0.1; 1; 10; 100; 1000; 2000 and 4000 $\mu g.ml^{-1}$).

3.3.2. Cell culture

3.3.2.1. Caco-2 cells

The human colon carcinoma cell line (Caco-2) was grown in DMEM-HG medium (Sigma) supplemented with 10% FBS (fetal bovine serum) and 1 mL.L^{-1} pen/strep (10 U/mL penicillin and 10 µg/ml streptomycin; Gibco) to promote cell growth and prevent cell culture microorganism contaminations. Cells were cultured in cell culture dishes 100mm x 20mm (Corning) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, Caco-2 cells were trypsinized with trypsin/EDTA solution (0.004 g trypsin in 0.1 g EDTA dissolved in 500 mL of PBS) at approximately 90-100% of confluence. These cells were plated in a 96-well plate with 1x10^4 cells/well density. After 24 h, cells(P35, P36 and P37 for PE and PVC; P37, P38 and P39 for PA)were treated with different concentrations of Polyethylene (PE powder; Sigma), Polyvinyl Chloride (PVC powder; Sigma) and Polyamide (Nylon-12 powder; Sigma).

3.3.2.2. HepG2 cells

The human hepatocellular carcinoma cell line (HepG2) was grown in HepG2 expansion media (Cellular Engineering Technologies) supplemented with 10 %FBS (fetal bovine serum) and 0.1 mL. L^{-1} pen/strep (100 U/mL penicillin and 100 µg/ml streptomycin; Gibco) to promote cell growth and prevent cell culture microorganism contaminations. Cells were cultured in 75 cm^2 bovine collagen-coated culture t-flask (Cell Applications, Inc.) and maintained at 37°C in a humidified atmosphere of 5% CO^2 and 95% air. For the experiments, HepG2 cells were trypsinized with trypsin/EDTA solution (0.004 g trypsin in 0.1 g EDTA dissolved in 500 mL of PBS) at approximately 70-80% of confluence. These cells were plated in collagen-coated 96-well plates (Zenbio) with a density of 1x10^4 cells/well. After 48 h, cells(P1, P2 and P3)were treated with different concentrations of Polyethylene (PE powder; Sigma), Polyvinyl Chloride (PVC powder; Sigma) and Polyamide (Nylon-12 powder; Sigma).

3.3.2.3. N27 cells

The immortalized rat mesencephalic dopaminergic cell line (N27) was grown in RPMI 1640 medium (Sigma) supplemented with 10% FBS (fetal bovine serum) and 1 mL.L^{-1} pen/strep (10 U/mL penicillin and 10 µg/ml streptomycin; Gibco) to promote cell growth and prevent cell culture microorganism contaminations. Cells were cultured in cell culture dishes 100mm x 20mm (Corning) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, N27 cells were trypsinized with trypsin/EDTA solution (0.004 g trypsin in 0.1 g EDTA dissolved in 500 mL of PBS) at approximately 70-80% of confluence. These cells were plated in a 96-well plate with 1x10^4 cells/well density. After 24 h, cells(P31, P32 and P33 for PE and PVC; P33, P34 and P35 for PA)were treated with different concentrations of

Polyethylene (PE powder; Sigma), Polyvinyl Chloride (PVC powder; Sigma) and Polyamide (Nylon-12 powder; Sigma).

3.3.3. Cell treatment with microplastics

In order to evaluate the cytotoxic effect of Polyethylene (PE powder; Sigma), Polyvinyl Chloride (PVC powder; Sigma) and Polyamide (Nylon-12 powder; Sigma) each cell line (Caco-2,HepG2 and N27)was exposed to different concentrations of these compounds for 24 h. Each concentration was tested in five replicates of three independent experiments (n=3).

3.3.4. Cell viability

Cell viability was evaluated after exposure by cell counting kit-8 (CCK-8; Dojindo Molecular Technologies). Cell counting kit-8 allows to determine the number of viable cells in cell proliferation and cytotoxicity assays. This kit uses highly water-soluble tetrazolium salt, the WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]), that produces a water-soluble formazan dye after its reduction. WST-8 is reduced by dehydrogenases in cells and gives origin to an orange product, which is capable to be dissolved in the culture medium. After exposure to PE, PVC and PA solutions, the culture medium was removed and 5 µL of CCK-8 solution plus 95 µL of culture mediumwas added to each well. The N27,Caco-2 and HepG2 cells were kept in the incubator with 5% CO_2 at 37°C, protected from light, for 2h, and then the absorbance was measured at 450 nm in a microplate spectrophotometer (xMArkTM Microplate Absorbance Spectrophotometer, BIO-RAD). Cell viability was calculated based on the relative absorbance compared with the control group (unexposed cells).

Chapter 4

4. Results and Discussion

4.1. Microplastics in environmental samples

4.1.1. Indoor Air

4.1.1.1. First sampling campaign

The amount of particles collected in each petri dish are presented in Table 7. The kitchen was the location with the highest mass of particles collected (2.3 mg against 0.9 mg in the bedroom). However, some particles were also foundin the blank (0.1 mg).

Table 7. Mass of particles collected during the first sampling campaign.

Sampling 1	Mass (g)		
	Initial	Final	Difference
Blank	63.2844	63.2845	0.0001
Bedroom	63.0121	63.0130	0.0009
Kitchen	62.9865	62.9888	0.0023

4.1.1.1.1. Confocal microscopy

After Nile Red staining, the samples were observed in the wide-field microscope. For each sample, five pictures were taken in different spots. The images were processed in ImageJ software, following the steps: 1) Image >Color> Channels ToolMore > Green; 2) Image > Adjust > Brightness/Contrast > Set > Minimum displayed value: 125 Maximum displayed value: 302; 3) Image > Type > RGB; 4) Analyse > Tools > Scale bar.

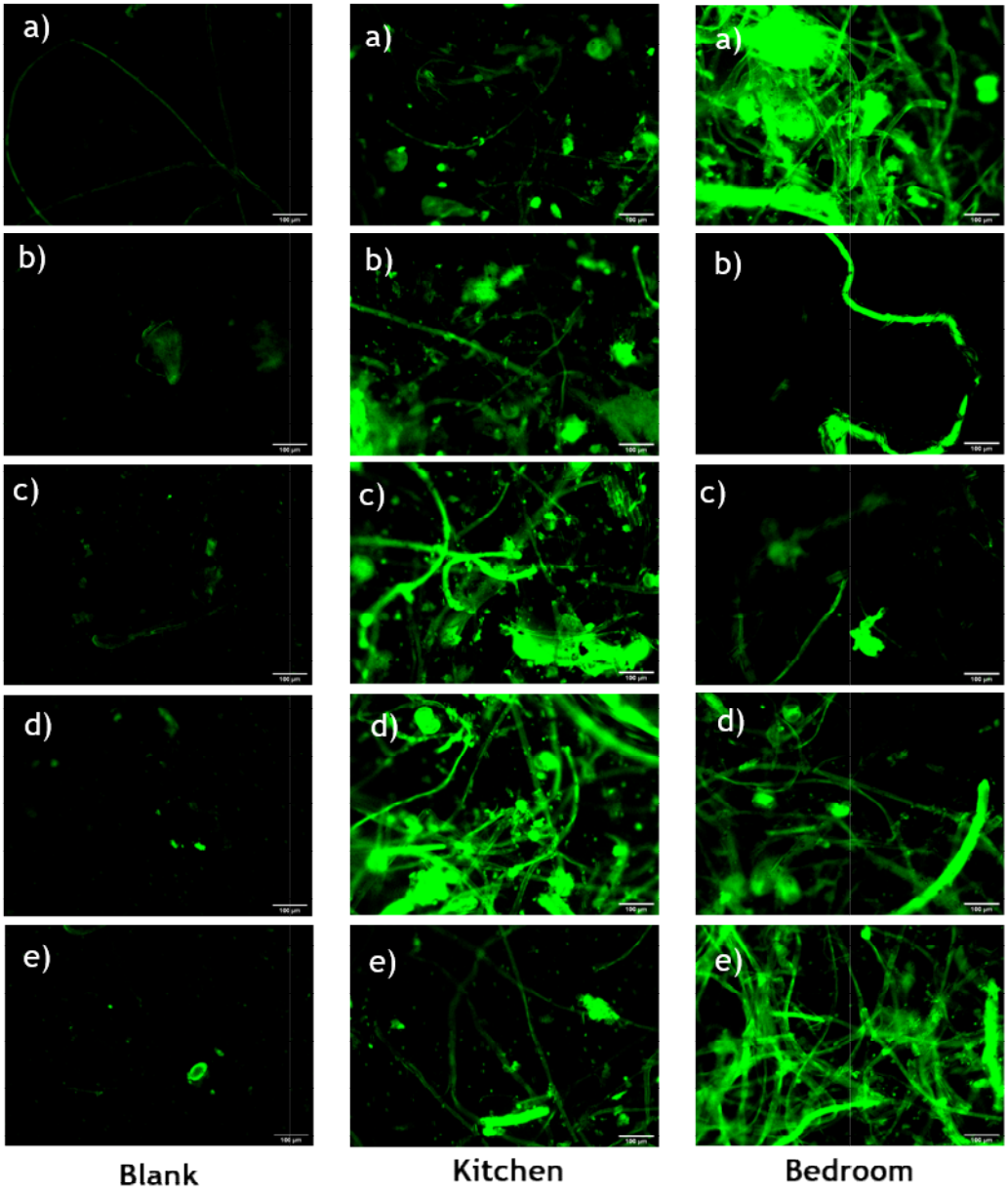


Figure 7.Images of the samples from the first campaign stained with Nile Red and processed in ImageJ software.

As it can be seen from Figure 7, some microplastics (particles) were observed in the blank. However, the amount of microplastics are almost negligible when compared with the ones observed in the kitchen sample (Figure 7 (middle)) and in the bedroom (Figure 7 (right)).It is also notorious from the figures that in the kitchen there are mostly particles with some fibres, whereas in the bedroom sample there are mainly fibres.

It should be stressed that, within the same sample, a large variation between the 5 replicates exists (e.g. Kitchend) versus e) or bedroom a) versus c)).

4.1.1.1.2. Fluorescenceintensity

For fluorescence quantification, the macro described in Annex 7.2 was run and the values of intensity were obtained for each replicate in the different samples analysed and are depicted in Figure 8. Also, a box plot graph was done (Figure 9).

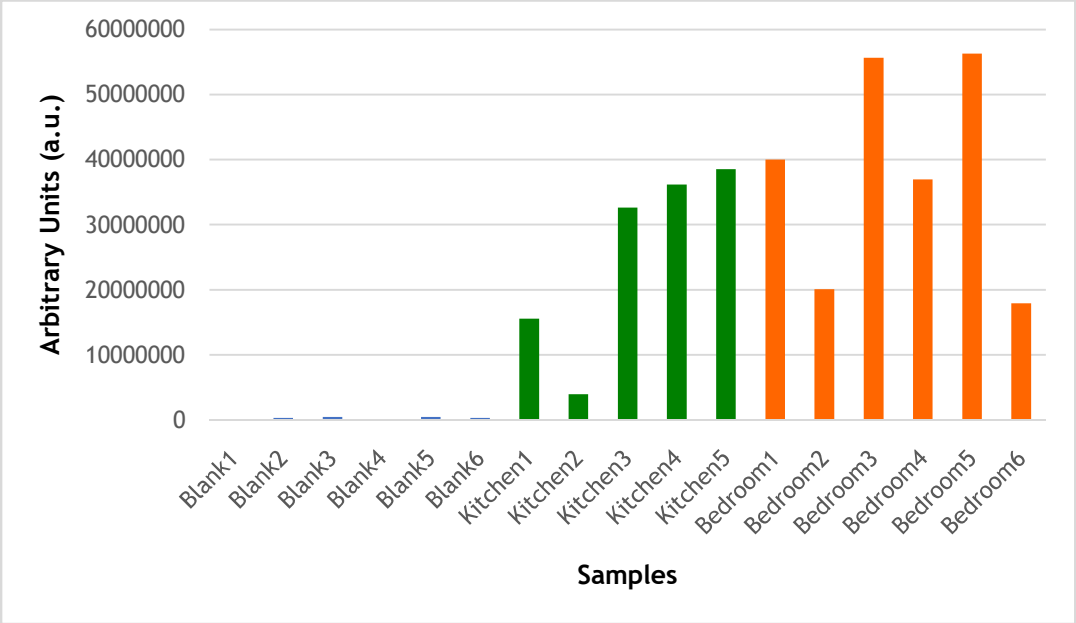


Figure 8. Fluorescence intensity calculated with ImageJ software for the first sampling campaign in the blank (blue bars), the kitchen (green bars) and the bedroom (orange bars).

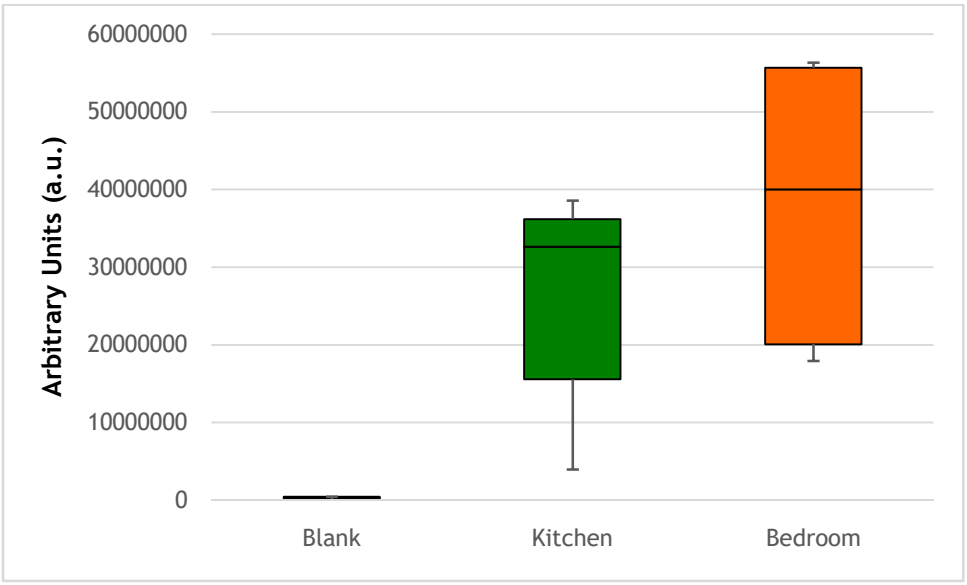


Figure 9. Box plot of the fluorescence intensity calculated with ImageJ software for the first sampling campaign.

The obtained results (Figure 8 and 9) demonstrate that the highest amount of fluorescence was obtained in the bedroom ($5.63x10^7$ ±$1.56x10^7$ a.u.) which contrasts with the fact that the highest amount (in grams) of particles was obtained for the kitchen (2.3 mg against 0.9 mg in the bedroom). This might be due to the fact that in the bedroom the MPs were essentially fibers (Figure 7 Bedroom e)) and in the kitchen, a higher amount of particles were found (Figure 7 Kitchen a)). Also, comparing the values for fluorescence intensity, the kitchen registered more consistent values (ranging from $3.93x10^6$ to $3.85x10^7$a.u.) between the various samples than the bedroom (ranging from $1.79x10^7$ to 5.63 $x10^7$ a.u.). Furthermore, more hydrophobic synthetic polymers and lower molecular weight ones such as PE, PP, PS and nylon (which are commonly used by the textile industry) exhibit higher fluorescence. On the other hand, high molecular weight polymers like PVC, PET, PC and PUR produce less fluorescence due to lower hydrophobicity and are mostly used in packaging, sheets and utensils [1,46] that are commonly used in the kitchen.

4.1.1.2. Second sampling campaign

The amount of particles collected in the kitchen are described in Table 8. It is notorious that, this time, the amount of particles collected was much lower (0.0001 mg) when compared with the first sampling campaign(2.3 mg), despite the fact that the sampling duration was the same (8 days).This happened because the house surveyed was cleaned in the day thatthe Petri dishes were removed and thus some particles might have been resuspended.Also, it is also notorious that, by working inside the vertical laminar flow chamber it was possible to have no particles in the field blank.

Table 8. Weighing of Petri dishes before and after the second sampling campaign.

Sampling 2	Mass (g)		
	Initial	Final	Difference
Blank	63.3695	63.3695	0.0000
Kitchen	63.3438	63.3439	0.0001

4.1.1.2.1. Confocal microscopy

The same protocol described in 4.1.1.1.1. was used and the images obtained in the microscope after Nile red staining are depicted in Figure 10, blank on the left, kitchen sample in the middle and the hydrophilic polycarbonate membranein the right. Overall, no contamination is observed in the filter membrane (Figure 10, (right)) whereas in the blank it is possible to visualize some MPs. This contrasts with the results previously described for the amount of particles detected in the field blank (0.0 mg). Thus, that contamination may come

from the sample´s handling during the treatment processes, which has one more step (density separation) than the previous campaign. This extra step increases the probability of particle deposition and contamination through laboratory items such as Milli-Q water container used to wash the petri dish into the filtration system[22]. In addition, the amount of particles in the kitchen sample was justslightly higher than the blank, beingthe occurrence of some fibers crucial to differentiate these two samples. Furthermore, there is an important difference between the amount of particles identified in the kitchen sample of both sampling campaigns that was due to the low amount of particles collected (0.1 mg) in this sampling campaign as described before.

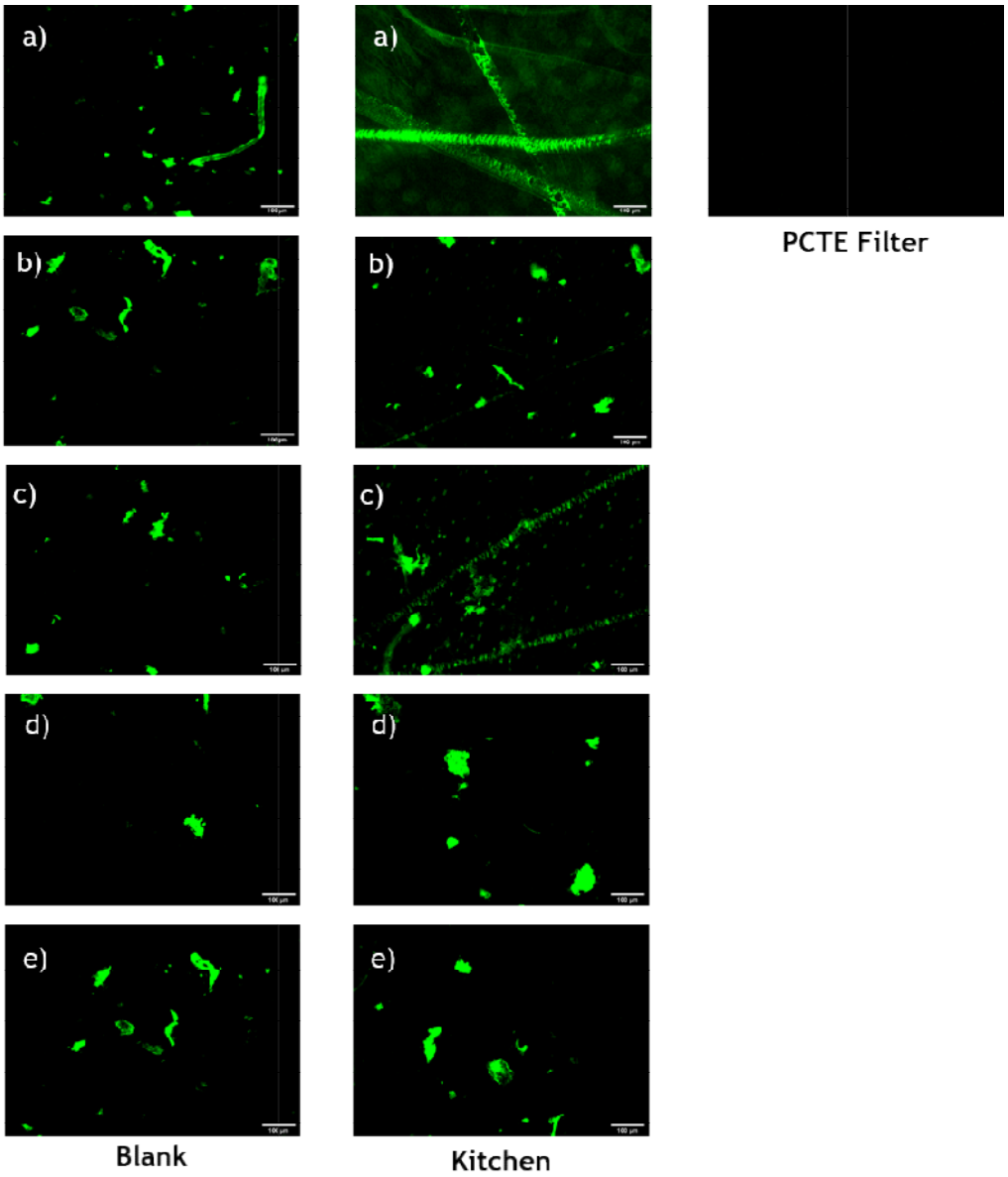


Figure 10. Images of the samples from the second campaign stained with Nile Red and processed in ImageJ software.

4.1.1.2.2. Fluorescenceintensity

The fluorescence intensity results obtained using the same protocol as the one described in 4.1.1.1.2. are depicted in Figure 11 and 12. Although the blank sample showed relatively low fluorescence intensity, it is noticeable the contamination with the maximum value of 5.17 $x10^6$ ± 1.32 $x10^6$a.u., as already mentioned above (4.1.1.2.1.). In fact, the intensity of Blank4E sample slightly exceeds the value registered for the Kitchen4E. Furthermore, in this sampling campaign, the maximum value for fluorescence in the kitchen was half the value of the first sampling. Also, the fluorescence of PCTE filter was null, as already expected because there were no MPs detected as seen in Figure 10.

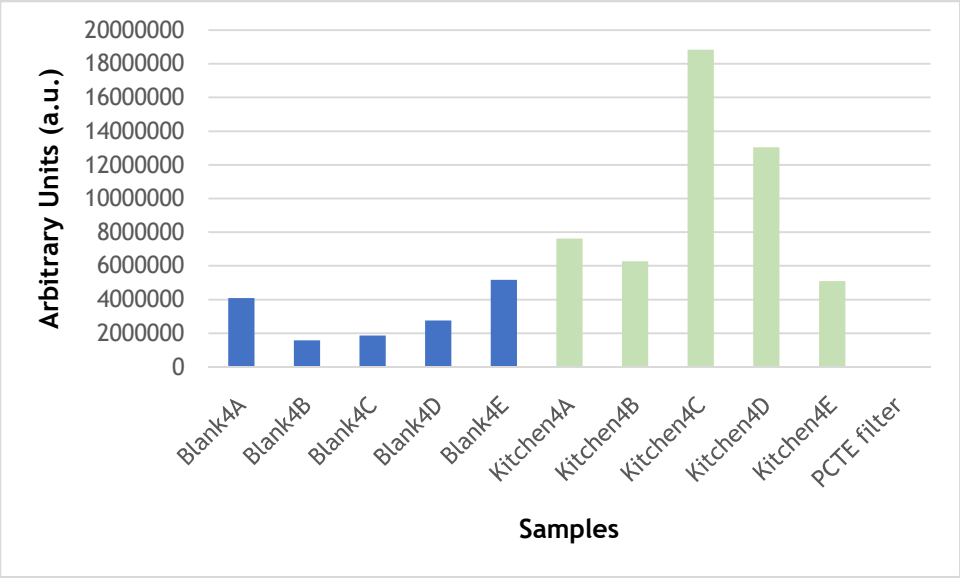


Figure 11. Fluorescence intensity calculated with ImageJ software for the second sampling campaign.

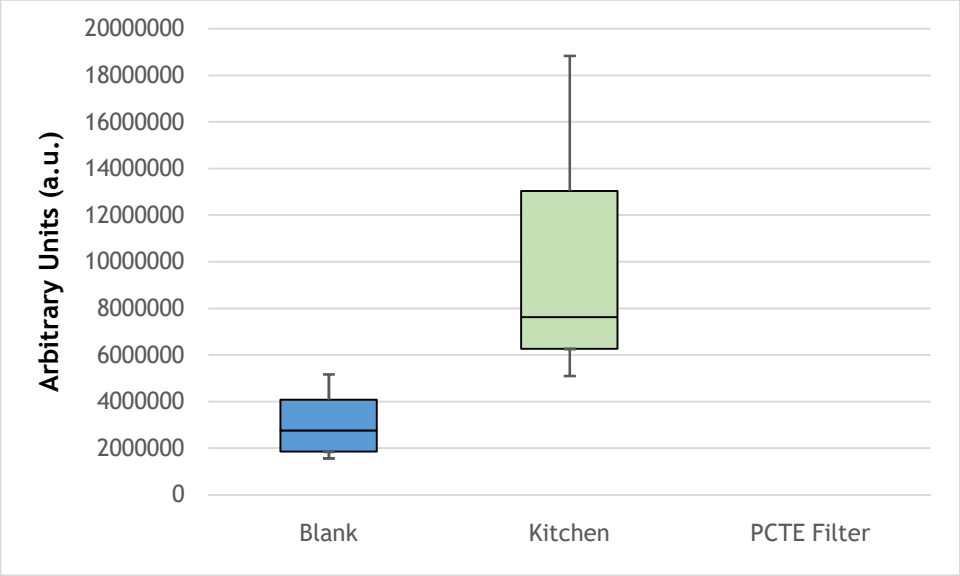


Figure 12. Boxplot of the fluorescence intensity calculated with ImageJ software for the second sampling campaign.

4.1.2. House Dust

Different amounts of house dust samples were treated with variable volumes of H_2O_2 in order to optimize these two parameters for future experiments as illustrated in Table 9. Indeed, given that only the fine fraction of dust is used (<63 µm) it is necessary a large amount of dustperform all the analysis and thus it is advantageous to use the minimum amount of sample possible. Also, the reduction of the number of reagents used turns the whole process less expensive and more sustainable.

Table 9. Combinations studied for H_2O_2 digestion.

Dust (g)	H_2O_2 (30%) (mL)
1.5 (<63 µm)	25.17
1.25 (<63 µm)	20.97
1.0 (<63 µm)	16.78
0.75 (<63 µm)	12.58
0.5 (<63 µm)	8.39

4.1.2.1.1. Confocal microscopy

The same protocol described in 4.1.1.1.1. was employed and the images obtained in the microscope after Nile red staining are depicted in Figure 13.By the analysis of this figure, it stands out that all samples are very homogeneous when compared toeach other even between 0.50 g and 1.50 g samples (e.g. 0.50 g b) and 1.50 g c)). Also, it is notorious from the figures that the samples are mostly particles without fibers, contrary to what was found previously (in air-born samples) and reported by Liu, et al. [91] where fibers accounted for 88.0% of dust samples taken in the bedroom and living room. This may be related to the density separation step that forced the fibers to settle in the bottom of the beaker because of their size and high density, being discarded soon after (only the top phase is analysed). In addition, there is no evidence that this sample had synthetic fibers.However, this is very uncommon [30,90,91].

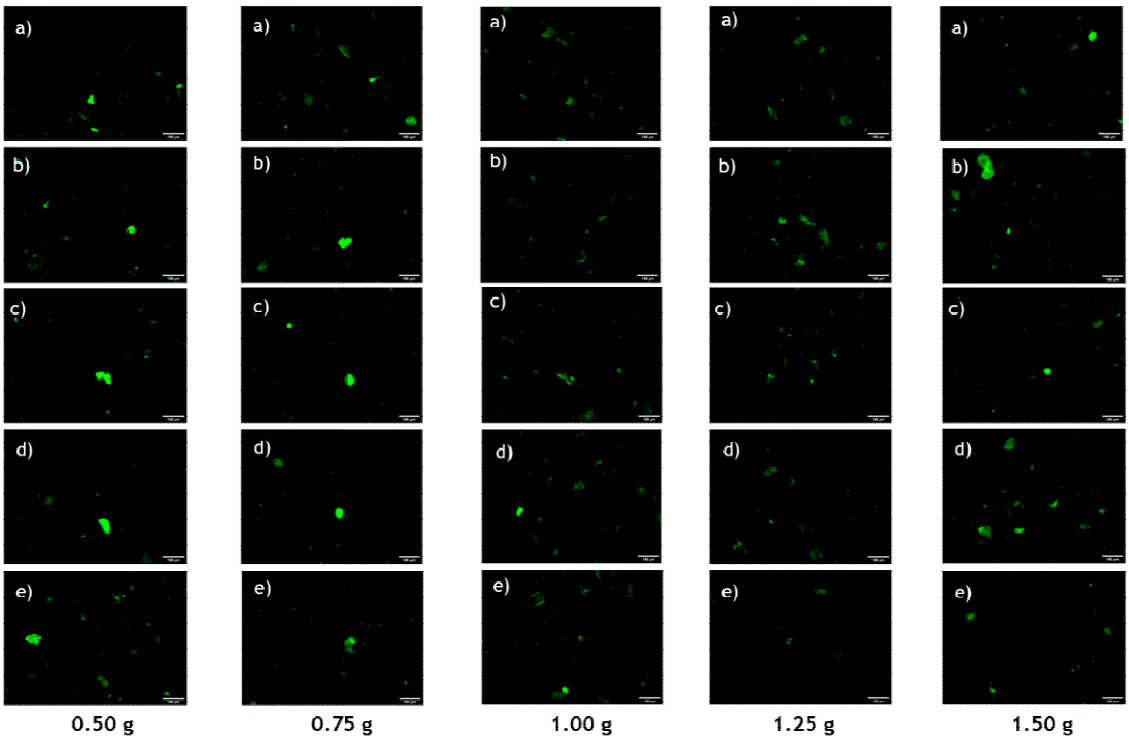


Figure 13. Images of the <63 µm dust samples at different concentrations stained with Nile Red and processed in ImageJ software.

4.1.2.1.2. Fluorescenceintensity

The fluorescence intensity results for house dust samples were obtained using the same protocol as described in 4.1.1.1.2. and are shown in Figure 14 and 15.

Alarge discrepancy in the values registered for the same sampleis perceptible (e.g. Sample1d and Sample1e or Sample3b and Sample3d). Hence, a higher amount of house dust sample is not reflected in higher values of fluorescence intensity. On the other hand, the intensity of the fluorescence is directly correlated to the type of polymer present in the sample as previously mentioned in 4.1.1.1.2. For example, more hydrophobic polymers such as PE, PP, PS, and nylon-6 are much more fluorescent than PUR, PC, PET, and PVC as described by other authors [46].

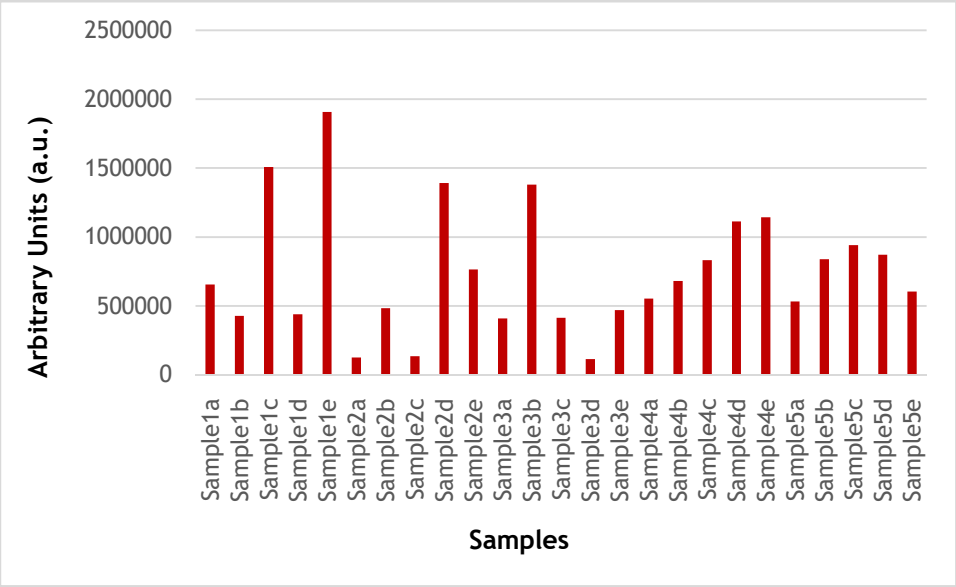


Figure 14. Fluorescence intensity calculated with ImageJsoftware for house dust samples. Sample 1: 1.5 g; Sample 2: 1.25 g; Sample 3: 1.00 g; Sample 4: 0.75 g; Sample 5: 0.50 g.

The results obtained demonstrate a high variability of fluorescence in the samples 1, 2 and 3 (1.5 g; 1.25 g and 1.00 g, respectively), while sample 4 and 5 (0.75 g and 0.5 g) were much more consistent. This suggests that 0.5 g of house dust might be appropriate for future studies because it uses a relatively low amount of sample without compromising the results (see figure 15).

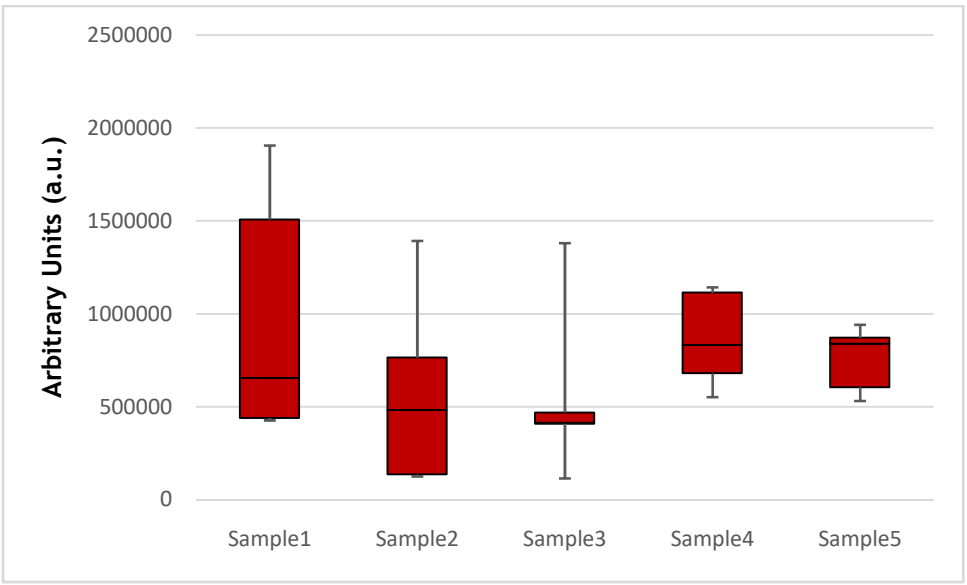


Figure 15. Boxplot of fluorescence intensity calculated with ImageJ software for house dust sampling.Sample 1: 1.5 g; Sample 2: 1.25 g; Sample 3: 1.00 g; Sample 4: 0.75 g; Sample 5: 0.50 g.

Overall, for air-born samples,a two-step treatment with density separation (NaCl)and oxidative digestion (H_2O_2) should not be performedbecause it could potentiate contamination byMPs present in the laboratory. Thus, samples only should be treated with H_2O_2, performing oxidative digestion of the lipids content. On the other hand, house dust samples need to be treated with both NaCl, for density separation, and H_2O_2, for oxidative digestion, because the sample´s matrix is much more complex and concentrates a lot of material that needs to be discarded before MPs analyses [143].

Also, the Nile Red technique proved to be efficient in MPs detection and quantification, however, it cannot identify the different MPs in the sample. Hence, this technique should be used as a preliminary approach to confirm the existence/non-existence of MPs and then resort to spectroscopy for MPs identification such as FTIR or Raman [47,86,141,144].

4.2. Ionic liquid-based method for sample treatment and density separation

4.2.1. "Single" phase systems

4.2.1.1. House dust

The behaviour of the dust in water and in each ionic liquid is shown in Figure 16. When mixed with water, the dust fraction completely sediments in the bottom of the test tube. When mixed with IL_1 ([emim][FAP]) two fractions of dust can be seen (Figure 16b) and c)). The separation of the dust sample into two fractions was also obtained with IL_2 ($[C_4mpy][NTf_2]$)(Figure 16 d) and e)).The separation was a result of the different density between house dust matrix particles, being expected to have MPs in the low density phase (top) and/or dispersed in the IL_2 ($C_4mpy][NTf_2]$).

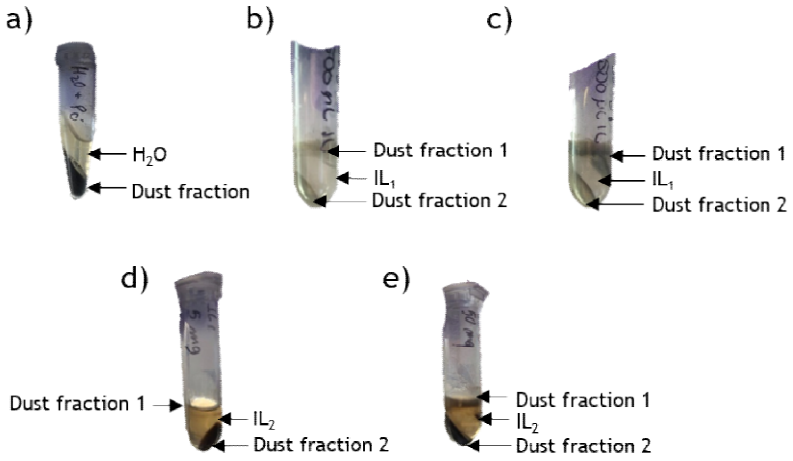


Figure 16. "Single" phase systems combinations for house dust, being a) S-50H-W; b) S-5H-IL_1; c) S-50H-IL_1; d) S-5H-IL_2; e) S-50H-IL_2 (S-Single phase; H-House dust;W-Water; IL_1-[emim][FAP]; IL_2-$[C_4mpy][NTf_2]$).

4.2.1.2. Microplastics

The behaviour of each MPs (PE, PVC and PA) in both ionic liquids is shown in Figure 17.Every MP stayed in the top phase of both ILs, which is in accordance with the densities of 0.94 $g.cm^{-3}$ for PE, 1.4 $g.cm^{-3}$ for PVC and 1.02 $g.cm^{-3}$ for PA.

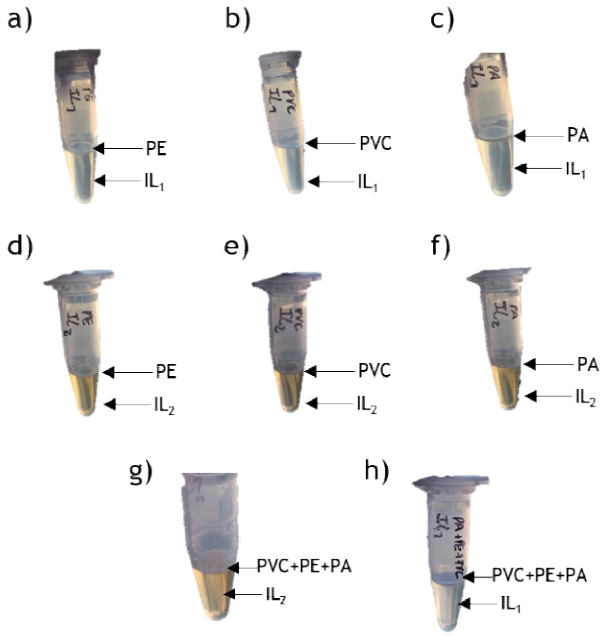


Figure 17. "Single" phase systems combinations for microplastics, being a) S-PE-IL1; b) S-PVC-IL1; c) S-PA-IL_1; d) S-PE-IL_2; e) S-PVC-IL_2; f) S-PA-IL_2 g) S-MP-IL_2 h) S-MP-IL_1 (S-Single phase; H-House dust; W-Water; IL_1-[emim][FAP]; IL_2-[C_4mpy][NTf_2]).

4.2.1.3. Microplastics and house dust

The behaviour of house dust deliberately contaminated with MPs (PE, PVC and PA) in both ionic liquids is shown in Figure 18.Two fractions of house dust can be seen, as described above (4.2.1.1.) with MPs present in the top fraction as also registered above (4.2.1.2.).

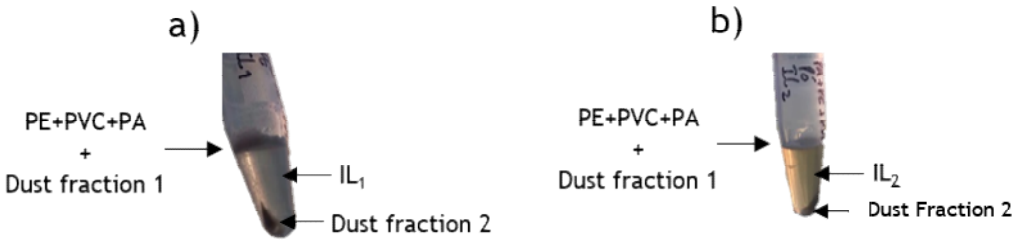


Figure 18."Single" phase systems combinations for microplastics, being a) S-MP-IL_1; b) S-MP-IL_2 (S-Single phase; H-House dust; W-Water; IL_1-[emim][FAP]; IL_2-[C_4mpy][NTf_2]).

4.2.2. "Biphasic" phase systems

4.2.2.1. House dust

The behaviour of house dust in the "biphasic" system is shown in Figure 19. The IL_1([emim][FAP]; d=1.71 g.cm^{-3}) and IL_2([C_4mpy][NTf_2]; d=1.41 g.cm^{-3}) when mixed merged into a single-phase system that we suspect to have an intermediate density (d=1.56 g.cm^{-3}) between both ILs as illustrated in Figure 19a). When it comes to [C_4mpy][NTf_2] and water, the behaviour of the system remains the same as described in 4.2.1.1. (two house dust fractions) (Figure 19a) and e)). However, that does not happen with[emim][FAP] and water, because a single house dust fraction was formed which is represented in Figure 19b) and c). Indeed, the organic content of house dust such as hair, dead skin cells and pollen [143] leads to a higher affinity to water which "forces" the dust to be in the interface between this two liquids and originates only one fraction of dust.

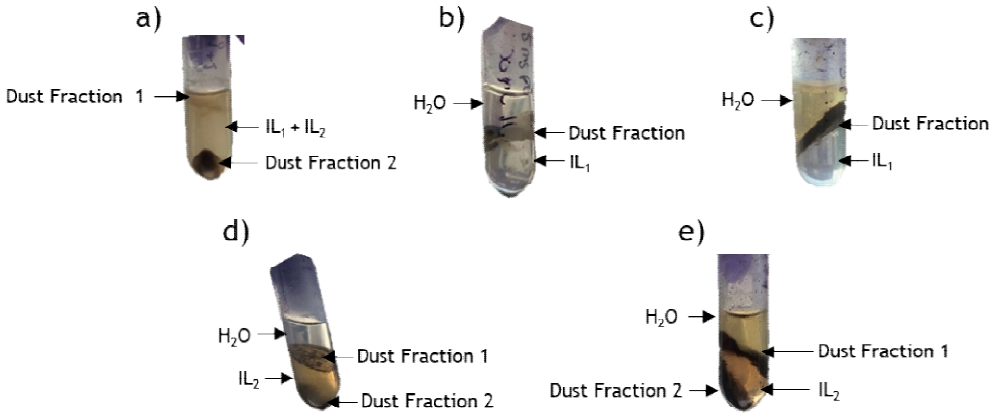


Figure 19. "Biphasic" systems combinations for house dust, being a) B-5H-IL_{1+2}; b) B-5H-IL_1-W; c) B-50H-IL_1-W; d) B-5H-IL_2-W; e) S-50H-IL_2-W (S-Single phase; H-House dust; W-Water; IL_1-[emim][FAP]; IL_2-[C_4mpy][NTf_2]).

4.2.2.2. Microplastics

The behaviour of MPs (PE, PVC and PA) in a "biphasic" system is shown in Figure 20. It is remarkable that all the MPs stayed in the interface between the two liquids which demonstrates their affinity towards the ILs. However, PE density is 0.94 g.cm^{-3} and it should be above the water phase, what doesn't happen (Figure 20a), d), g) and h)).

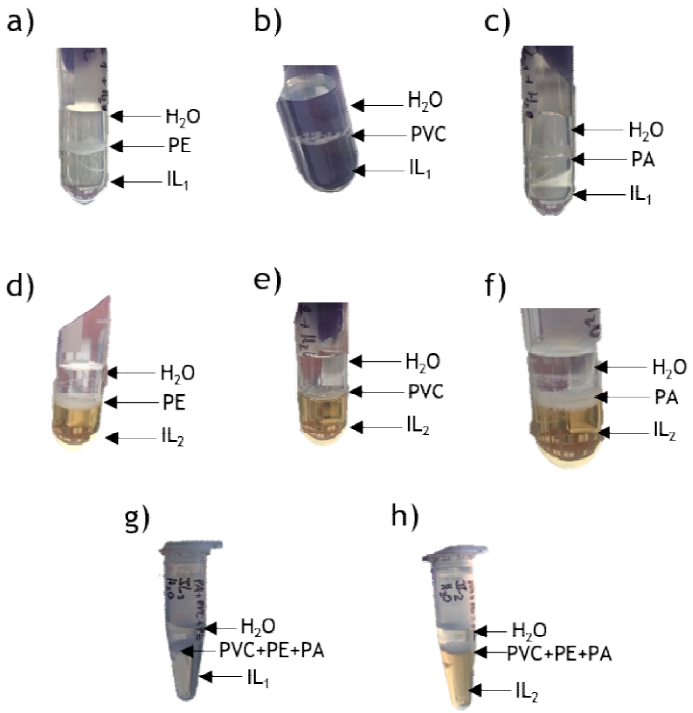


Figure 20. "Biphasic" systems combinations for microplastics, being a) B-PE-IL_1-W; b) B-PVC-IL_1-W; c) B-PA-IL_1-W; d) B-PE-IL_2-W; e) B-PVC-IL_2-W; f) B-PA-IL_2-W g) B-MP-IL_1-W h) B-MP-IL_2-W (S-Single phase; H-House dust; W-Water; IL_1-[emim][FAP]; IL_2-[C_4mpy][NTf_2]).

4.2.2.3. Microplastics and House dust

The behaviour of house dust deliberately contaminated with MPs (PE, PVC and PA) in "the biphasic" systems is shown in Figure 21.In [emim][FAP] samples, the house dust originates two different fractions and the MPs (PVC and PA) stayed in the interface (between ([emim][FAP] and water) (Figure 21 b) and c)), but PE stayed on the top of the system and only formed one house dust fraction (Figure 21a)). In [C_4mpy][NTf_2] samples, a single fraction was formed in the interface with a mixture of house dust and MPs (PVC and PA) (Figure 21e) and f)). However, PE, once again, was on the top of the system, as seen in Figure 20 d).

On the other hand, the mixtures with all MPs (PE, PVC and PA) originated a two-fraction separation Figure 21 g) and h) with the MPs in the interface.

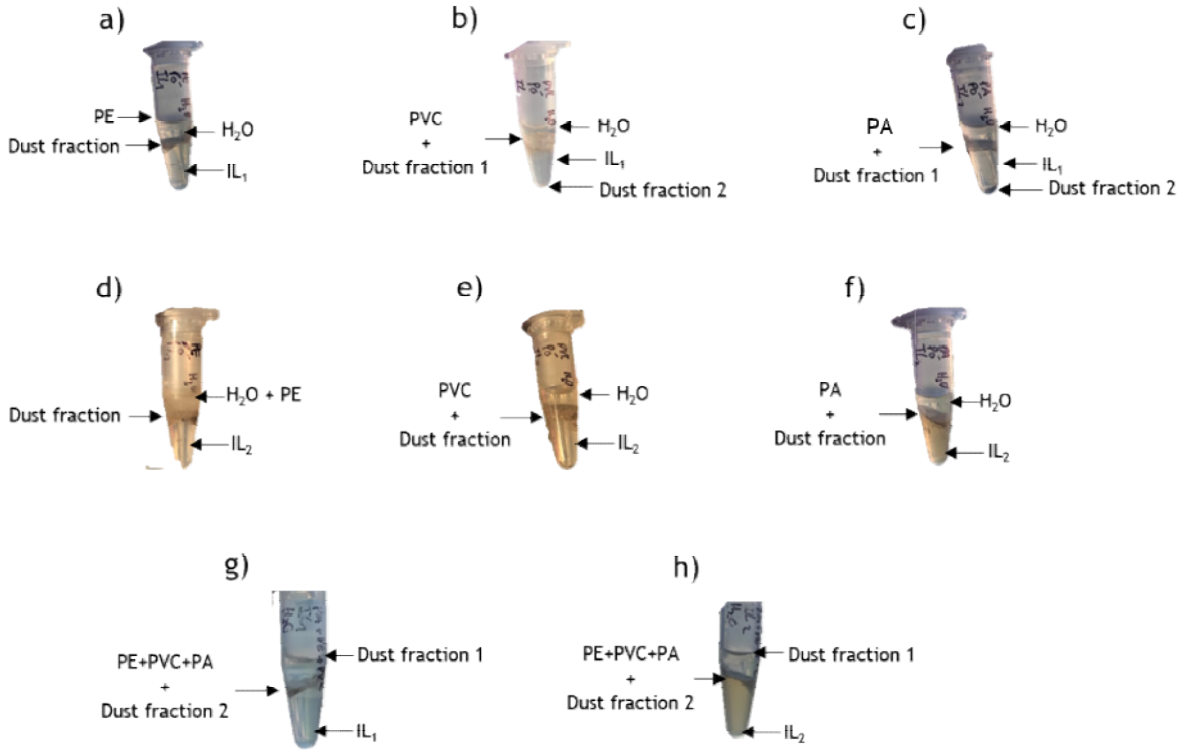


Figure 21."Biphasic" systems combinations for microplastics, being a) B-PE-5H-IL_1-W; b) B-PVC-5H-IL_1-W; c) B-PA-5H-IL_1-W; d) B-PE-5H-IL_2-W; e) B-PVC-5H-IL_2-W; f) B-PA-5H-IL_2-W g) B-MP-5H-IL_1-W h) B-MP-5H-IL_2-W (S-Single phase; H-House dust; W-Water; IL_1-[emim][FAP]; IL_2-[C_4mpy][NTf_2]).

These experiments above were performed to understand which systems are better to separate the MPs in house dust. Thus, the [emim][FAP] was discarded in future studies due to his density (1.71 g.cm^{-3}) being very high which doesn't add any benefit toMPs separation.

4.2.3. Raman Spectroscopy

4.2.3.1. Controls

4.2.3.1.1. Microplastics

Firstly, controls for PE, PVC, PA were analysed. The Raman spectra for each polymer were registered for further comparison with those obtained with ILs and real samples. The results are depicted in Figure 22 and Table 10.

The controls were used to obtain the characteristic Raman spectrum of each commercial MPs, in which different vibrational bands were selected for each polymer such as 1130 and 2860 cm^{-1} for PE, 2940 cm^{-1} for PVC and 1641 and 2917 cm^{-1} for PA (Their assignment are detailed in Table 10). The areas of these bands can be integrated, to create Raman maps. Then, leading to a clear identification of the MPs in the samples.

The microscopic optic image (left) in Figure 22 shows that PVC MPs are notable larger then PE and PA. Furthermore, we found some particles that are inherent to the hydrophilic polycarbonate membranes as shown in Figure 23.

Table 10. Raman bands obtained forPE, PVC and PApolymers.

Polymers	Raman (cm^{-1})	Assigment
PE[145]	1130	$\nu_{sym}(CC)$
	2848	$\nu_{sym}(CH_2)$
PVC[146]	2940	$\nu_{asym}(CH_2)$
PA[147]	1641	ν(C=O) and Amide I
	2917	$\nu_{sym}(CH_2)$

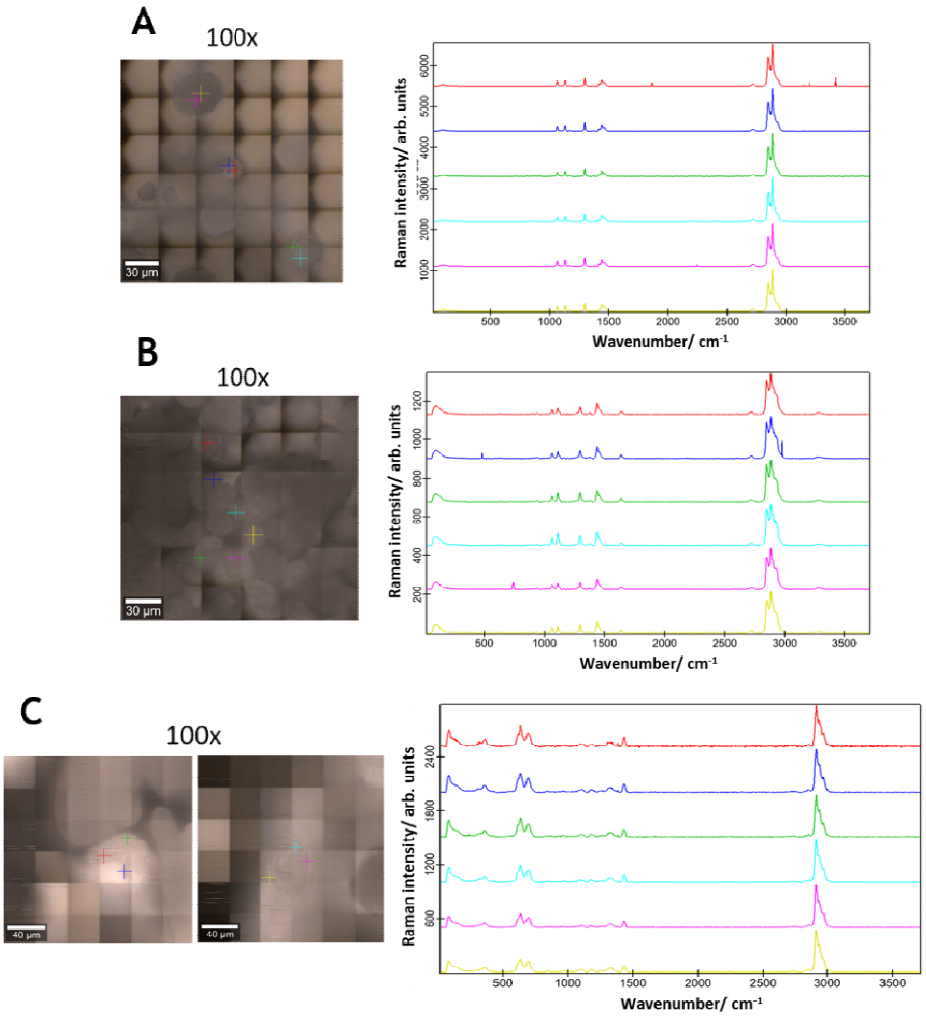


Figure 22. Stitching image (100x objective) and Raman spectra ofcommercial samples ofPE (A), PA (B) and PVC (C) particles.

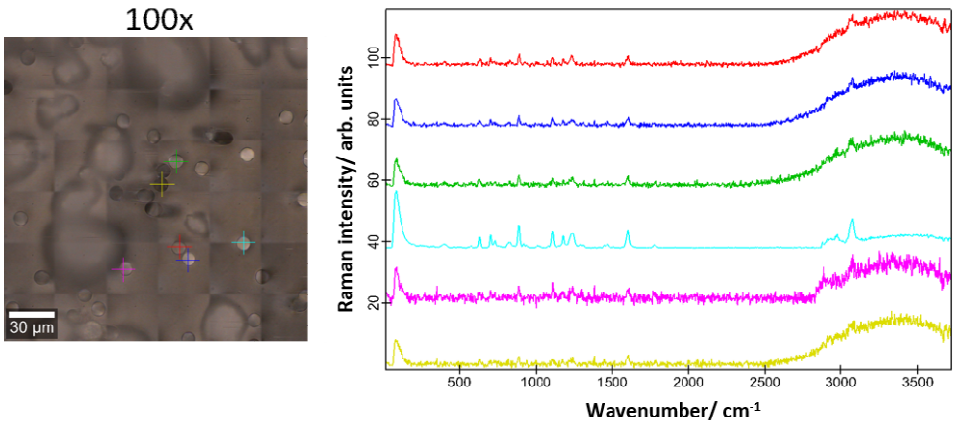


Figure 23. Stitching image and Raman spectra for particles inherent to the Hydrophilic polycarbonate membranes.

4.2.3.1.2. Untreated house dust sample

A non-treated house dust sample (<63 µm) was analysed as illustrated in Figure 26. It is clearly observable the occurrence of many different spectra that reflects the high complexity of this sample. For example, we interpretedthe cyan spectrum asstarch, the orange spectrum corresponding to amorphous carbon particles and the pink spectrum correspondingto calcium carbonate [143,148].

Furthermore, it should be stressed that house dust samples are very difficult and sensitive to analyse because of the high amount of organic material which requires the power of the laser to be low (7.5mW instead of the typical 15 mW).

Some studies have identified flame retardants (e.g. PBDEs; TDCIPP -Tris(1,3-dichloro-2-propyl)phosphate), proteins (e.g. keratin) and phthalates (e.g. DEHP - di(2-ethylhexyl)phthalate; BBzP - butyl benzyl phthalate) in house dust through Raman spectroscopy. However, none of these compounds or similar was found in this sample, at least in the selected area[149-153].

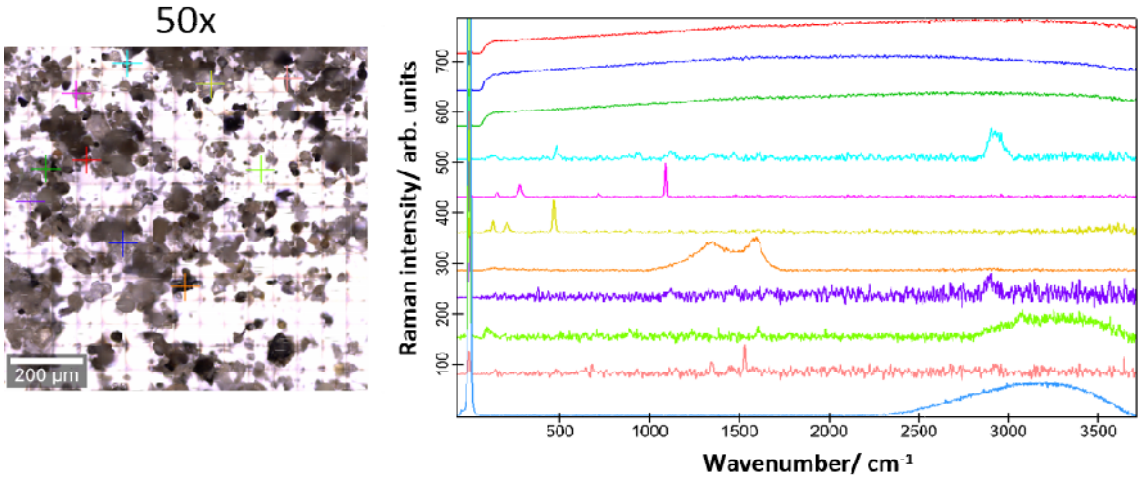


Figure 24. Stitching image and Raman spectraof control house dust.

4.2.3.2. Sample treatment with ILs (Trial 1)

In the first trial (with 1.5ml test tubes), twodifferent phases were analysed: i) the topphaseand ii) the bottom phase. According to the densities of each MP, we expectedto find PE in the topphase (d=0.94 g.cm^{-3}) and PVC and PA in the bottom phase (d=1.4 g.cm^{-3} and d=1.02 g.cm^{-3}, respectively).

At first, the samples with commercial MPs treated with [C_4mpy][NTf_2] and followed by density separation with Milli-Q waterwere analysed. For these samples,the Raman imaging and

spectra were registeredfor the two phases. Afterwards, real dust samples were studied. For real dust samples, the two phases were analysed and image-stitching and Raman spectra were obtained.

4.2.3.2.1. Commercial MPs

The top phase was extracted and vacuum filtered by hydrophilic polycarbonate membrane (10.0 µm pore size, 25 mm diameter; Sigma). From this filter, a specific area was selected and analysed by Raman imaging. The density of PE is 0.94 $g.cm^{-3}$ and therefore it was expected to obtain mainly PE in this sample, thus the characteristic band of PE assigned to the stretching vibration of the CC at 1135 cm^{-1} was monitored. However, other polymers were detected such as PA(blue Raman spectrum), PE (green Raman spectrum) and an unknown polymer (red Raman spectrum), which was not one of the commercial MPs used as control (Figure 25). According to PublicSpectra [148], this unknown polymer was identified aspolystyrene (PS)with 97.28% of similarity to a standard reference. The results disclose that all three MPs are found in the top phase as marked in green (PE), red (PS) and blue (PA), with PA being the most abundant one.

For the bottom phase of the sample deliberately contaminated with MPs, it was expected to obtain mainly PVC (1.4 $g.cm^{-3}$) and PA (1.02 $g.cm^{-3}$), thus those were the bands monitored (2940 cm^{-1} and 1641 cm^{-1}, respectively) (Figure 25).However, the presence of PE was also detected and may be due to the Milli-Q water washing step carried out at the end of the filtration step, which dragged out PE particles that were in the walls of the ampoule. From the combined Raman image, the presence of PE (green), PVC (red) and PA (blue) are clearly identified, with PA being the most abundant.

The results suggest that PA microplastics were resuspended along the 1.5 mL tube because their density (1.02 $g.cm^{-3}$) is similar tothat of water, used in the second-density separation. Hence, PA is dispersed which explains the great number of particles found. The amount of PE in the top phase unexpected since its density is lower than water (0.94 $g.cm^{-3}$) and thus there should be more PE MPs in that phase. We suspect that the PE adhered to the walls of the tube and thus was not quantified. The PS found was interpreted as contamination caused by handling during the sample preparation process.

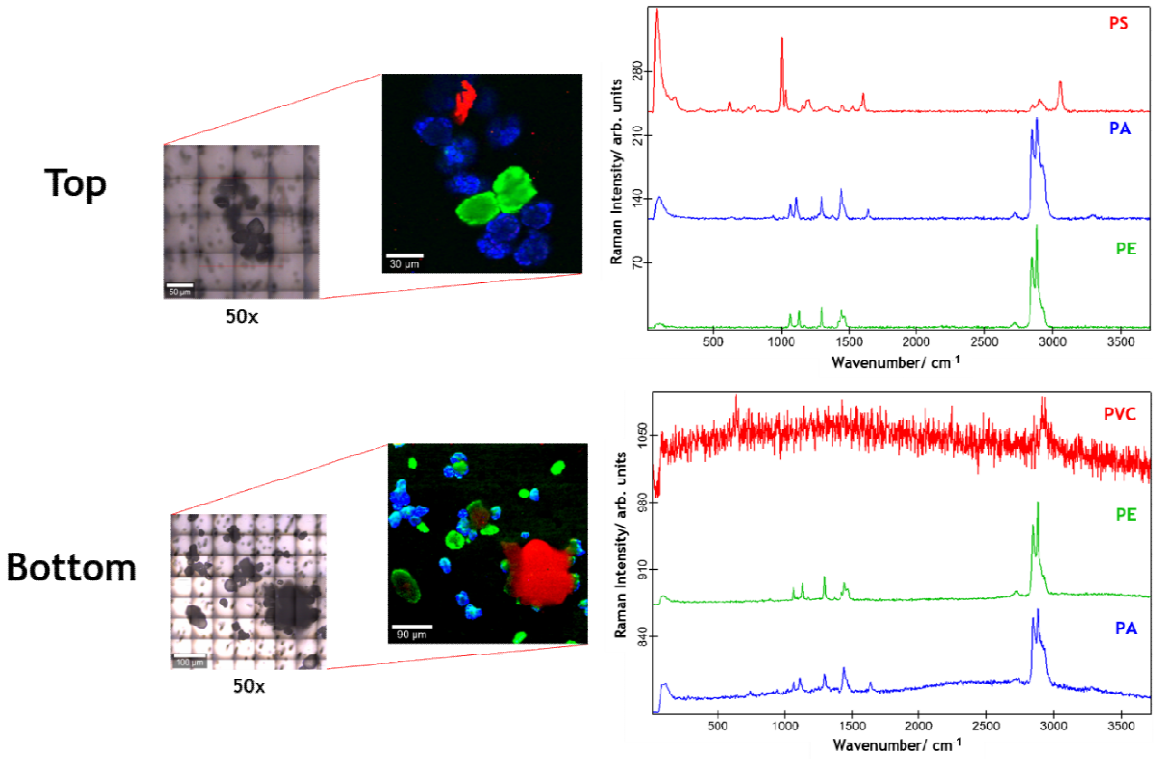


Figure 25. Stitching images (50x objective) and combined Raman images obtained by using three different Raman spectra for the MPs (Top: red for PS; blue for PA and green for PE; Bottom: red for PVC; green for PEand blue for PA). Raman image of the top phase of MPs separated through test tubes was obtained by using the integrated intensity of the Raman bands of PS at 1006 cm^{-1}, PE at 1135 cm^{-1}, and PA at 1641 cm^{-1}. For the bottom phase, the Raman image was obtained by using the integrated intensity of the Raman bands of PVC at 2940 cm^{-1}, PE at 1133 $cm^{-1}$and PA at 1641 cm^{-1}.

4.2.3.2.2. House Dust

The same procedure described above was employed on the house dust sample to evaluate the separation of the microplastics in both phases using Raman spectroscopy. Raman imaging was not performed in this sample because has high amount of material and high sensitivity to the laser as mentioned in 4.2.2.3.

In the top phase, it was expected to found PE particles as the major component due to its density (0.94 $g.cm^{-3}$), however, any PE particles were detected in this phase (Figure 26).By the Raman spectra, it was found PA (d=1.02 $g.cm^{-3}$) (light green spectrum) and other components such as amorphous carbon (purple Raman spectrum), polycarbonate particles from the filter membrane (red, pink, green and light green Raman spectra) and calcium carbonate (blue Raman spectrum).

The absence of PE MPs can be related to the nature of this sample since it is possible the absence of this polymer even before any treatment. Also, these particles could have been also stuck in the tube wall, as previously mentioned. Thus, for trial 2, a different approach with a combination of house dust and commercial MPs was proposed.

In the bottom phase, it was expected to find PVC and PA due to their density of 1.4 $g.cm^{-3}$ and 1.02 $g.cm^{-3}$ respectively. Nevertheless, no MPs were found in this sample as illustrated in Figure 26. These findings support the theory mentioned for PE in the top phase and added the fact of the inexistence of PVC and PA in the sample.

Similar to what happened in the top phase, it was detected amorphous carbon (pink spectrum) because the bottom phase corresponds to the high-density phase which is in accordance with the density of the carbon. In addition, the density of polyvinylpyrrolidone is 1.2 $g.cm^{-3}$, which makes the occurrence of this polymer (salmon spectrum) [148] concur with density fundamentals.

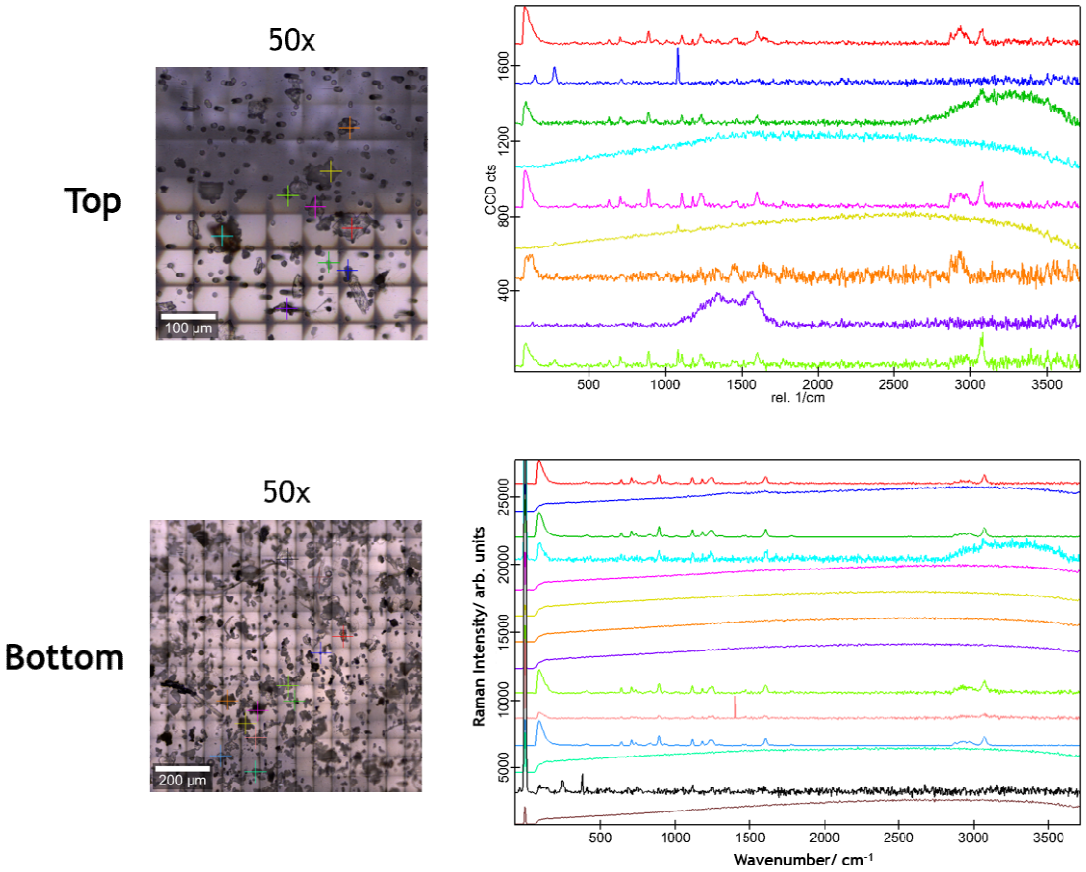


Figure 26. Stitching image (50x objective) and Raman spectra of the top and bottom phase from house dust sample separated through test tubes.

4.2.3.3. Trial 2 (Ampoules)

In the second trial (with ampoules), thephases analysed of the density separation first step were the pellet (bottom phase with house dust) and the ionic liquid - $[C_4mpy][NTf_2]$(discarded). From the second step, three different phases were analysed i) the top phase (expected to contain PE; d=0.94 $g.cm^{-3}$), ii) the mid-bottom phase (expected to contain PA; d=1.02 $g.cm^{-3}$) and iii) the bottom phase (expected to contain PVC; d=1.4 $g.cm^{-3}$). However, for the mixture of house dust and commercial MPs, the mid-bottom and bottom phaseswere filtered together.

This analysis was performed for samples only with commercial MPs (PE, PVC and PA), for house dust samples and for a mixture of commercial MPs and house dust.

4.2.3.3.1. Commercial MPs

Annex 7.3illustrates the IL - $[C_4mpy][NTf_2]$ phase (discarded) of the sample with commercial MPs. Two different spectra were found, one from the polycarbonate particles from the filter membrane (e.g. red Raman spectrum) and other unknown (e.g. green Raman spectrum).It should be noted that no MPs were found in this sample which demonstrates the success of the first separation step.

In order to evaluate if the unknown Raman spectrum could be from the ionic liquid itself, a Raman spectrum was acquired from a liquid sample of $[C_4mpy][NTf_2]$and the results are shown in Annex 7.4 The red spectrum shows the particles found in the IL and the blue spectrum corresponds to the liquid IL.

The same procedure described in 4.2.3.2.1. was employed for the top phase deliberately contaminated with commercial MPs and separated through ampoules. The density of PE is 0.94 $g.cm^{-3}$ and sinceit was expected to mainly obtain this polymerin this sample, the characteristic band of PE (1130 cm^{-1}) was monitored. However, we detected another polymer, PA (Figure 27). The combined Raman image allows to identify the presence of PE (blue) and PA (red), with PA being the most abundant. Theoccurrence of PA MPs can once again be explained by their resuspension ability in water, due to adensity of 1.02 $g.cm^{-3}$.

The same procedure described in 4.2.3.2.1. was employed for the mid-bottom phase deliberately contaminated with commercial MPs and separated through ampoules. The density of PA is 1.02 $g.cm^{-3}$and, expecting to obtain mostly PA in this sample, the characteristic band of PA (1641 cm^{-1}) was monitored. It was clearly identified in Figure 27 the presence of PA (red) and calcium carbonate (blue), with PA being the most abundant one. In addition, it should be noted that no PVC or PE MPs were found in this sample, at least in the selected area.

The same procedure described in 4.2.3.2.1. was employed for the bottom phase deliberately contaminated with commercial MPs and separated through ampoules. The density of PVC is 1.4 g.cm^{-3} and therefore it was expected to obtain itin this sample. However, instead of finding this polymer, we found many PA (e.g. red Raman spectra in Figure 28) and some PE MPs (e.g. blue Raman spectra in Figure 28).

The presence of PE in this phase can be explained by the fact that these MPs were probably in the ampoule walls and when we washed it with Milli-Q water, to ensure that no material remains in the ampoule, the PE MPs were dragged out with PA and PVC particles.Furthermore, the lack of PVC may be related to the sample selected area where the analysis was performed.

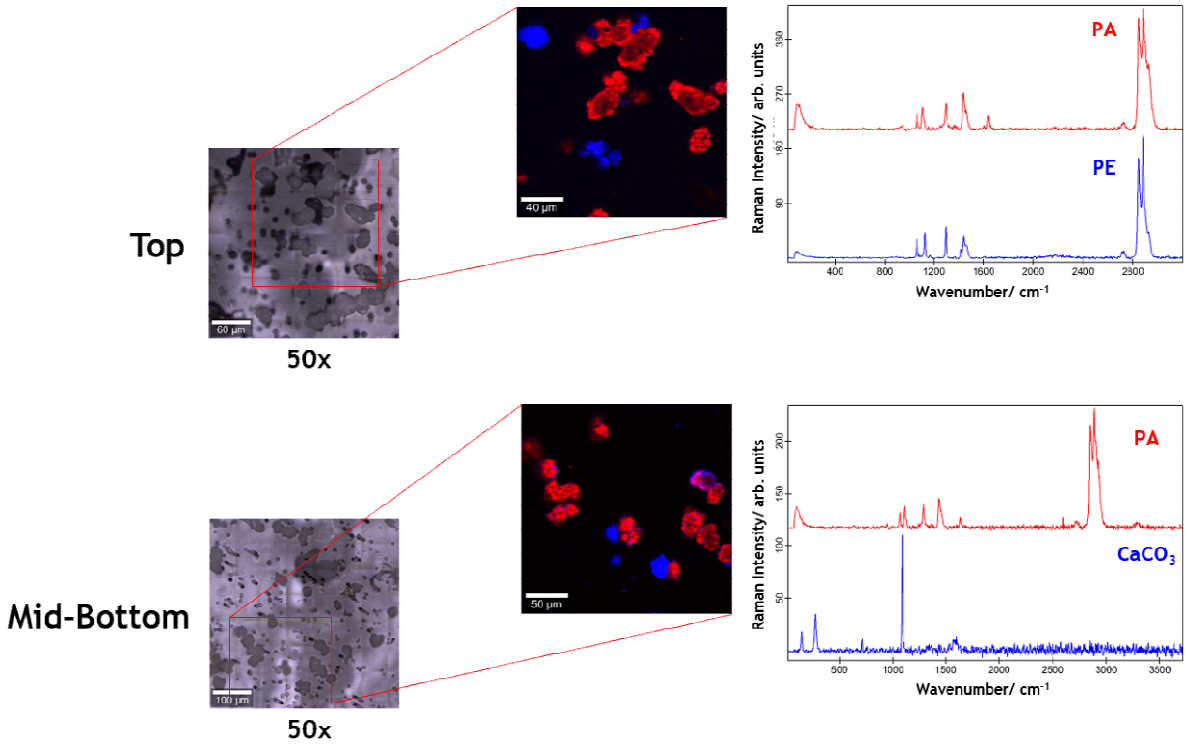


Figure 27. Stitching images (50x objective) and combined Raman images obtained by using two different Raman spectra for the MPs (Top: red for PA and blue for PE; Mid-Bottom: red for PAand blue for $CaCO_3$. The Raman image of the top phase of commercial MPs separated through test tubes was obtained by using the integrated intensity of the Raman bands of PE at 1130 cm^{-1}and PA at 1641 cm^{-1}. For the mid-bottom phase, the Raman image was obtained by using the integrated intensity of the Raman bands of PA at 1641 cm^{-1}and $CaCO_3$ at 1086 cm^{-1}.

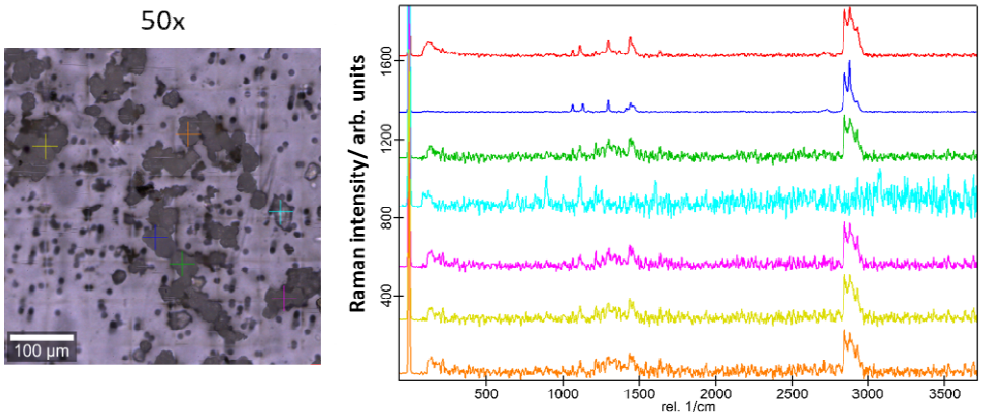


Figure 28. Stitching image (50x objective) and Raman spectra of the bottom phase from commercial MPs separated through ampoules.

4.2.3.3.2. House Dust

The same procedure described in 4.2.3.2.1. was employed for the pellet phase from house dust separated through the ampoule, it was expected that this phase had the high-density house dust particles and did not include MPs, working like a "purification" step as it allows to separate and concentrate particles of no interest. Annex. 7.5 confirms the lack of MPs in this phase being only calcium carbonate found (light green spectrum) [143,148]. Also, it is noticeable from the image on the left that the high amount of sample made the analysis of each individual particle difficult.

For the $[C_4mpy][NTf_2]$ rich phase (discarded) from house dust separated through ampoules, none MP was expected. However, an unknown polymer (Annex 7.6 green and red Raman spectra) was found, this was further identified as PET [154]. The density of PET (d=1.38 $g.cm^{-3}$) is very similar to the$[C_4mpy][NTf_2]$ (d=1.41 $g.cm^{-3}$) which might lead to the retention and further mixture of these two species.

The presence of MPs on the top phase from the house dust sample separated through ampouleswas evaluated using the same procedure described in 4.2.3.2.1. (Figure 29).Several Raman spectra were acquired in different spots of the sample and it was possible to identify PE particles (red Raman spectrum), calcium carbonate (e.g. green Raman spectrum) and alsopolycarbonate particles from the filter membrane (e.g. cyan Raman spectrum). Due to the high amount of sample, some of the Raman spectra were inconclusive (absence of Raman bands and high fluorescence).

The same procedure described in 4.2.3.2.1. was employed for the mid-bottom phase from house dust separated through ampoules. The density of PA is 1.02 $g.cm^{-3}$ and therefore it was expected to obtain PA in this sample and thus the characteristic band of PA (1641 cm^{-1}) was monitored. Moreover, we also found calcium carbonate in this sample (Figure 30). In the combined Raman image, it is clearly identified the presence of PA (red) and calcium

carbonate (blue), with PA being the most abundant. Furthermore, it should be noted that no PVC or PE was found in this sample.

For the bottom fraction of the from house dust separated through ampoules,it was expected to obtain PVC (density=1.4 $g.cm^{-3}$). However, no PVC MPs were found in this sample, as shown in Figure 31, and this absence is probably due to the nature of the original house dust sample that did not have this polymer. This was already confirmed in the untreated house dust sample (4.2.3.1.2.) and in trial sample 1 (4.2.3.2.2.).

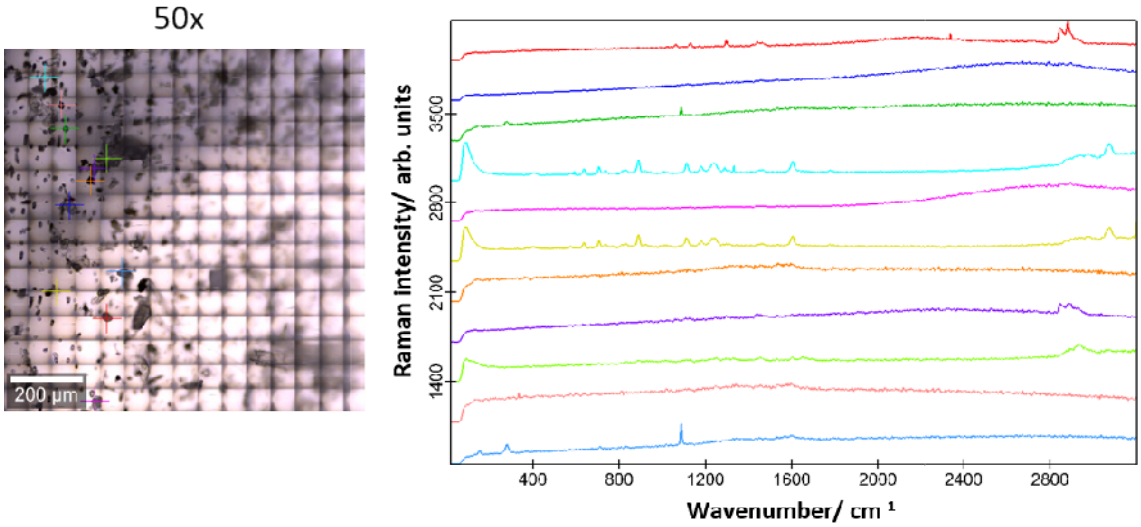


Figure 29. Stitching image (50x objective) and Raman spectrum of the top phase from house dust separated through ampoules.

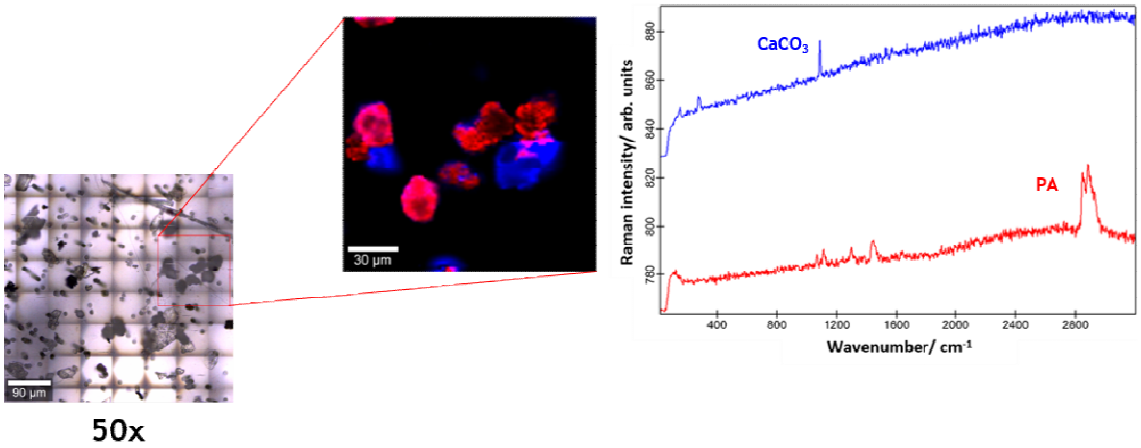


Figure 30.Stitching image (10x objective) and combined Raman image obtained by using two different Raman spectra for MPs (red for PA and blue for $CaCO_3$). The Raman image of the mid-bottom phase from

the house dust separated through ampoules was obtained by using the integrated intensity of the Raman bands of PA at 2906 cm^{-1} and $CaCO_3$ at 1636 cm^{-1}.

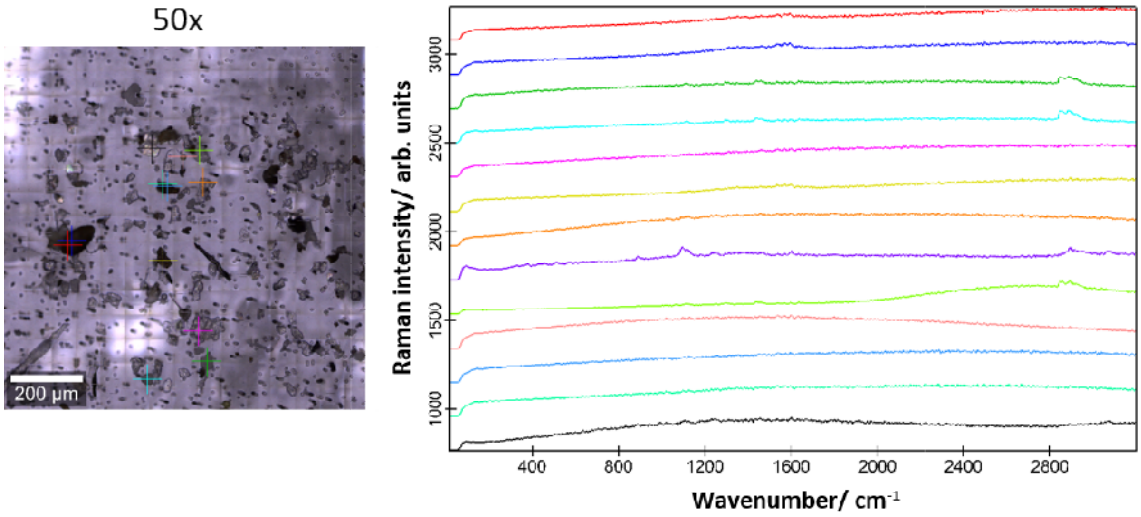


Figure 31. Stitching image(50x objective) and Raman spectrum of the bottom phase from house dust separated through ampoules.

4.2.3.3.3. Commercial MPs and House Dust

The filter obtained for the pellet sample could not be analysed due to the high amount of material, beingimpossible to identifyand/or isolate the particles.Thusfrom this sample,only the IL([C_4mpy][NTf_2])and supernatant were analysed.

The Raman spectra of the[C_4mpy][NTf_2] from the mixture of commercial MPs and house dust <63 µm sample separated through ampoules was done (Annex. 7.7). Although we didn't detect any MPs in this sample.

For the top phase from the mixture of house dust and commercial MPs separated through ampoules, the characteristic band of PE (1135 cm^{-1})was monitored as this was the MP likely to occur in this phase based in its density (0.94 $g.cm^{-3}$). However, other polymers such as PA were also detected by Raman spectroscopy. The combined Raman image presented in Figure 32, it is clearly identified the presence of PE (red) and PA (blue), with PA being the most abundant one. This occurrence of PA MPs can once again be explained by their resuspension ability in water, due to the density of 1.02 $g.cm^{-3}$.

The bottom phase from the mixture of house dust and commercial MPs separated through ampoules was also analysed for the presence of MPs namely PVC and PA, which are the MPs with higher density (PVC - 1.4 $g.cm^{-3}$ and PA - 1.02 $g.cm^{-3}$). [1]and the results are illustrated in Figure 41. The Raman image presented in Figure 32 demonstrates not only the presence and

distribution of PVC (blue) and PA particles (pink) but also PE particles (green). This could be explained by the washing process used in this method as mentioned in 4.2.3.3.1.

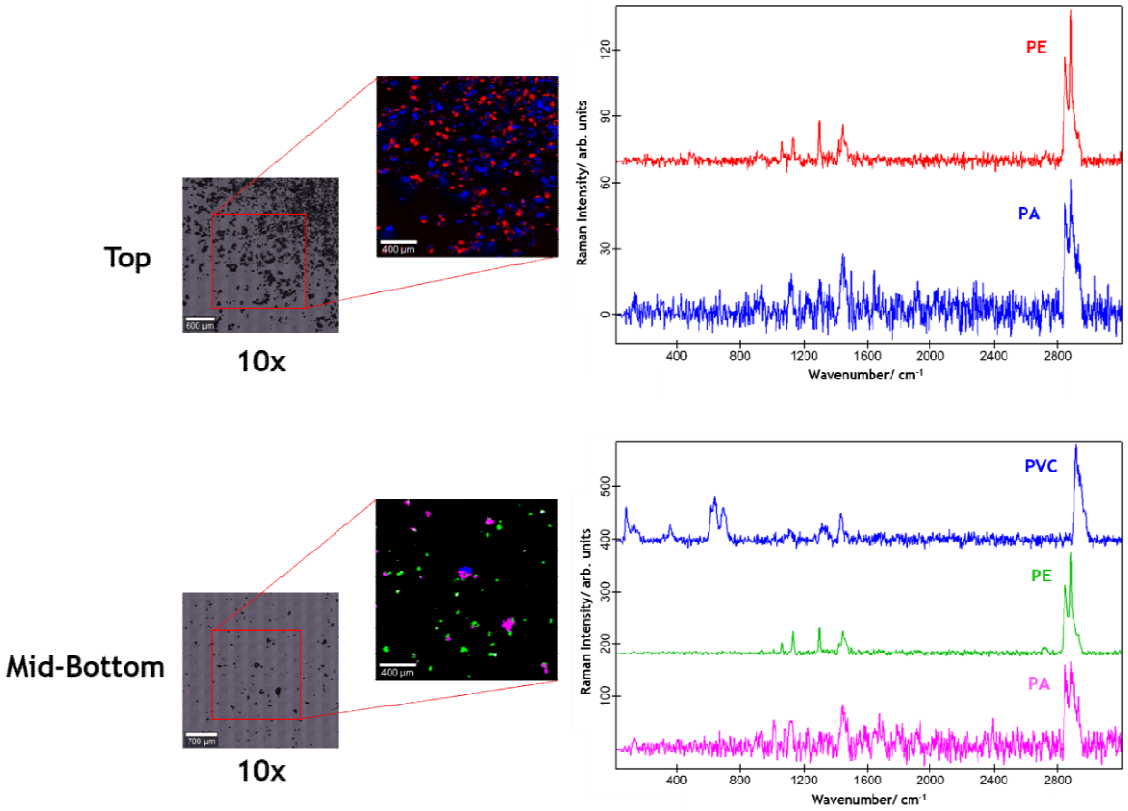


Figure 32. Stitching image (10x objective) and combined Raman image obtained by using two different Raman spectra for MPs (red for PE and blue for PA). The Raman image of top phase from the mixture of house dust and commercial MPs separated through ampoules was obtained by using the integrated intensity of the Raman bands of PE at 1130 cm^{-1} and PA at 1111 cm^{-1}. For the bottom phase, the Raman image was obtained by using the integrated intensity of the Raman bands of PVC at 2934 cm^{-1}, PE at 1130 cm^{-1}, and PA at 1111 cm^{-1}.

Overall, both approaches (trial 1 and trial 2) were not very successful in the separation of low-density MPs (PE – d=0.94 $g.cm^{-3}$) and high-density MPs (PVC – d=1.4 $g.cm^{-3}$). In addition, PE (d=0.94 $g.cm^{-3}$) and PA MPs (d=1.02 $g.cm^{-3}$) had some variations in behaviour, being detected both in the top phase with low-density particles and in the bottom phase with high density particles.

Furthermore, we expected trial 2 to be more efficient, which did not happen as the problems to separate PE and PA in the top and bottom phases still occurred, althoughit was able to isolate PA in the mid-bottom phase. Because theprocedure usingampoules is very time consuming and higher amounts of chemicals are necessary, its use is not recommended for future studies.

Yet, Raman spectroscopy proved to be efficient to identify MPs in house dust samples. However, since it is an expensive technique (due to time and specialized labour demands) should be applied to samples that already havethe presence of MPsconfirmed.

4.3. Microplastics Toxicity

4.3.1. Caco-2 cells

The Caco-2 cell line is a well-established model of the intestinal epithelial barrier, being this cancer derived cell line used in the toxicological evaluation of several compounds [155]. In addition, many studies reported that MPs have the ability to interact with intestinal cells, being nano plastics capable to be transported through the digestive tract [156-159].

The viability of Caco-2 cells exposed to PE, PVC and PA solutions (0.01; 0.1; 1; 10; 100; 2000; 4000mg.L^{-1}) during 24h and evaluated by CCK-8 is shown in Figure 33.

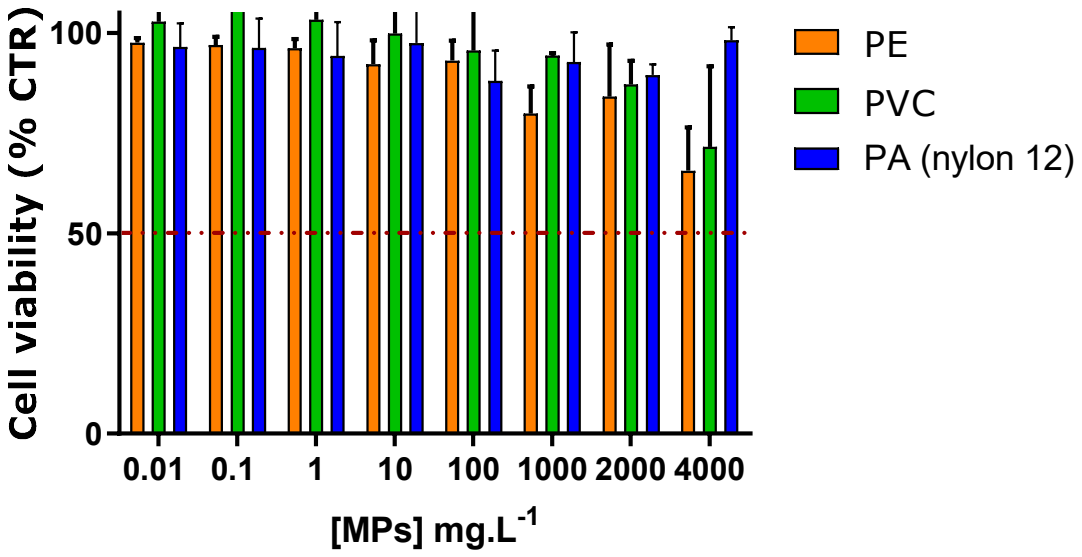


Figure 33. Data obtained from CCK-8 assay for PA (polyamide), PE (polyethylene) and PVC (polyvinyl chloride) on human epithelial colorectal adenocarcinoma cells (Caco-2). Data represented as live cells content normalized to the control (unexposed cells) of 5 replicates of three independent experiments (n=3). The x-axis represents the concentrations to which cells were exposed during 24h.

After exposure, PA barely changes the cell viability of Caco-2 cells and PE and PVC MPs induce a slight reduction of cell viability that starts from the 100 mg.L^{-1}concentration up to4000

mg.L^{-1} (highest concentration), but even that does notexceed 35%. Similar results were obtained by other authors with the same cell line [159,160].

The low microplastic toxicity on Caco-2 cells could be explained by the additive composition of the plastics or by a low extraction of plastic additives [109]. In addition, for PE and PVC the viability is decreasing slightly as the concentration increases. Thus, in order to be able to obtain a more robust dose-response curve, higher concentrations should have been tested. Yet, we decided not to test higher concentrations as those correspond to an unrealistic exposure scenario.

4.3.2. HepG2 cells

As well as Caco-2 cells, the HepG2 cell line is a well-established model of the human hepatocyte carcinoma, commonly used in toxicological studies [161]. However, these cells were never submitted to MPs.

The viability of HepG2 cells exposed to PE, PVC and PA solutions (0.01; 0.1; 1; 10; 100; 1000 mg.L^{-1}) during 24h and evaluated by CCK-8 is shown in Figure 34.

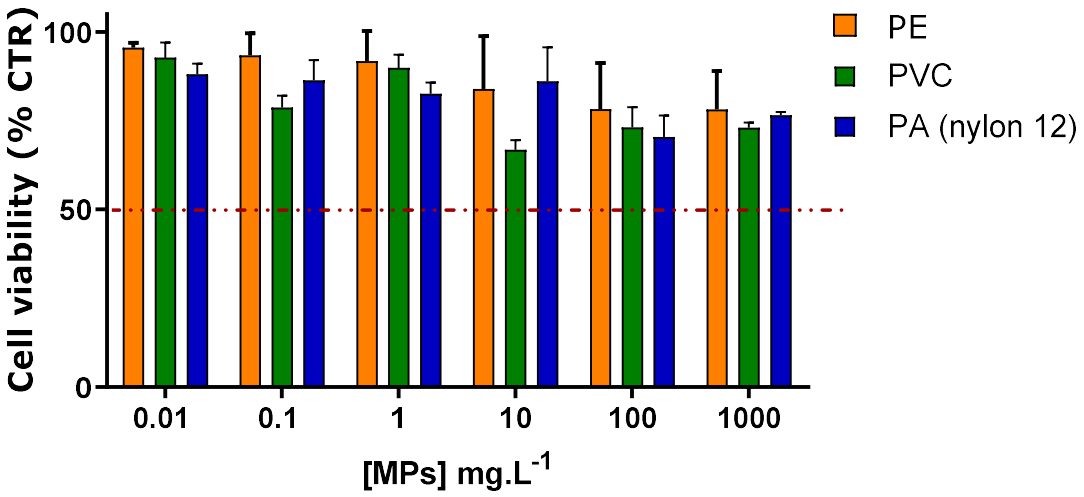


Figure 34. Data obtained from CCK-8 assay for PE (polyethylene), PVC (polyvinyl chloride) and PA (polyamide) on human hepatocellular carcinoma cell line (HepG2). Data represented as live cells content normalized to the control (unexposed cells) of 5 replicates of three independent experiments (n=3). The x-axis represents the concentrations to which cells were exposed during 24h.

After 24h of exposure toPE, PVC and PA decreased cell viability was registered, being most notorious for 10 mg.L^{-1} of PVC. Of the three MPs tested, PVC was the most toxic towards the HepG2 cell line as previously registered for Caco-2 cells. Again, it was not possible to

calculate the EC_{50} as we could not obtain a clear dose-response curve within the tested concentrations.

Furthermore, it was recently discovered by Stock, et al. [162] that PE (d=0.95 $g.cm^{-3}$) particles under normal cultivation conditions were not cytotoxic because they float and therefore were not in contact with cells. However, when incubated in inverted cell model these particles were cytotoxic to HepG2 cells, demonstrating the necessity to adapt the cell culture conditions to the physicochemical properties of the particles.

4.3.3. N27 cells

The N27 cell line is a dopaminergic neuron model used for several studies of neurodegenerative disorders caused by several compounds [163]. Yet, MPs toxicity has never been evaluated in this cell line.

The viability of N27 cells exposed to PE, PVC and PA solutions (0.01; 0.1; 1; 10; 100; 2000; 4000 $mg.L^{-1}$) during 24h and evaluated by CCK-8 is shown in Figure 35.

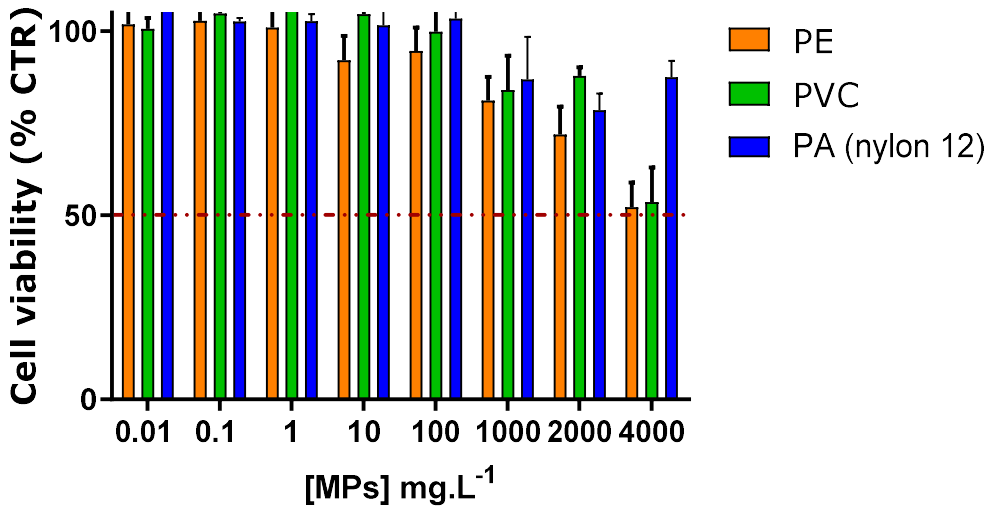


Figure 35 Data obtained from CCK-8 assay for PE (polyethylene) and PVC (polyvinyl chloride) on immortalized rat mesencephalic dopaminergic cell line (N27). Data represented as live cells content normalized to the control (unexposed cells) of 5 replicates of three independent experiments (n=3). The x-axis represents the concentrations to which cells were exposed during 24h.

After 24h of exposure, PE and PVC solutions induceda reduction of cell viability, particularly from 100 $mg.L^{-1}$ onwards. In fact, a 50% reduction on cell viability was observed for PE and PVC at the highest concentration tested. Such results demonstrate that dopaminergic neurons are much more sensitive than epithelial intestinal cell and then hepatocytes.

Chapter 5

5. Conclusions and future research

This work focused on the study of the indoor environment, namely the analysis of microplastics levels in airborne and dust samples and also the toxicity of the most common MPs. Due to the lack of information about these particles and techniques currently available to address reliable and cost-efficient methods for their analysis, we attempted to implementboth for airborne and dust samples some strategies for MPs isolation and identification already described in the literature for other matrices [46,164]. The treatment of samples such as house dustrequireda two-step approach with NaCl (density separation) and H_2O_2 (oxidative digestion)due to thehigh content of non-target materials present in this kind of samples. On the other hand, for air-born samples, a single step of oxidative digestion with H_2O_2 is enough. Nile Red demonstrated to be a goodmethod to detectMPs'presence in samples and,as importantly, to quantify total MPs content. Indeed, this technique should be performed asthe first approach tosample analysis since it is highly time-effective. However,the identification of individual microplastics requiresRaman spectroscopy. This technique allows the identification and characterization of each particle present in the sample but being time consuming and requiring specialized personnel should only be performed if the presence of MPs isconfirmed.

In order to improve the sample treatment and avoid the digestion step, anew method for isolation and purification of MPs was developed. This method demonstrated to be very promising because it is much more cost-effective than those previously described.However, the new protocol for phases separation and extraction needs to be improved and further replication tests are necessary to fully understand the behavior of MPs in these conditions.

For further work, we propose that cell lines should be exposed to a mixture of MPs with additives (as well as those adsorbing environmental contaminants, such as PAHs and PCBs) because commercial additive-free MPs used in testing do not reflect the real MPs to whom biota and humans are continuously exposed. Also, MPs originated from larger plastic items (Secondary MPs), by facing a process of deterioration which exposed and provided secondary ramification of the constituting molecules to link with environmental contaminants, might possess higher toxicity[6]. In addition, reactive oxygen species (ROS) quantification of these particles should also be performed.

6. Bibliography

Uncategorized References

1 Plastics Europe. Plastics - the Facts 2019. (2019).

2 da Costa, J. P., Santos, P. S. M., Duarte, A. C. & Rocha-Santos, T. (Nano)plastics in the environment - Sources, fates and effects. *Sci Total Environ***566-567**, 15-26, doi:10.1016/j.scitotenv.2016.05.041 (2016).

3 Oliveira, M., Almeida, M. & Miguel, I. A micro(nano)plastic boomerang tale: A never ending story? *TrAC Trends in Analytical Chemistry***112**, 196-200, doi:10.1016/j.trac.2019.01.005 (2019).

4 Hartmann, N. B. *et al*. Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris. *Environ Sci Technol***53**, 1039-1047, doi:10.1021/acs.est.8b05297 (2019).

5 Prata, J. C., da Costa, J. P., Lopes, I., Duarte, A. C. & Rocha-Santos, T. Environmental exposure to microplastics: An overview on possible human health effects. *Sci Total Environ***702**, 134455, doi:10.1016/j.scitotenv.2019.134455 (2020).

6 Rochman, C. M. *et al*. Rethinking microplastics as a diverse contaminant suite. *Environ Toxicol Chem***38**, 703-711, doi:10.1002/etc.4371 (2019).

7 Browne, M. A., Galloway, T. & Thompson, R. Microplastic--an emerging contaminant of potential concern? *Integr Environ Assess Manag***3**, 559-561, doi:10.1002/ieam.5630030412 (2007).

8 Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E. & Svendsen, C. Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Sci Total Environ***586**, 127-141, doi:10.1016/j.scitotenv.2017.01.190 (2017).

9 Jiang, J.-Q. Occurrence of microplastics and its pollution in the environment: A review. *Sustainable Production and Consumption***13**, 16-23, doi:10.1016/j.spc.2017.11.003 (2018).

10 Lusher, A. L., Welden, N. A., Sobral, P. & Cole, M. Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Analytical Methods***9**, 1346-1360, doi:10.1039/c6ay02415g (2017).

11 Blettler, M. C. M., Abrial, E., Khan, F. R., Sivri, N. & Espinola, L. A. Freshwater plastic pollution: Recognizing research biases and identifying knowledge gaps. *Water Res***143**, 416-424, doi:10.1016/j.watres.2018.06.015 (2018).

12 Thompson, R. C. *et al*. Lost at sea: where is all the plastic? *Science***304**, 838, doi:10.1126/science.1094559 (2004).

13 Arthur, C., Baker, J. E. & Bamford, H. A. Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington Tacoma, Tacoma, WA, USA. (2009).

14 Koehler, A. *et al.SOURCES, FATE AND EFFECTS OF MICROPLASTICS IN THE MARINE ENVIRONMENT: A GLOBAL ASSESSMENT*. (2015).

15 Costa, M. F., Pinto da Costa, J. & Duarte, A. C. "Sampling of micro(nano)plastics in environmental compartments: How to define standard procedures?". *Current Opinion in Environmental Science & Health***1**, 36-40, doi:10.1016/j.coesh.2017.10.001 (2018).

16 Hale, R. C. Analytical challenges associated with the determination of microplastics in the environment. *Analytical Methods***9**, 1326-1327, doi:10.1039/c7ay90015e (2017).

17 Schwaferts, C., Niessner, R., Elsner, M. & Ivleva, N. P. Methods for the analysis of submicrometer- and nanoplastic particles in the environment. *TrAC Trends in Analytical Chemistry***112**, 52-65, doi:10.1016/j.trac.2018.12.014 (2019).

18 Helm, P. A. Improving microplastics source apportionment: a role for microplastic morphology and taxonomy? *Analytical Methods***9**, 1328-1331, doi:10.1039/c7ay90016c (2017).

19 European Parlament. Microplastics: sources, effects and solutions. (2019).

20 IUPAC. *Compendium of polymer terminology and nomenclature : IUPAC recommendations, 2008*. (RSC Pub. : IUPAC, 2009).

21 Rocha-Santos, T. & Duarte, A. C. A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. *TrAC Trends in Analytical Chemistry***65**, 47-53, doi:10.1016/j.trac.2014.10.011 (2015).

22 Wesch, C. *et al*. Assuring quality in microplastic monitoring: About the value of clean-air devices as essentials for verified data. *Sci Rep***7**, 5424, doi:10.1038/s41598-017-05838-4 (2017).

23 Lithner, D., Larsson, A. & Dave, G. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci Total Environ***409**, 3309-3324, doi:10.1016/j.scitotenv.2011.04.038 (2011).

24 ISO. (2013).

25 Ivleva, N. P., Wiesheu, A. C. & Niessner, R. Microplastic in Aquatic Ecosystems. *Angew Chem Int Ed Engl***56**, 1720-1739, doi:10.1002/anie.201606957 (2017).

26 European Chemicals Agency. Vol. (2012).

27 Wright, S. L. & Kelly, F. J. Plastic and Human Health: A Micro Issue? *Environ. Sci. Technol.***51**, 6634-6647, doi:10.1021/acs.est.7b00423 (2017).

28 Ory, N. C., Sobral, P., Ferreira, J. L. & Thiel, M. Amberstripe scad Decapterus muroadsi (Carangidae) fish ingest blue microplastics resembling their copepod prey along the coast of Rapa Nui (Easter Island) in the South Pacific subtropical gyre. *Sci Total Environ***586**, 430-437, doi:10.1016/j.scitotenv.2017.01.175 (2017).

29 Besley, A., Vijver, M. G., Behrens, P. & Bosker, T. A standardized method for sampling and extraction methods for quantifying microplastics in beach sand. *Mar Pollut Bull***114**, 77-83, doi:10.1016/j.marpolbul.2016.08.055 (2017).

30 Dris, R. *et al*. A first overview of textile fibers, including microplastics, in indoor and outdoor environments. *Environ Pollut***221**, 453-458, doi:10.1016/j.envpol.2016.12.013 (2017).

31 Dehaut, A. *et al*. Microplastics in seafood: Benchmark protocol for their extraction and characterization. *Environ Pollut***215**, 223-233, doi:10.1016/j.envpol.2016.05.018 (2016).

32 Dris, R. *et al*. Microplastic contamination in an urban area: a case study in Greater Paris. *Environmental Chemistry***12**, 592-599, doi:10.1071/en14167 (2015).

33 Qiu, Q. *et al*. Extraction, enumeration and identification methods for monitoring microplastics in the environment. *Estuarine, Coastal and Shelf Science***176**, 102-109, doi:10.1016/j.ecss.2016.04.012 (2016).

34 Silva, A. B. *et al*. Microplastics in the environment: Challenges in analytical chemistry - A review. *Anal Chim Acta***1017**, 1-19, doi:10.1016/j.aca.2018.02.043 (2018).

35 Cole, M., Lindeque, P., Halsband, C. & Galloway, T. S. Microplastics as contaminants in the marine environment: a review. *Mar Pollut Bull***62**, 2588-2597, doi:10.1016/j.marpolbul.2011.09.025 (2011).

36 Muller, Y. K. *et al*. Microplastic analysis-are we measuring the same? Results on the first global comparative study for microplastic analysis in a water sample. *Anal Bioanal Chem*, doi:10.1007/s00216-019-02311-1 (2019).

37 Woodall, L. C. *et al*. Using a forensic science approach to minimize environmental contamination and to identify microfibres in marine sediments. *Mar Pollut Bull***95**, 40-46, doi:10.1016/j.marpolbul.2015.04.044 (2015).

38 De Witte, B. *et al*. Quality assessment of the blue mussel (Mytilus edulis): comparison between commercial and wild types. *Mar Pollut Bull***85**, 146-155, doi:10.1016/j.marpolbul.2014.06.006 (2014).

39 Hidalgo-Ruz, V., Gutow, L., Thompson, R. C. & Thiel, M. Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environ Sci Technol***46**, 3060-3075, doi:10.1021/es2031505 (2012).

40 Duis, K. & Coors, A. Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on personal care products), fate and effects. *Environ Sci Eur***28**, 2, doi:10.1186/s12302-015-0069-y (2016).

41 Chiang, R., Rand-Meir, T., Makino, R. & Hager, L. P. Compound X. An intermediate in enzymatic halogenation. *J Biol Chem***251**, 6340-6346 (1976).

42 Galgani, F. *et al.Guidance on Monitoring of Marine Litter in European Seas*. (2013).

43 Frias, J. P., Gago, J., Otero, V. & Sobral, P. Microplastics in coastal sediments from Southern Portuguese shelf waters. *Mar Environ Res***114**, 24-30, doi:10.1016/j.marenvres.2015.12.006 (2016).

44 Remy, F. *et al*. When Microplastic Is Not Plastic: The Ingestion of Artificial Cellulose Fibers by Macrofauna Living in Seagrass Macrophytodetritus. *Environ Sci Technol***49**, 11158-11166, doi:10.1021/acs.est.5b02005 (2015).

45 Van Cauwenberghe, L. & Janssen, C. R. Microplastics in bivalves cultured for human consumption. *Environ Pollut***193**, 65-70, doi:10.1016/j.envpol.2014.06.010 (2014).

46 Erni-Cassola, G., Gibson, M. I., Thompson, R. C. & Christie-Oleza, J. A. Lost, but Found with Nile Red: A Novel Method for Detecting and Quantifying Small Microplastics (1 mm to 20 mum) in Environmental Samples. *Environ Sci Technol***51**, 13641-13648, doi:10.1021/acs.est.7b04512 (2017).

47 Renner, G., Schmidt, T. C. & Schram, J. Analytical methodologies for monitoring micro(nano)plastics: Which are fit for purpose? *Current Opinion in Environmental Science & Health***1**, 55-61, doi:10.1016/j.coesh.2017.11.001 (2018).

48 Bergmann, M., Gutow, L. & Klages, M. *Marine Anthropogenic Litter*. (2015).

49 Prata, J. C., da Costa, J. P., Duarte, A. C. & Rocha-Santos, T. Methods for sampling and detection of microplastics in water and sediment: A critical review. *TrAC Trends in Analytical Chemistry***110**, 150-159, doi:10.1016/j.trac.2018.10.029 (2019).

50 Quinn, B., Murphy, F. & Ewins, C. Validation of density separation for the rapid recovery of microplastics from sediment. *Analytical Methods***9**, 1491-1498, doi:10.1039/c6ay02542k (2017).

51 Nuelle, M.-T., Dekiff, J. H., Remy, D. & Fries, E. A new analytical approach for monitoring microplastics in marine sediments. *Environmental Pollution***184**, 161-169, doi:10.1016/j.envpol.2013.07.027 (2014).

52 Maes, T., Jessop, R., Wellner, N., Haupt, K. & Mayes, A. G. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. *Sci Rep***7**, 44501, doi:10.1038/srep44501 (2017).

53 Wagner, J. *et al*. Novel method for the extraction and identification of microplastics in ocean trawl and fish gut matrices. *Analytical Methods***9**, 1479-1490, doi:10.1039/c6ay02396g (2017).

54 Courtene-Jones, W., Quinn, B., Murphy, F., Gary, S. F. & Narayanaswamy, B. E. Optimisation of enzymatic digestion and validation of specimen preservation methods for the analysis of ingested microplastics. *Analytical Methods***9**, 1437-1445, doi:10.1039/c6ay02343f (2017).

55 Claessens, M., Van Cauwenberghe, L., Vandegehuchte, M. B. & Janssen, C. R. New techniques for the detection of microplastics in sediments and field collected organisms. *Mar Pollut Bull***70**, 227-233, doi:10.1016/j.marpolbul.2013.03.009 (2013).

56 Catarino, A. I., Thompson, R., Sanderson, W. & Henry, T. B. Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues. *Environ Toxicol Chem***36**, 947-951, doi:10.1002/etc.3608 (2017).

57 Karami, A. *et al*. A high-performance protocol for extraction of microplastics in fish. *Sci Total Environ***578**, 485-494, doi:10.1016/j.scitotenv.2016.10.213 (2017).

58 Cole, M. *et al*. Isolation of microplastics in biota-rich seawater samples and marine organisms. *Sci Rep***4**, 4528, doi:10.1038/srep04528 (2014).

59 Kuhn, S. *et al*. The use of potassium hydroxide (KOH) solution as a suitable approach to isolate plastics ingested by marine organisms. *Mar Pollut Bull***115**, 86-90, doi:10.1016/j.marpolbul.2016.11.034 (2017).

60 Munno, K., Helm, P. A., Jackson, D. A., Rochman, C. & Sims, A. Impacts of temperature and selected chemical digestion methods on microplastic particles. *Environ Toxicol Chem***37**, 91-98, doi:10.1002/etc.3935 (2018).

61 Karlsson, T. M. *et al*. Screening for microplastics in sediment, water, marine invertebrates and fish: Method development and microplastic accumulation. *Marine Pollution Bulletin***122**, 403-408, doi:10.1016/j.marpolbul.2017.06.081 (2017).

62 Karbalaei, S., Hanachi, P., Walker, T. R. & Cole, M. Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environ Sci Pollut Res Int***25**, 36046-36063, doi:10.1007/s11356-018-3508-7 (2018).

63 Andrady, A. L. Microplastics in the marine environment. *Mar Pollut Bull***62**, 1596-1605, doi:10.1016/j.marpolbul.2011.05.030 (2011).

64 Rillig, M. C. Microplastic in Terrestrial Ecosystems and the Soil? *Environ. Sci. Technol.***46**, 6453-6454, doi:10.1021/es302011r (2012).

65 Steinmetz, Z. *et al*. Plastic mulching in agriculture. Trading short-term agronomic benefits for long-term soil degradation? *Sci. Total. Environ.***550**, 690-705, doi:10.1016/j.scitotenv.2016.01.153 (2016).

66 Napper, I. E., Bakir, A., Rowland, S. J. & Thompson, R. C. Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. *Mar. Pollut. Bull.***99**, 178-185, doi:10.1016/j.marpolbul.2015.07.029 (2015).

67 von Moos, N., Burkhardt-Holm, P. & Köhler, A. Uptake and Effects of Microplastics on Cells and Tissue of the Blue Mussel Mytilus edulis L. after an Experimental Exposure. *Environ. Sci. Technol.***46**, 11327-11335, doi:10.1021/es302332w (2012).

68 Zitko, V. & Hanlon, M. Another source of pollution by plastics: Skin cleaners with plastic scrubbers. *Mar. Pollut. Bull.***22**, 41-42, doi:10.1016/0025-326X(91)90444-W (1991).

69 Moore, C. J. Synthetic polymers in the marine environment: A rapidly increasing, long-term threat. *Environ. Res.***108**, 131-139, doi:10.1016/j.envres.2008.07.025 (2008).

70 Murphy, F., Ewins, C., Carbonnier, F. & Quinn, B. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. *Environ. Sci. Technol.***50**, 5800-5808, doi:10.1021/acs.est.5b05416 (2016).

71 Carr, S. A., Liu, J. & Tesoro, A. G. Transport and fate of microplastic particles in wastewater treatment plants. *Water Res.***91**, 174-182, doi:10.1016/j.watres.2016.01.002 (2016).

72 Magnusson, K. & Norén, F. Screening of microplastic particles in and down-stream a wastewater treatment plant. 22 (2014).

73 Prata, J. C. Microplastics in wastewater: State of the knowledge on sources, fate and solutions. *Mar. Pollut. Bull.***129**, 262-265, doi:10.1016/j.marpolbul.2018.02.046 (2018).

74 Mintenig, S. M., Int-Veen, I., Loder, M. G. J., Primpke, S. & Gerdts, G. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. *Water Res***108**, 365-372, doi:10.1016/j.watres.2016.11.015 (2017).

75 Gies, E. A. *et al*. Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada. *Marine Pollution Bulletin***133**, 553-561, doi:10.1016/j.marpolbul.2018.06.006 (2018).

76 Zubris, K. A. V. & Richards, B. K. Synthetic fibers as an indicator of land application of sludge. *Environ. Pollut.***138**, 201-211, doi:10.1016/j.envpol.2005.04.013 (2005).

77 Nizzetto, L., Futter, M. & Langaas, S. Are Agricultural Soils Dumps for Microplastics of Urban Origin? *Environ. Sci. Technol.***50**, 10777-10779, doi:10.1021/acs.est.6b04140 (2016).

78 Barnes, D., Galgani, F., Thompson, R. & Barlaz, M. Accumulation and fragmentation of plastic debris in global environments. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences***364**, 1985-1998, doi:10.1098/rstb.2008.0205 (2009).

79 Dris, R., Gasperi, J., Saad, M., Mirande, C. & Tassin, B. Synthetic fibers in atmospheric fallout: A source of microplastics in the environment? *Mar Pollut Bull***104**, 290-293, doi:10.1016/j.marpolbul.2016.01.006 (2016).

80 Peng, J., Wang, J. & Cai, L. Current understanding of microplastics in the environment: Occurrence, fate, risks, and what we should do. *Integr Environ Assess Manag***13**, 476-482, doi:10.1002/ieam.1912 (2017).

81 Weinstein, J. E., Crocker, B. K. & Gray, A. D. From macroplastic to microplastic: Degradation of high-density polyethylene, polypropylene, and polystyrene in a salt marsh habitat. *Environmental Toxicology and Chemistry***35**, 1632-1640, doi:10.1002/etc.3432 (2016).

82 Hurley, R. R. & Nizzetto, L. Fate and occurrence of micro(nano)plastics in soils: Knowledge gaps and possible risks. *Current Opinion in Environmental Science & Health***1**, 6-11, doi:10.1016/j.coesh.2017.10.006 (2018).

83 Napper, I. E. & Thompson, R. C. Release of synthetic microplastic plastic fibres from domestic washing machines: Effects of fabric type and washing conditions. *Mar Pollut Bull***112**, 39-45, doi:10.1016/j.marpolbul.2016.09.025 (2016).

84 Belzagui, F., Crespi, M., Alvarez, A., Gutierrez-Bouzan, C. & Vilaseca, M. Microplastics' emissions: Microfibers' detachment from textile garments. *Environ Pollut***248**, 1028-1035, doi:10.1016/j.envpol.2019.02.059 (2019).

85 De Falco, F. *et al.* Evaluation of microplastic release caused by textile washing processes of synthetic fabrics. *Environ Pollut***236**, 916-925, doi:10.1016/j.envpol.2017.10.057 (2018).

86 Browne, M. A. *et al*. Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environ Sci Technol***45**, 9175-9179, doi:10.1021/es201811s (2011).

87 Clayton, G. W. *et al*. Polymer seed coating of early- and late-fall-seeded herbicide-tolerant canola (Brassica napus L.) cultivars. *Canadian Journal of Plant Science***84**, 971-979, doi:10.4141/p03-185 (2004).

88 Huerta Lwanga, E. *et al*. Microplastics in the Terrestrial Ecosystem: Implications for Lumbricus terrestris (Oligochaeta, Lumbricidae). *Environ Sci Technol***50**, 2685-2691, doi:10.1021/acs.est.5b05478 (2016).

89 Scarascia-Mugnozza, G., Sica, C. & Russo, G. Plastic Materials in European Agriculture: Actual Use and Perspectives. *Journal of Agricultural Engineering***42**, doi:10.4081/jae.2011.28 (2012).

90 Zhang, J., Wang, L. & Kannan, K. Microplastics in house dust from 12 countries and associated human exposure. *Environ Int***134**, 105314, doi:10.1016/j.envint.2019.105314 (2020).

91 Liu, C. *et al*. Widespread distribution of PET and PC microplastics in dust in urban China and their estimated human exposure. *Environ Int***128**, 116-124, doi:10.1016/j.envint.2019.04.024 (2019).

92 Sommer, F. *et al*. Tire Abrasion as a Major Source of Microplastics in the Environment. *Aerosol and Air Quality Research***18**, 2014-2028, doi:10.4209/aaqr.2018.03.0099 (2018).

93 Kole, P. J., Lohr, A. J., Van Belleghem, F. & Ragas, A. M. J. Wear and Tear of Tyres: A Stealthy Source of Microplastics in the Environment. *Int J Environ Res Public Health***14**, 1265, doi:10.3390/ijerph14101265 (2017).

94 Davidson, K. & Dudas, S. E. Microplastic Ingestion by Wild and Cultured Manila Clams (Venerupis philippinarum) from Baynes Sound, British Columbia. *Arch Environ Contam Toxicol***71**, 147-156, doi:10.1007/s00244-016-0286-4 (2016).

95 Catarino, A. I., Macchia, V., Sanderson, W. G., Thompson, R. C. & Henry, T. B. Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal. *Environ Pollut***237**, 675-684, doi:10.1016/j.envpol.2018.02.069 (2018).

96 Foekema, E. M. *et al*. Plastic in north sea fish. *Environ Sci Technol***47**, 8818-8824, doi:10.1021/es400931b (2013).

97 Halstead, J. E., Smith, J. A., Carter, E. A., Lay, P. A. & Johnston, E. L. Assessment tools for microplastics and natural fibres ingested by fish in an urbanised estuary. *Environ Pollut***234**, 552-561, doi:10.1016/j.envpol.2017.11.085 (2018).

98 Horton, A. A., Jurgens, M. D., Lahive, E., van Bodegom, P. M. & Vijver, M. G. The influence of exposure and physiology on microplastic ingestion by the freshwater fish Rutilus rutilus (roach) in the River Thames, UK. *Environ Pollut***236**, 188-194, doi:10.1016/j.envpol.2018.01.044 (2018).

99 Renzi, M. & Blaskovic, A. Litter & microplastics features in table salts from marine origin: Italian versus Croatian brands. *Mar Pollut Bull***135**, 62-68, doi:10.1016/j.marpolbul.2018.06.065 (2018).

100 Yang, D. *et al*. Microplastic Pollution in Table Salts from China. *Environ Sci Technol***49**, 13622-13627, doi:10.1021/acs.est.5b03163 (2015).

101 Liebezeit, G. & Liebezeit, E. Synthetic particles as contaminants in German beers. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess***31**, 1574-1578, doi:10.1080/19440049.2014.945099 (2014).

102 Kosuth, M., Mason, S. A. & Wattenberg, E. V. Anthropogenic contamination of tap water, beer, and sea salt. *PLoS One***13**, e0194970, doi:10.1371/journal.pone.0194970 (2018).

103 Lu, L. *et al*. Interaction between microplastics and microorganism as well as gut microbiota: A consideration on environmental animal and human health. *Sci Total Environ***667**, 94-100, doi:10.1016/j.scitotenv.2019.02.380 (2019).

104 da Costa, J. P., Duarte, A. C. & Rocha-Santos, T. A. P. in *Characterization and Analysis of MicroplasticsComprehensive Analytical Chemistry* 1-24 (2017).

105 Ogonowski, M., Gerdes, Z. & Gorokhova, E. What we know and what we think we know about microplastic effects – A critical perspective. *Current Opinion in Environmental Science & Health***1**, 41-46, doi:10.1016/j.coesh.2017.09.001 (2018).

106 Anbumani, S. & Kakkar, P. Ecotoxicological effects of microplastics on biota: a review. *Environ Sci Pollut Res Int***25**, 14373-14396, doi:10.1007/s11356-018-1999-x (2018).

107 Galloway, T. S. in *Marine Anthropogenic Litter* (eds Melanie Bergmann, Lars Gutow, & Michael Klages) 343-366 (Springer International Publishing, 2015).

108 Pannetier, P. *et al*. Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches: Part II-adverse effects on Japanese medaka early life stages. *Environmental Pollution***248**, 1098-1107, doi:10.1016/j.envpol.2018.10.129 (2019).

109 Pannetier, P. *et al*. Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on beaches: Part I-adverse effects on fish cell line. *Environ Pollut***248**, 1088-1097, doi:10.1016/j.envpol.2018.12.091 (2019).

110 Kirstein, I. V. *et al*. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. *Mar Environ Res***120**, 1-8, doi:10.1016/j.marenvres.2016.07.004 (2016).

111 Prata, J. C. Airborne microplastics: Consequences to human health? *Environ Pollut***234**, 115-126, doi:10.1016/j.envpol.2017.11.043 (2018).

112 Wang, F. *et al*. Interaction of toxic chemicals with microplastics: A critical review. *Water Res***139**, 208-219, doi:10.1016/j.watres.2018.04.003 (2018).

113 Zhan, Z. *et al*. Sorption of 3,3′ ,4,4′ -tetrachlorobiphenyl by microplastics: A case study of polypropylene. *Marine Pollution Bulletin***110**, 559-563, doi:10.1016/j.marpolbul.2016.05.036 (2016).

114 Yeo, B. G. *et al*. POPs monitoring in Australia and New Zealand using plastic resin pellets, and International Pellet Watch as a tool for education and raising public awareness on plastic debris and POPs. *Marine Pollution Bulletin***101**, 137-145, doi:10.1016/j.marpolbul.2015.11.006 (2015).

115 Karapanagioti, H. K., Endo, S., Ogata, Y. & Takada, H. Diffuse pollution by persistent organic pollutants as measured in plastic pellets sampled from various beaches in Greece. *Marine Pollution Bulletin***62**, 312-317, doi:10.1016/j.marpolbul.2010.10.009 (2011).

116 Wagner, M. *et al*. Microplastics in freshwater ecosystems: what we know and what we need to know. *Environ Sci Eur***26**, 12, doi:10.1186/s12302-014-0012-7 (2014).

117 Harrison, J. P., Sapp, M., Schratzberger, M. & Osborn, A. M. Interactions Between Microorganisms and Marine Microplastics: A Call for Research. *Marine Technology Society Journal***45**, 12-20, doi:10.4031/MTSJ.45.2.2 (2011).

118 McCormick, A., Hoellein, T. J., Mason, S. A., Schluep, J. & Kelly, J. J. Microplastic is an Abundant and Distinct Microbial Habitat in an Urban River. *Environmental Science & Technology***48**, 11863-11871, doi:10.1021/es503610r (2014).

119 de Sá, L. C., Oliveira, M., Ribeiro, F., Rocha, T. L. & Futter, M. N. Studies of the effects of microplastics on aquatic organisms: What do we know and where should we focus our efforts in the future? *Science of The Total Environment***645**, 1029-1039, doi:10.1016/j.scitotenv.2018.07.207 (2018).

120 Lambert, S. & Wagner, M. in *Freshwater Microplastics : Emerging Environmental Contaminants?* (eds Martin Wagner & Scott Lambert) 1-23 (Springer International Publishing, 2018).

121 Jamieson, A. J. *et al*. Microplastics and synthetic particles ingested by deep-sea amphipods in six of the deepest marine ecosystems on Earth. *R Soc Open Sci***6**, 180667, doi:10.1098/rsos.180667 (2019).

122 Naidu, S. A., Ranga Rao, V. & Ramu, K. Microplastics in the benthic invertebrates from the coastal waters of Kochi, Southeastern Arabian Sea. *Environ Geochem Health***40**, 1377-1383, doi:10.1007/s10653-017-0062-z (2018).

123 Prata, J. C., da Costa, J. P., Lopes, I., Duarte, A. C. & Rocha-Santos, T. Effects of microplastics on microalgae populations: A critical review. *Sci Total Environ***665**, 400-405, doi:10.1016/j.scitotenv.2019.02.132 (2019).

124 Lusher, A. L., McHugh, M. & Thompson, R. C. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Mar Pollut Bull***67**, 94-99, doi:10.1016/j.marpolbul.2012.11.028 (2013).

125 Mao, Y. *et al*. Phytoplankton response to polystyrene microplastics: Perspective from an entire growth period. *Chemosphere***208**, 59-68, doi:10.1016/j.chemosphere.2018.05.170 (2018).

126 Lu, L., Wan, Z., Luo, T., Fu, Z. & Jin, Y. Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice. *Science of The Total Environment***631-632**, 449-458, doi:10.1016/j.scitotenv.2018.03.051 (2018).

127 Wang, C. *et al*. Bioaccumulation in the gut and liver causes gut barrier dysfunction and hepatic metabolism disorder in mice after exposure to low doses of OBS. *Environment International***129**, 279-290, doi:10.1016/j.envint.2019.05.056 (2019).

128 Moreno, J. Prevotella copri and the microbial pathogenesis of rheumatoid arthritis. *Reumatología Clínica (English Edition)***11**, 61-63, doi:10.1016/j.reumae.2014.11.005 (2015).

129 Workneh, M., Wang, F., Romagnoli, M., Simner, P. J. & Carroll, K. Bypass graft infection and bacteremia caused by Anaerostipes caccae: First report of human infection caused by a recently described gut anaerobe. *Anaerobe***42**, 98-100, doi:10.1016/j.anaerobe.2016.09.005 (2016).

130 Revel, M., Châtel, A. & Mouneyrac, C. Micro(nano)plastics: A threat to human health? *Current Opinion in Environmental Science & Health***1**, 17-23, doi:10.1016/j.coesh.2017.10.003 (2018).

131 Salim, S. Y., Kaplan, G. G. & Madsen, K. L. Air pollution effects on the gut microbiota: a link between exposure and inflammatory disease. *Gut Microbes***5**, 215-219, doi:10.4161/gmic.27251 (2014).

132 Vianello, A., Jensen, R. L., Liu, L. & Vollertsen, J. Simulating human exposure to indoor airborne microplastics using a Breathing Thermal Manikin. *Sci Rep***9**, 8670, doi:10.1038/s41598-019-45054-w (2019).

133 Eschenbacher, W. L. *et al*. Nylon flock-associated interstitial lung disease. *American journal of respiratory and critical care medicine***159**, 2003-2008, doi:10.1164/ajrccm.159.6.9808002 (1999).

134 Kremer, A. M., Pal, T. M., Boleij, J. S. M., Schouten, J. P. & Rijcken, B. Airway hyper-responsiveness and the prevalence of work-related symptoms in workers exposed to irritants. *American Journal of Industrial Medicine***26**, 655-669, doi:10.1002/ajim.4700260508 (1994).

135 Warheit, D. B. *et al*. Potential Pulmonary Effects of Man-Made Organic Fiber (MMOF) Dusts. *Critical Reviews in Toxicology***31**, 697-736, doi:10.1080/20014091111965 (2001).

136 Atis, S. *et al*. The respiratory effects of occupational polypropylene flock exposure. *European Respiratory Journal***25**, 110-117, doi:10.1183/09031936.04.00138403 (2005).

137 Chang, X., Xue, Y., Li, J., Zou, L. & Tang, M. Potential health impact of environmental micro- and nanoplastics pollution. *J Appl Toxicol***40**, 4-15, doi:10.1002/jat.3915 (2020).

138 Van Tienhoven, E. A., Korbee, D., Schipper, L., Verharen, H. W. & De Jong, W. H. In vitro and in vivo (cyto)toxicity assays using PVC and LDPE as model materials. *J Biomed Mater Res A***78**, 175-182, doi:10.1002/jbm.a.30679 (2006).

139 Schirinzi, G. F. *et al*. Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells. *Environ Res***159**, 579-587, doi:10.1016/j.envres.2017.08.043 (2017).

140 Mercier, F., Glorennec, P., Thomas, O. & Le Bot, B. Organic contamination of settled house dust, a review for exposure assessment purposes. *Environ Sci Technol***45**, 6716-6727, doi:10.1021/es200925h (2011).

141 Araujo, C. F., Nolasco, M. M., Ribeiro, A. M. P. & Ribeiro-Claro, P. J. A. Identification of microplastics using Raman spectroscopy: Latest developments and future prospects. *Water Res***142**, 426-440, doi:10.1016/j.watres.2018.05.060 (2018).

142 Espinosa, C., Garcia Beltran, J. M., Esteban, M. A. & Cuesta, A. In vitro effects of virgin microplastics on fish head-kidney leucocyte activities. *Environ Pollut***235**, 30-38, doi:10.1016/j.envpol.2017.12.054 (2018).

143 Gustafsson, A., Krais, A. M., Gorzsas, A., Lundh, T. & Gerde, P. Isolation and characterization of a respirable particle fraction from residential house-dust. *Environ Res***161**, 284-290, doi:10.1016/j.envres.2017.10.049 (2018).

144 Elert, A. M. *et al*. Comparison of different methods for MP detection: What can we learn from them, and why asking the right question before measurements matters? *Environ Pollut***231**, 1256-1264, doi:10.1016/j.envpol.2017.08.074 (2017).

145 Kida, T., Hiejima, Y. & Nitta, K.-h. Raman Spectroscopic Study of High-density Polyethylene during Tensile Deformation. *International journal of Experimental Spectroscopic Techniques***1**, 1-6, doi:10.35840/2631-505X/8501 (2016).

146 Prokhorov, K. *et al*. Raman Spectroscopy Evaluation of Polyvinylchloride Structure. *Journal of Physics: Conference Series***691**, 012001, doi:10.1088/1742-6596/691/1/012001 (2016).

147 Vaskova, H., Manas, D., Ovsik, M., Manas, M. & Stanek, M. in *Proc. 16th WSEAS International Conference on Circuits& Systems, Kos Island, Greece*. 329-332.

148 PublicSpectra. <https://publicspectra.com/SpectralSearch> (2019).

149 Wagner, J., Ghosal, S., Whitehead, T. & Metayer, C. Morphology, spatial distribution, and concentration of flame retardants in consumer products and environmental dusts using scanning electron microscopy and Raman micro-spectroscopy. *Environ Int***59**, 16-26, doi:10.1016/j.envint.2013.05.003 (2013).

150 Rauert, C. *et al*. Test chamber and forensic microscopy investigation of the transfer of brominated flame retardants into indoor dust via abrasion of source materials. *Sci Total Environ***493**, 639-648, doi:10.1016/j.scitotenv.2014.06.029 (2014).

151 Kolarik, B. *et al*. The concentrations of phthalates in settled dust in Bulgarian homes in relation to building characteristic and cleaning habits in the family. *Atmospheric Environment***42**, 8553-8559, doi:10.1016/j.atmosenv.2008.08.028 (2008).

152 Voloshina, O. V., Shirshin, E. A., Lademann, J., Fadeev, V. V. & Darvin, M. E. Fluorescence detection of protein content in house dust: the possible role of keratin. *Indoor Air***27**, 377-385, doi:10.1111/ina.12326 (2017).

153 Ghosal, S. & Wagner, J. Correlated Raman micro-spectroscopy and scanning electron microscopy analyses of flame retardants in environmental samples: a micro-analytical tool for probing chemical composition, origin and spatial distribution. *Analyst***138**, 3836-3844, doi:10.1039/c3an00501a (2013).

154 Klein, M. & Fischer, E. K. Microplastic abundance in atmospheric deposition within the Metropolitan area of Hamburg, Germany. *Sci Total Environ***685**, 96-103, doi:10.1016/j.scitotenv.2019.05.405 (2019).

155 Neves, C. *et al*. Simultaneous Separation of Antioxidants and Carbohydrates From Food Wastes Using Aqueous Biphasic Systems Formed by Cholinium-Derived Ionic Liquids. *Front Chem***7**, 459, doi:10.3389/fchem.2019.00459 (2019).

156 Bouwmeester, H., Hollman, P. C. & Peters, R. J. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. *Environ Sci Technol***49**, 8932-8947, doi:10.1021/acs.est.5b01090 (2015).

157 Mahler, G. J. *et al*. Oral exposure to polystyrene nanoparticles affects iron absorption. *Nat Nanotechnol***7**, 264-271, doi:10.1038/nnano.2012.3 (2012).

158 Collard, F. *et al*. Microplastics in livers of European anchovies (Engraulis encrasicolus, L.). *Environ Pollut***229**, 1000-1005, doi:10.1016/j.envpol.2017.07.089 (2017).

159 Wu, B., Wu, X., Liu, S., Wang, Z. & Chen, L. Size-dependent effects of polystyrene microplastics on cytotoxicity and efflux pump inhibition in human Caco-2cells. *Chemosphere***221**, 333-341, doi:10.1016/j.chemosphere.2019.01.056 (2019).

160 Wu, S., Wu, M., Tian, D., Qiu, L. & Li, T. Effects of polystyrene microbeads on cytotoxicity and transcriptomic profiles in human Caco-2 cells. *Environ Toxicol***n/a**, doi:10.1002/tox.22885 (2019).

161 Ramirez, T. *et al*. Prediction of liver toxicity and mode of action using metabolomics in vitro in HepG2 cells. *Archives of Toxicology***92**, 893-906, doi:10.1007/s00204-017-2079-6 (2018).

162 Stock, V., Böhmert, L., Dönmez, M. H., Lampen, A. & Sieg, H. An inverse cell culture model for floating plastic particles. *Analytical Biochemistry*, 113545, doi:10.1016/j.ab.2019.113545 (2019).

163 Heusinkveld, H. J. & Westerink, R. H. S. Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity. *Toxicology in Vitro***45**, 81-88, doi:10.1016/j.tiv.2017.07.030 (2017).

164 Schymanski, D., Goldbeck, C., Humpf, H. U. & Furst, P. Analysis of microplastics in water by micro-Raman spectroscopy: Release of plastic particles from different packaging into mineral water. *Water Res***129**, 154-162, doi:10.1016/j.watres.2017.11.011 (2018).

7. Annexes

7.1. List of articles obtained froma Scopus search using the keywords “Microplastic” and “Toxicity”(October 10th, 2019).

https://www.scopus.com/results/results.uri?cc=10&sort=plff&src=s&nlo=&nlr=&nls=&sid=da1ab68f913930a6bc273c527e37cfd&sot=b&sdt=cl&cluster=scosubjabbr%2c%22PHAR%22%2ct%2bsosubtype%2c%22ar%22%2ct&sl=36&s=TITLE-ABS-KEY%28microplastic+toxicity%29&ss=plff&ps=r-f&editSaveSearch=&origin=resultslist&zone=resultslist

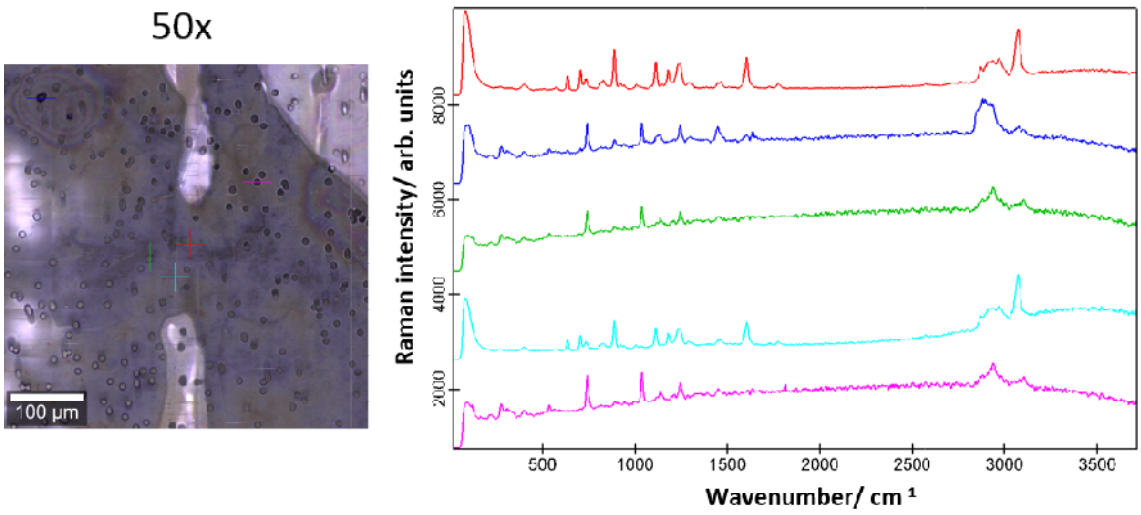
It should be noted that this list may currently return a higher number of articles than those shown in the linked list because of the timeline gap.

7.2. ImageJ script for automated microplastic detection and quantification

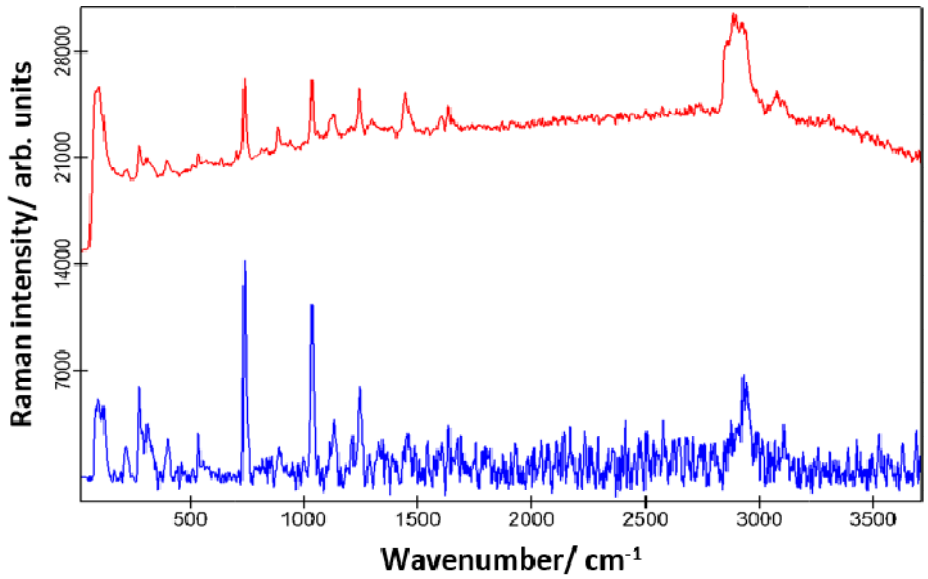
```
dir = getDirectory("Choose a source directory");
dir2 = getDirectory("Chose a output directory");

setBatchMode(true);
list = getFileList(dir);
for (i = 0; i<list.length; i++)
action(dir, dir2, list[i]);
setBatchMode(false);

function action(dir, dir2, filename) {
open(dir + filename);
run("Subtract Background...", "rolling=1500");
run("8-bit");
setAutoThreshold("Default");
//run("Threshold...");
setThreshold(9, 175);
//setThreshold(9, 175);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Analyze Particles...", "size=400-Infinity display clear include");
saveAs("Results", dir2 + filename + "results.csv");
run("Clear Results");
close();
}
```

Adaptedfrom[46]

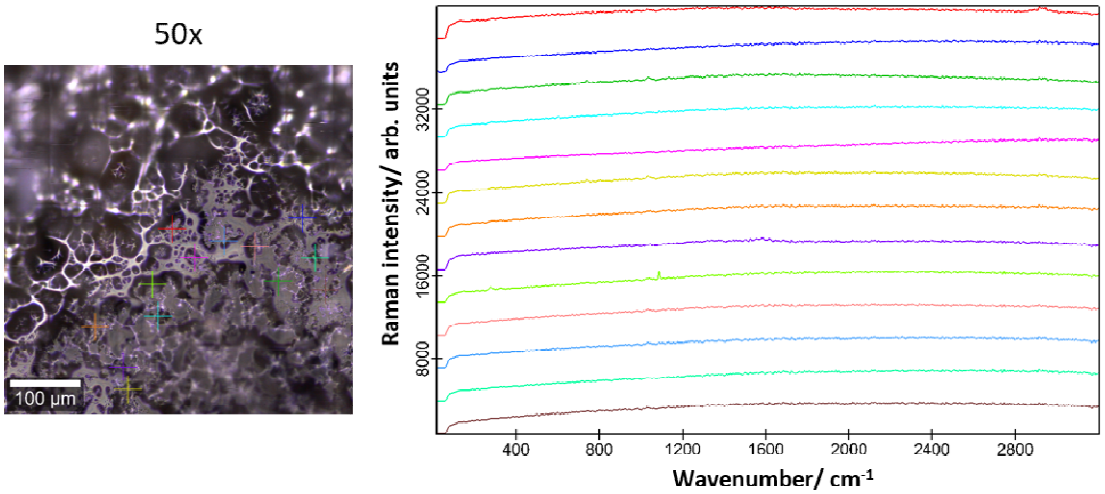
7.3. Stitching image (50x objective) and Raman spectra of [C_4mpy][NTf_2] rich phase from commercial MPs separated through ampoules.



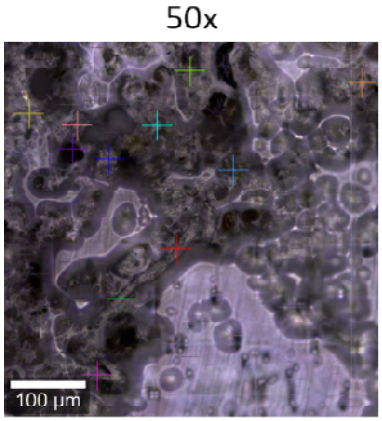
7.4. **Comparison of Raman spectra for $[C_4mpy][NTf_2]$, the red shows $[C_4mpy][NTf_2]$ particles and blue the liquid$[C_4mpy][NTf_2]$.**

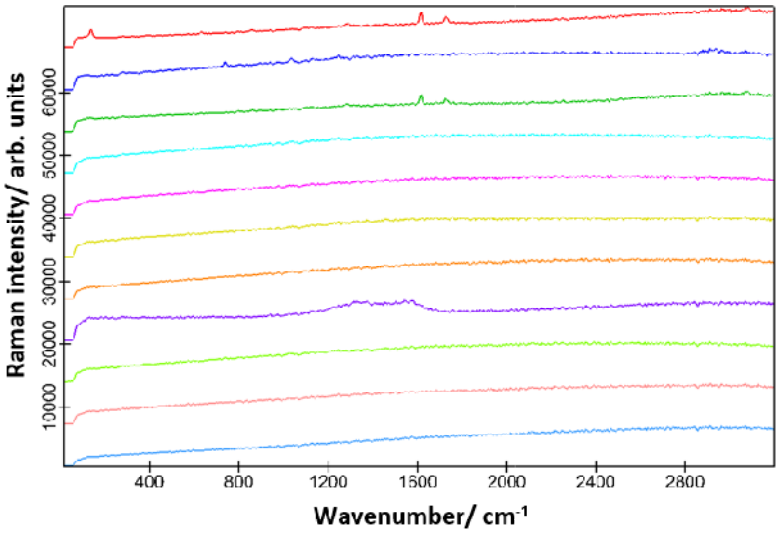


7.5. Stitching image (50x objective) and Raman spectrum of the pellet from house dust separated through ampoules.



7.6. Stitching image (50x objective) and Raman spectra of [C_4mpy][NTf_2] rich phase from house dust separated through ampoules.





7.7. Stitching image (50x objective) and Raman spectrum of $[C_4mpy][NTf_2]$ from commercial MPs and house dust separated through ampoules.