Tutor/s

Dra. Anna Maria de Juan Capdevila
*Departament d'Enginyeria Química i Química Analítica*

Dr. Joaquim Jaumot Soler
*Departament de Química Ambiental IDAEA-CSIC*

Grau de Química

# Treball Final de Grau

**Microplastics determination in environmental samples using a fluorescence-based approach.**

**Determinació de microplàstics mitjançant tècniques fluorimètriques en mostres ambientals**

Albert Menéndez Pedriza

*June of 2019*

UNIVERSITAT DE BARCELONA

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

*Si tingués mil idees i només una resultés ser bona, estaria satisfet*

Alfred Nobel

En primer lloc, agrair als meus dos tutors: al Dr. Joaquim Jaumot per tota la seva ajuda i dedicació en la realització d'aquest treball, i a la Dra. Anna de Juan per haver-me guiat quan ho he necessitat durant el transcurs d'aquest projecte.

Agrair de forma especial al Marc i la Míriam, no només per la seva ajuda, sinó també pel tracte rebut durant tot aquest temps. M'heu fet sentir com un més del grup. Fer una menció especial també a la Judith per tota la seva ajuda en la realització d'aquest treball.

Donar gràcies també a la meva família, en especial als meus pares. Valorar també el suport incondicional que sempre he tingut per part d'en Josep, en Sergi, en Sergio i en David.

Finalment, donar gràcies també a la Montse i la Helena Gonell, sense elles aquest treball mai hagués estat possible.

# REPORT

# CONTENTS

# 1. SUMMARY

In the last decades, plastic environmental contamination has increased significantly. This phenomenon is especially dangerous when it comes to microplastics due to their small size range. Microplastics are defined as synthetic polymers with an upper size limit of 5 mm. This type of plastic debris produces important environmental damages, especially in the marine environment, where microplastics can be ingested by marine wildlife.

For this reason, the qualitative and quantitative determination of microplastics in environmental samples has become a critical issue. This task has become one of the most important research fields for the scientific community due to the matrices complexity, their small size and their natural degradation under environmental conditions. Therefore, there is an urgent need for the development and the implementation of an analytical methodology that allows to find the microplastics determination in a fast, easy and cost-effective way.

In 2010, A. L. Andrady proposed to perform the determination of microplastics using a fluorescence-based approach. Solvatochromic dyes such as Nile Red, allow microplastics to be grouped by polymer polarity and provides the potential to do primary polymer classification. The main problem for the routine implementation of this method is the relatively high cost of Nile Red. In this project, the development and optimization of an analytical fluorescence-based approach using Nile Red as a dye are analyzed. To test the selectivity and efficiency of this analytical approach, different polymer studies were carried out. Moreover, different low-cost fluorescent dyes like Nile Blue A, Eosin Y and Crystal Violet were also tested. The efficiency of the microplastic determination using the analytical fluorescence-based technique developed with Nile Red and the other analyzed dyes were compared.

**Keywords**: microplastics, marine environmental contamination, fluorescent-based approach, Nile Red, Nile Blue.

# 2. RESUM

En els últims anys, la contaminació del medi ambient amb grans quantitats de plàstic s'ha incrementat de forma significativa. La contaminació produïda per part dels microplàstics és especialment greu. Aquests són petites porcions de plàstic de fins a un màxim 5 mm de diàmetre. Degut a la seva grandària, els microplàstics produeixen danys importants en els diferents ecosistemes, especialment en el medi marí, on gran quantitat de fauna els pot arribar a ingerir.

Per tot l'exposat anteriorment, és necessària la determinació qualitativa i quantitativa de microplàstics en mostres ambientals d'una forma ràpida, senzilla i econòmica. Degut a l'enorme complexitat de les matrius, la grandària que presenten aquests tipus de plàstics i la possible degradació que poden patir, aquesta tasca s'ha convertit en un dels majors reptes que existeixen en l'actualitat per a la comunitat científica. Per tant, l'urgent desenvolupament i implementació d'un metodologia analítica que permeti realitzar aquest tipus de determinacions és una tasca realment complexa.

L'any 2010, el Dr. Andrady va proposar un mètode basat en el canvi de la fluorescència observada en certs colorants quan interaccionen amb els diferents microplàstics, com ocorre en el cas del vermell de nil. Aquest colorant té el gran avantatge de presentar un fort caràcter solvatocròmic, fet que li permet poder classificar els diferents tipus de plàstics segons la seva polaritat. El desavantatge principal d'aquest colorant és el seu cost relativament elevat, fet que dificulta la seva implementació en un mètode de rutina. En aquest treball, es presenta el desenvolupament i posterior optimització d'un mètode capaç de poder determinar microplàstics en mostres ambientals mitjançant la seva detecció fluorimètrica degut a la prèvia tinció amb el vermell de nil. Posteriorment, aquest mètode s'ha estudiat utilitzant altres colorants que podrien ser útils per a la determinació de microplàstics utilitzant el mètode analític desenvolupat, com serien el blau de nil, l'eosina Y o el violeta de cristall.

**Paraules clau**: microplàstics, contaminació de l'ecosistema marí, mètode de fluorescència, vermell de nil, blau de nil.

# 3. INTRODUCTION

Nowadays, one of the most important industries in the world is the plastic factory. Plastic global production has increased significantly over the past decades (1.7 million tons in the 1950s to 348 million tons in 2017[1]). Plastics are ideal for a large variety of applications due to their versatility, durability, light-weight and their low-cost production. Many different types of plastic are globally produced, but the most common ones are Polyethylene (PE, high and low density), Poly(vinyl chloride) (PVC), Polypropylene (PP), Polystyrene (PS), Poly(ethylene terephthalate) (PET), Polyurethane (PUR), and Nylon[2].

The continuous increase in synthetic production and poor management in plastic debris through the last years, has led to plastics currently become one of the biggest portions of the municipal waste. In recent years, several studies have revealed that plastic debris accounts for 60-80% of marine litter[3]. It is also known that approximately 8 million tons of plastic waste end up in the marine environment every year[4]. The exact amount of plastic litter that can be found in the oceans has not been reliably calculated, but it is estimated that between 5 to 50 trillion plastic fragments are currently floating on the ocean's surface[5].

Hence, marine litter has become a global environmental problem, due to its persistence, ubiquity and toxic potential. For that reason, one of the most critical and important research fields for the scientific community is the identification and quantification of plastics in environmental samples. This is especially remarkable for microplastics (MPs) due to their small size range. Figure 1 corroborates this reality. As it is shown, the number of academic publications about microplastics has increased almost exponentially over the last years.

Another remarkable fact that summarizes the increasing importance of microplastics in the modern society could be that the Fundéu BBVA, which is assessed by the *Real Academia Española* (RAE), has chosen this word as the word of the year 2018[6].

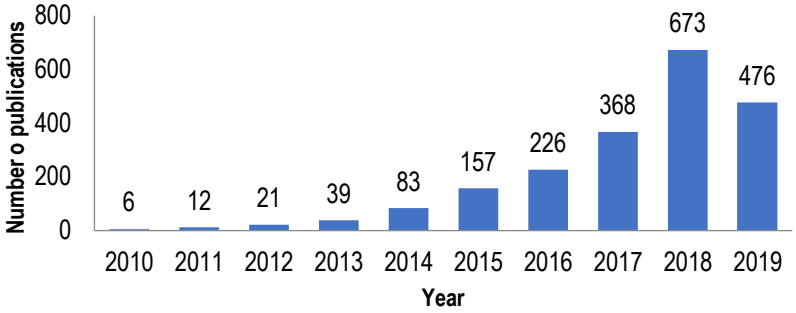



Figure 1. Publications by year (2010 –2019) using the term 'microplastics' in Web of Science. (checked on June 7th 2019)

### 3.1 MICROPLASTICS: DEFINITION AND CLASSIFICATION

Plastic debris can be divided into macroplastics, mesoplastics, microplastics and nanoplastics depending on their size range[7]. Small pieces of floating plastics in the ocean surface were firstly reported in the scientific literature in the early 1970s, but it was not until 2009 that the National Oceanic and Atmospheric Administration (NOAA) defined 'microplastics' as synthetic polymers with an upper size limit of 5 mm[8]. Below 1 µm scale, plastics should be defined as nanoplastics, the least known part of the marine waste. In table 1, the full classification of plastics by size is shown[9–11]. Microplastics can be categorized into primary and secondary microplastics. Manufactured plastics for industrial or domestic applications of microscopic scale are classified as primary microplastics. They are used in facial cleansers, toothpaste, cosmetics (e.g. shower gels) or textiles. Primary microplastics can be used in medicine, for example as vectors for drugs. Virgin plastic pellets are also considered as primary microplastics[9,10]. The estimate ranges of the global release of primary microplastic into the marine environment are between 0.8 and 2.5 Mtons/year according to an optimistic or pessimistic scenario respectively[11]. It is estimated that they represent between 15-31% of the total MPs in the oceans.

*Table1. Classification of plastics by their size.*

| ***Size Range*** | **Terminology** | |
|---|---|---|
| *>20cm* | Macroplastic | |
| *5-20cm* | Mesoplastic | |
| *1-5mm* | Large | Microplastics |
| *1-1000µm* | Small | |
| *<1000µm* | Nanoplastic | |

Larger plastic waste in the ocean and land can be fragmented into smaller particles over time due to degradation processes (i.e. physical, chemical and biological). These types of

microplastics are categorized as secondary microplastics. A variety of factors control their rate of fragmentation. The different degradation types can be described as biodegradation (i.e. the action of living organisms), photodegradation (i.e. light radiation), thermooxidative degradation (slow oxidative breakdown at moderate temperatures), thermal degradation (i.e. high temperatures) and hydrolysis (i.e. water)[1]. A combination of environmental factors and properties of the polymers influences the degradation process of macroplastics to microplastics.

UV radiation commonly starts the photooxidative degradation of exposed polymers in the marine environment. After that, other types of degradation such as thermooxidative degradation can occur without the need for exposure to UV-radiation.

It is important to mention that degradation initiated by solar UV radiation is a very effective mechanism in plastics exposed in the air or lying on a beach surface. Instead, it is much less efficient when the plastic material is floating in seawater. The lower temperatures and the lower oxygen concentration in water environments could explain this behavior. Degradation of plastic debris in oceans can be slowed down by fouling effects too[1]. For this reason, the most common place for the generation of microplastics in the marine environment is the beaches sand.

## 3.2 MICROPLASTICS SOURCES AND DISTRIBUTION IN THE MARINE ENVIRONMENT

Microplastics can be from land and ocean provenance. The land-based sources contribute to the 80% of the total microplastic debris in the marine environment[10]. The fact that the main types of degradation are much more efficient on land sources than in water environment could be a possible explanation for it. Microplastics formed on terrestrial sources can enter the marine environment via different pathways. Primary microplastics can be introduced in aquatic nature through industrial or domestic drainage principally. Secondary microplastics can be introduced in marine habitat via storms, sewers, wind, currents or overland flow. Sewage sludge could be another possible source of microplastic litter[9]. All this information is represented in Figure 2.

Microplastics have been found in almost every marine habitat around the world, even in Arctic and Antarctic waters, probably transported by ocean currents and wind. They can be suspended in the water column, surface waters, coastal waters, estuaries, rivers, beaches and deep-sea sediments.

Suspended in the water column, microplastics can be trapped in ocean currents and accumulated in central oceanic regions. The nature and inherent properties of MPs like shape

density or size influence their distribution in the ocean. The localization of the MPs source, ocean currents, wind and the subsequent complex interaction of physical, chemical and biological processes are also decisive. Currently, there is growing information about all these aspects. Many studies about the abundance and composition of microplastics have been carried out in all the continents. Even so, there are still major uncertainties about the spatial and temporal distribution of microplastics [8,11].

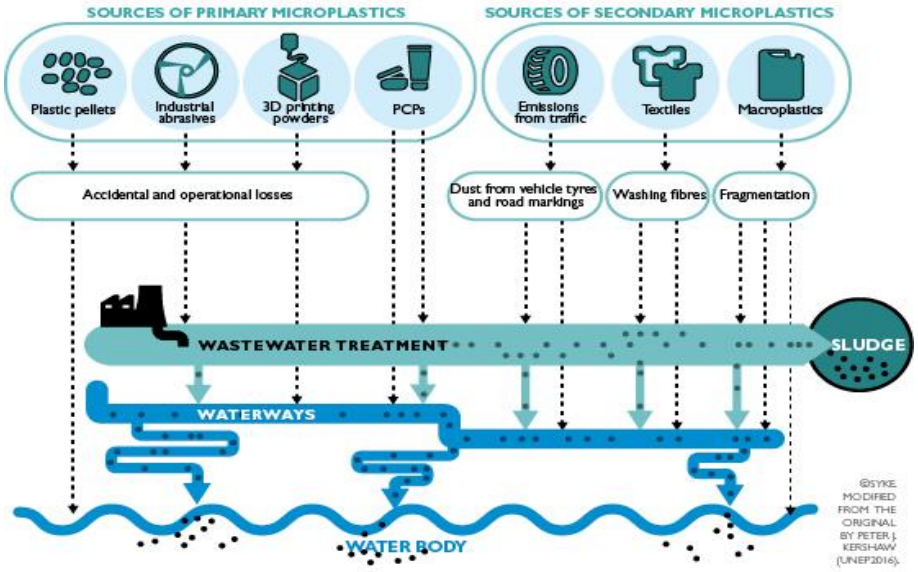



Figure 2. Summary of possible MPs land-sources. (With permission from Peter Kershaw and the Finnish Environment Institute)[12].

## 3.3 ENVIRONMENTAL IMPACT OF MICROPLASTICS

The potential harm that MPs can impose on ecosystems, marine organisms or humans explains the recent interest of the scientific community in this type of plastic debris. The environmental impacts of MPs can be classified in physical, chemical and biological effects.

Physical impacts include the entanglement and the ingestion of microplastics. Usually, entanglement is commonly related to macroplastics. Intake does not directly impose fatal effects. Over time, it can cause death by toxicity through many different mechanisms (chemical and biological impacts). Ingestion of microplastics can be found in almost all trophic levels, from zooplankton to whales[8].

The toxicity of microplastics can come from the polymeric compounds used in the production of plastics, as well as from additives added during their production in order to improve physical properties such as color, density, resistance or hardness. For example, PVC must have some plasticizers like phthalates and bisphenol A to reduce photodegradation. Other compounds can include some heavy metals, like chromium and cadmium.

Microplastics can also absorb organic pollutants from seawater. Due to the large surface area to volume ratio of microplastics, MPs tend to absorb waterborne contaminants like persistent organic pollutants (POPs), including polychlorinated biphenyl (PCBs), dichlorobiphenyl trichloroethane (DDTs) and polycyclic aromatic hydrocarbon (PAHs), or heavy metals. They are absorbed in higher concentrations than in the marine environment, making them much more toxic and deadlier. In addition, microplastics that have absorbed POPs can contaminate other ecosystems across ocean currents or wind.

The danger posed by the high concentration of POPs is particularly significant. POPs are very stable halogenated organic compounds with high lipid solubility. For that reason, they can accumulate in fatty tissues once they are ingested by marine organisms, causing several issues, even death.

Furthermore, this material ingested by marine biota can be transported along the food chain, and even reach humans. Toxicological effects of ingestion of microplastics have also been examined in many different studies[13–16]. These studies show that ingestion of plastics produces changes in the metabolism, endocrine function, behavior or reproduction.

## 3.4 MICROPLASTICS ANALYSIS IN ENVIRONMENTAL SAMPLES

In order to determine the real ecological and biological risks of microplastics, there is a pressing need to develop and implement standardized protocols for the analytical process to determine microplastics in different environmental matrices. Particularly, for MPs found in seawater, freshwater and sediments.

### 3.4.1. General sampling methods

Collecting appropriate samples is the first critical step to quantify microplastics from the marine environment. Sampling methods can be categorized into selective sampling, bulk

sampling and volume-reduced sampling[4]. Ocean currents or sample sediment type must be considered to choose an appropriate sampling method.

### 3.4.2. Sample extraction and purification

Firstly, microplastics must be separated from the initial matrix to simplify their identification/quantification. Many different methods are used to isolate microplastics from the initial matrix, such as filtration through size fractionation or sieving through size exclusion, but the most common one is the density flotation. In this technique, microplastics are separated from high-density sand, mud sediment and other sample matrices employing a high-density solution. NaCl solution (~1.20 g/cm$^3$) is the most used solution in the separation process due to its low cost and lack of toxicity. To separate high-density microplastics like PVC, NaI solutions (~1.80 g/cm$^3$), or $ZnCl_2$ (1.50-1.70 g/cm$^3$) can be used despite its high level of toxicity[17].

Subsequently, a purification approach must be performed to remove all the organic matter present on the surface of microplastics. To prevent the overestimation of synthetic particles in environmental samples, it is critically important that biological materials like wood, chitin, or shells are also eliminated. In conclusion, this process is very important since it allows clear identification of microplastics in environmental samples. Most common purification methods use a 30-35% hydrogen peroxide ($H_2O_2$) digestion[18]. Enzymatic digestion can also be used[9].

### 3.4.3. Identification and quantification techniques

There are many different analytical techniques used to carry out a qualitative and quantitative analysis of microplastics in environmental samples. Identification methods are based on the physical or chemical behavior of microplastics. Commonly, the combination of two different analytical approaches has been widely used, due to the difficulty to identify microplastics of various sizes, shapes and polymer types from complex environmental samples using only one analytical method. Usually, the microplastic analysis consists of two different steps: physical characterization of potential microplastics (e.g. microscopy) followed by chemical analysis for the confirmation of plastics (e.g. spectroscopy).

The most common quantification technique for microplastics is visual counting. Large plastics can be sorted out directly, but smaller-sized ones need further observation, normally using a microscope or the naked eye. This process is necessary, especially when the purification process

is not completely optimized. Electron microscopy (SEM-EDS) can be also applied when the microplastic size is <100 μm[19].

Afterwards, the characterization of microplastics is performed to confirm the synthetic procedure of the particles selected in the previous step. The most important approaches are Raman spectroscopy[9,20] and Fourier-transform infrared spectroscopy (FT-IR)[3,7,21]. Other approaches such as liquid chromatography (HPLC)[22], and gas chromatography coupled to mass spectrometry (Pyro-GC/MS)[18,23], are also used.

Quantitative data is needed to illustrate the abundance of microplastics in environmental samples. The concentration units "particles per $m^2$" or "particles per $m^3$" are widely used to characterize microplastics in the surface water, meanwhile "particles per $m^3$" is the habitual concentration unit used to quantify microplastic concentrations in the water column[18].

In Table 2, the advantages and limitations of the most common methods applied nowadays in the identification and quantification of microplastics in environmental matrices are presented.

*3.4.3.1 Emerging approaches*

Currently, the main problem existing in the microplastics determination in environmental samples is the non-existent of standardized, efficient, low-cost, effective, capable and robust methods for the quantification and identification of microplastics. This is the principal reason for the scientific community to improve and develop new analytical approaches in order to reduce time, costs, and efforts to detect these plastics in complex environmental samples. Some new analytical approaches recently tested are NMR[24], NIR[25,26], tagging method[9] or thermogravimetry[27].

Another promising approach proposed by A. L. Andrady in 2010, consists of the use of fluorescence lipophilic dyes such as Nile Red (NR) to stain microplastics. Performing these methods, microplastics visualization under the microscope is facilitated. The development of this analysis has not been followed until now[28]. Fluorescence staining approaches are very selective, fast, simple and inexpensive methods that can be very helpful to determine, in a qualitative and quantitative way, the microplastics in complex environmental samples as a routine method.

Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a lipophilic fluorescent dye which allows the *in-situ* staining of hydrophobic molecules, like lipids. For this reason, Nile Red is widely used in many different applications such as the determination of the lipid content in animal

Table 2. Summary of advantages and limitations of commonly used analytical methods for analysis of microplastics.

| ***Method*** | | **Advantages** | **Limitations** |
|---|---|---|---|
| *Visual Counting* | *Microscopy counting* | - Microplastics can be identified quickly, easily and in low cost-effective way | - May not provide accurate information<br>- Difficult to differentiate microplastic from other particles with similar size<br>-High possibility of under or overestimating the abundance of microplastics (especially for microplastics <1mm in size)<br>- No chemical confirmation |
| | SEM-EDS | -Provides a clear and high-magnification images of plastic particles<br>-Elemental composition of particles can be determined | - A high cost but effective technique<br>- Requires laborious sample preparation steps and substantial amount of time for examination<br>- No detailed information for identification is available |
| Spectroscopic Method | Raman spectroscopy | - Only available to analyze particles <1-2 µm<br>- Non-destructive analysis<br>- Reduction of false negative data<br>- No possibility of false positive data by chemical confirmation<br>- Functional non-polar groups and symmetric bonds can be easily identified | - Several interferences of fluorescence from organic and inorganic impurities<br>- Expensive instrument<br>- Requires laborious sample pre-treatment steps and substantial amount of time for the whole particle identification |
| | FT-IR | - Can detect microplastics down to 10-20µm<br>- Non-destructive analysis<br>- Reduction of false negative data<br>- No possibility of false positive data by chemical confirmation<br>- Functional polar groups of the polymers can be easily identified | - Expensive instrument<br>- Sample must be pre-treated to eliminate IR-active water<br>-Non-transparent particles could be difficult to analyze<br>- Spectrum is susceptible to variations by the surface condition of samples<br>- An expert with experience in interpreting spectra is needed |
| *Chromatography* Method | Pyro-GC-MS | - Simultaneous analysis of polymer type and additive chemicals<br>- Pre-treatment of the sample is not required<br>- Sensitive and reliable approach | - Destructive method<br>- Information about number, size or shape of microplastics is not obtained<br>- Representative of the sample may be compromised<br>- Complex instrumental and data processing<br>- Pyrolysis database available only for some selected polymers |
| | HPLC-SEC-RI | - The recoveries of polymers are quite good<br>- Quantification results can be achieved | - Information about number, size or shape of microplastics is not acquired<br>- Only a small amount of sample can be evaluated per run<br>- Only specific polymers can be analyzed by this method |

cells and microorganisms, for detecting intracellular lipid droplets as well as for flow cytofluorometry[29] or in polymer chemistry to stain synthetic polymers[20]. It is also used to probe the microenvironment of polymers, xerogels, liquid crystals and zeolites[30] because of its solvatochromic behavior (Figure 3).

Figure 3. Solvatochromic behavior of Nile Red can be easily seen when it is dissolved in different solvents. From left to right: hexane, tert-butyl methyl ether, chloroform, acetone, ethanol, methanol, acetone-water (1:1) and water

Because of this solvatochromic response, NR emission spectra shifts depend on its environmental polarity. That fact allows NR fluorescence approaches categorize microplastics according to their general hydrophobicity/polarity. That evidence can be seen in Thomas Maes *et al*. study[28]. They could also provide a good indicator to evaluate changes in surface properties due to oxidation or biofouling in the environment. Ingestion studies of microplastics and their effects by biota could also be carried out using this kind of methods. The main problem of Nile Red is its relatively high-cost, making these methods difficult to be implemented as a routine technique. For that reason, in this research, more cost-effective dyes will be tested in order to determine their efficiency and applicability in routine fluorescence staining methods comparing with NR.

# 4. OBJECTIVES

The main aim of this project is the development of a fluorescence-based analytical approach for the microplastic determination in environmental samples. This study attempts to establish a standardized protocol for the analysis of microplastics in complex matrices. To achieve that, some minor objectives must be accomplished:

- Optimization of the staining conditions for the chosen method using Nile Red considering experimental factors such as polarity of the solvent, time of incubation, the concentration of the dye and time of heating.
- Evaluation of the optimized NR staining method using various microplastics types (polyethylene, polypropylene and polystyrene).
- Assessment of the staining procedure using alternatives dyes (Nile Blue A, Crystal Violet and Eosin Y).
- Apply the optimized method in environmental samples, considering different types of experiments: a study of industrial plastics, plastics in complex matrices (sediments) and photodegraded plastics.

# 5. EXPERIMENTAL SECTION

## 5.1. CHEMICALS AND REAGENTS

The different fluorescent dyes tested are:

- Nile Red, CAS 7385-67-3, TCI
- Crystal Violet, CAS 548-62-9, Sigma Aldrich, (certified by biological stain commission)
- Nile Blue A, CAS 3625-57-8, Sigma Aldrich, (dye content ≥ 75%)
- Eosin Y, CAS 15086-94-9, Sigma Aldrich, (dye content ~ 99%)

Cospheric (USA) provide the microplastics used as references during the experimental part. Microplastics have the following specifications:

Table 3. Specifications are given by Cospheric about MPs used in the experiments.

| **Name** | **MPs type** | **Color** | **Particle size** [µm] | **Density** [g/cm$^3$] | **Quantity** |
|---|---|---|---|---|---|
| $PE_g$ | polyethylene | Fluorescent Green | 425-500 | 1.00 | 10g |
| $PE_s$ | polyethylene | Clear | 710-850 | 0.96 | 10g |
| $PE_l$ | polyethylene | Clear | 1700-2000 | 0.96 | 10g |
| PP | polypropylene | White | 2400-2500 | 0.90 | 100 spheres |
| PS | polystyrene | Clear | 1890-2100 | 1.06 | 100 spheres |

The various solvents employed are:

- Acetone for analysis-ACS-ISO-Reag.Ph.Eur-Reag.USP, Carlo-Erba Reagents
- Propan-2-ol for analysis ACS-Rreag.Ph.Eur-Reag.USP, Carlo-Erba Reagents
- Chloroform for analysis ISO-ACS-Reag.Ph.Eur-Reag.USP, Stabilized with ethanol, Carlo-Erba Reagents
- Methanol for liquid chromatography, Merck
- Ethanol absolute for analysis ACS-ISO-Reag.Ph.Eur., Emsure
- Hexane for gas chromatography, Merck
- Milli-Q water

Other reagents used are:

- Tween 80, CAS 9005-65-6, Sigma Aldrich.
- Potassium chloride, CAS 7447-40-7, Sigma Aldrich

## 5.2. EQUIPMENT

The diverse apparatus used during this project are:

- Agilent Cary Eclipse Fluorescence Spectrometer (Agilent Technologies, USA)
- Atlas Suntest CPS (Atlas, USA)
- Plant Grow Chambers MLR-352-H (Panasonic, Japan)
- 3D Sunflower Mini-Shaker (Biosan, Latvia)
- Conterm drying oven (J.P. Selecta, Spain)
- Sample Concentrator (Techne, UK)
- Milli-Q Integral 3 purification system (Merck, USA)

## 5.3. STAINING PROCEDURE

Different approaches were tested to optimize the staining of microplastics by Nile Red. Standard $PE_s$ were employed in the optimization of the staining procedure. The "Aqueous" staining method was used as described by Thomas Maes *et al.*[28], with some adaptations. "Organic" staining approach was based on the approach described by Matthias Tamminga *et al.*[31].

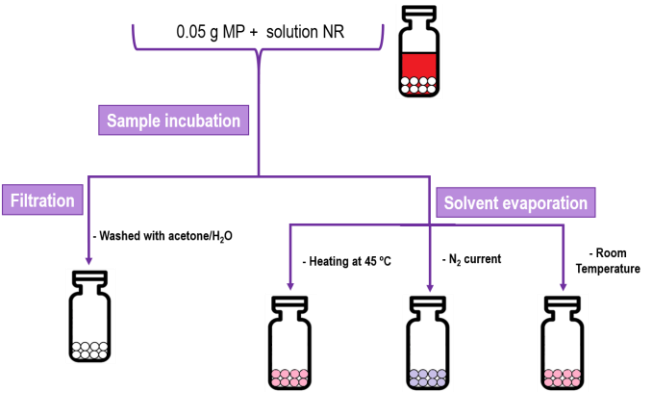



Figure 4. Generic scheme of "Organic" staining method.

The general scheme of "Organic" method is summarized in Figure 4. In the "Organic" method, staining was carried out analyzing diverse solvents (acetone, acetone-water (1:1), methanol,

ethanol, hexane, and chloroform) at varying concentrations of NR (10, 100, 500 and 1000 µg/mL). Many solvent volumes were also tested (300 µL, 0.5, 1 and 2 mL). As it is shown, after the sample incubation two different procedures were analyzed. The filtration step was evaluated using acetone or water. On the other hand, the solvent was also evaporated directly using three different procedures: at room temperature, heating MPs at 45 ºC or using an $N_2$ current.

The "Aqueous" method procedure is represented in Figure 5. In this procedure, once sample incubation had finished, diverse filtration options were evaluated. MPs were washed with acetone or water. After that, MPs were dried at room temperature or 45 ºC.

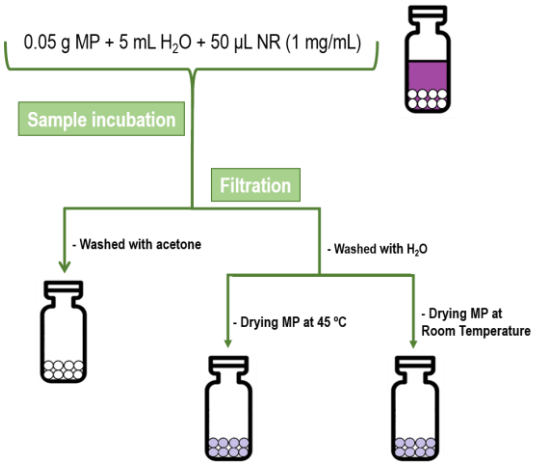



Figure 5. General scheme of "Aqueous" staining method.

Preliminary results showed that MPs were better stained using the "Aqueous" method. The optimization of this method was carried out testing seven different adsorption times (15 min, 30 min, 60 min, 120 min, 4h, 6h and overnight) in the incubation step. Time of heating at 45 ºC was also optimized (1h, 2h, 3h, 4h, 5h and overnight). After that, other dyes (Nile Blue A, Crystal Violet, Eosin Y) were tested using the previously optimized conditions for NR.

## 5.4. APPLICATION OF THE MPs STAINING METHOD TO ENVIRONMENTAL SAMPLES

### 5.4.1. Samples and studies

Different types of environmental samples were analyzed during this project. All the sediment samples used in this project were collected in the *Castelldefels* beach. Diverse plastic types were used in the experiments performed as spiked samples. Plastics types can be classified in:

- Reference microplastics (see table 3).
- Industrial plastics (PET bottle, PP and PS plastic glasses), which were ground until their size scale was appropriate.

The different studies carried out in this project can be divided into:

- Staining efficiency evaluation: In these experiments, both types of plastic were used. Reference samples (without sediments) and sediment spiked samples were assessed.
- Assessment of the staining method in photodegraded plastics: Only standard microplastics were used. Reference plastics were evaluated in a high-intensity photodegradation process (30 min and 6 hours at 400 W/m$^2$ (1h at 400 W/m$^2$ is equivalent to 40 days of UV daylight dose in Madrid[32])) and a low-intensity photodegradation process using an environmental chamber (see more details in the Appendix 1). Reference samples (without sediments) and sediment samples were studied.

### 5.4.2. Sediment samples treatment

To separate the microplastics in sediment samples, a density flotation step was performed before the staining method was carried out. In this project, a KCl (sat.) solution (~1.18 g/cm$^3$) was used. The workflow performed is detailed below.

The staining method was directly carried out without filtrating the sediments. Preliminary tests performed showed that no interferences of the MPs adsorbing process were caused by the sediments. Once the staining process was finished, a visual classification of the different types of MPs was carried out. After that, following the optimized "Aqueous" procedure, MPs were dried at 45 ºC. To test the density flotation efficiency, a recovery study using the reference microplastics was performed.

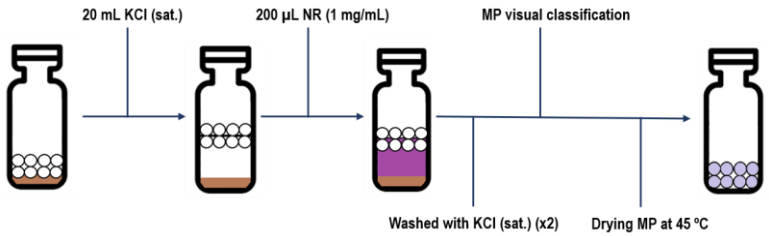



Figure 6. General scheme of the sediment samples treatment.

## 5.5. FLUORESCENCE MEASUREMENTS

### 5.5.1. Theoretical background

Luminescence is the emission of light from an atom or molecule and it happens because of electronically excited states. For that reason, luminescence follows the absorption of electromagnetic energy and can be divided into two categories depending on the nature of the excited state: fluorescence and phosphorescence.

Fluorescence can be described as a type of luminescence phenomenon in which light emission goes from the first excited electronic singlet level ($S_1$) to its ground electronic level ($S_0$). On the other hand, phosphorescence is the emission of light which arises from its triplet excited state ($T_1 \rightarrow S_0$). In this case, the energy provided by the photon, makes the electron undergo to a metastable level. This phenomenon explains why the emission rate in fluorescence ($10^8$ $s^{-1}$) has higher values than in phosphorescence (1-$10^3$ $s^{-1}$). All these facts are summarized in the Jablonski's diagram. These diagrams depict the changes in the energy of a molecule or atom when it absorbs and emits photons.

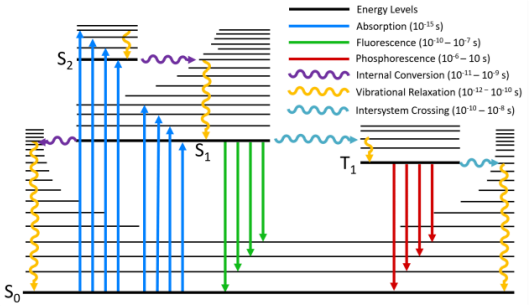



Figure 7. Jablonski's diagram (With permission of Edinburgh Instruments)[33].

Fluorescent chemical compounds are also called fluorophores. Fluorescence typically occurs in aromatic compounds which displays a number of general attributes[34,35]:

- Fluorescence always occurs at longer wavelengths than the excitation (Stokes shift).
- The shape of emission spectra does not change as the excitation wavelengths are varied (Kasha's rule).
- Excitation spectra have the same shape as absorption spectra.
- Emission spectra are a mirror image of the absorption band of the least frequency in a good approximation (Mirror image rule).

### 5.5.2. Instrumental measurements

Fluorescence measurements are typically carried out analyzing the emitted perpendicular fluorescence by a sample. In other words, the light source is in a 90-degree angle with the detector. Four-faced transparency quartz cuvettes are widely used. In this project, microplastics fluorescence analysis is performed employing wellplates. Although initially they were designed for cell cultures and cell assays, nowadays, this type of fluorescence measurement is commonly applied for intensity analysis of many samples. For example, these systems are commonly used in the drug discovery field. In Figure 8, a scheme of the two operating procedures of the fluorescence measurements are presented: either with the light beam hitting the sample surface (1) or illuminating the plate under the sample cell (2).

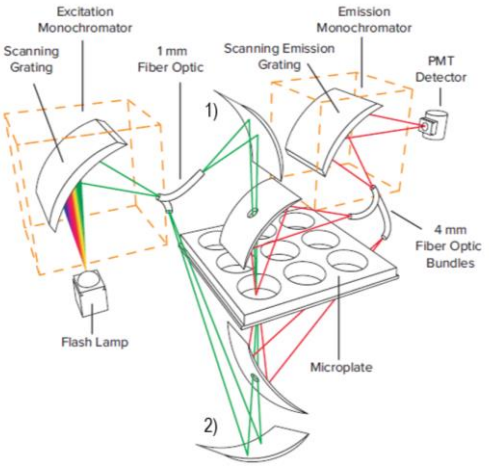



Figure 8. Representation of fluorescence measurement using a microplate (With permission of Molecular Devices)[36].

Another important fact worth commenting is that MPs fluorescence measurements are conducted in the solid-state. Usually, solid systems do not allow to apply fluorescence approaches for analytical applications. Measuring MPs in solution involves many extraction problems and measurement difficulties. The reproducibility of experiments is also compromised using this type of measurements. On the other hand, solid analysis allows much easier extraction steps such as filtration or solvent stage. That fact was shown on the preliminary studies performed.

# 6. DYES AND MPS FLUORESCENCE PRELIMINARY STUDIES

Several staining approaches were tested with the aim of optimizing the microplastics dyeing workflow. Firstly, how fluorescence measurements would be carried out had to be solved. Typically, the fluorescence of compounds is analyzed in solution. Despite that, in the literature, most microplastics fluorometric analysis were performed in solid-state using many filter imaging methods[5,28,37]. As microplates were used in this project, preliminary studies had to be done to assess the suitability of the approach.

With that purpose, the fluorescence spectra of the diverse dyes used during this project were measured in solution. As it was expected, good results and reproducibility were obtained. Spectra registered for NR using solvents with different polarity are presented in Figure 9.

As it is commented in Erni-Cassola *et al.* work, these results agree with previous studies performed about the solvatochromic behavior of Nile Red, which favors detection of strongly hydrophobic samples at short excitation wavelengths. Hydrophilic samples are ideally visualized at longer excitation (i.e. 515-560 nm) and emission wavelengths (≥ 590 nm)[5].

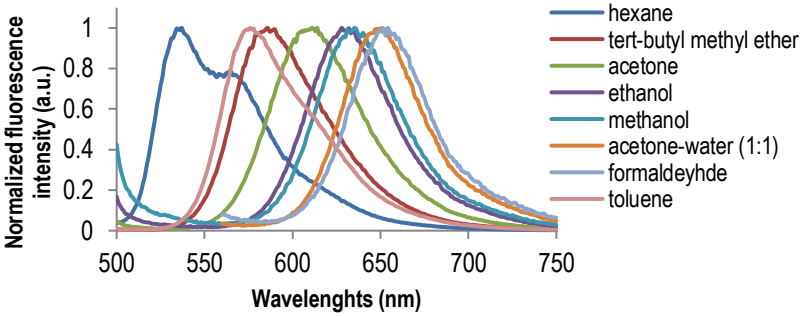



Figure 9. NR solvatochromic behavior is shown. All the spectra were observed at $\lambda_{ex}$=490 nm, excepts formaldehyde and acetone-water (1:1) which were recorded at $\lambda_{ex}$=540 nm.

Fluorescence spectra of NR in the various solvents could be justified appealing to the mirror image rule, which is related to the Franck-Condon principle. As T. J. Zuehlsdorff commented on

his work Nile Red experimental absorption spectrum in the visible light region is characterized by a single strong feature using polar solvents such as acetone or alcohols. Furthermore, in non-polar solvents like cyclohexane or hexane, the single absorption band is found to exhibit two separate identifiable peaks with a tail. These spectral differences can be explained looking at NR structure. The dye consists of a 2-diethylamino group connected to an aromatic ring system via a single C-N bond, as is shown in Figure 10. The $S_1$ state corresponding to a HOMO-LUMO transition with strong oscillator strength dominates its absorption and fluorescence spectra. Different quantum chemistry studies have shown that rotation around the C-N bond is a key feature influencing the dye's absorption and fluorescence spectra[38]. These facts were experimental proved, as is seen in Figure 9. Results from other studies performed in this project confirmed that NR excitation spectra have the same shape than the absorption spectra. It was also confirmed that fluorescence spectra, to a good approximation, are a mirror image of the absorption spectra.

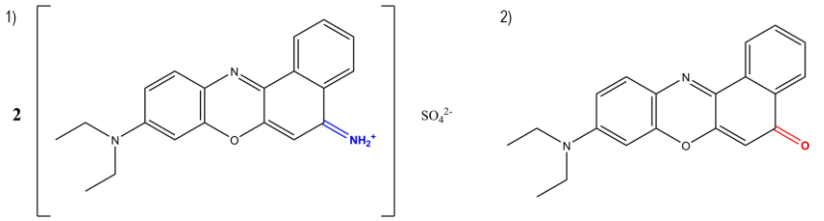



Figure 10. Structures of Nile Blue A (1) and Nile Red (2).

Fluorescence spectra of the other dyes tested during this project were also measured. Eosin Y and Nile Blue A fluorescence spectra are shown in the Appendix 2 and 3.

After that, fluorescent microplastics studies started with different measurements of $PE_g$. These measurements were performed in solution and in solid-state. Results achieved indicated that measuring microplastics in solutions was less reproducible than in the solid-state. This fact was caused by the poor reproducibility obtained in the transfer of MPs to the microplate. Hydrophobic interactions could explain this fact. In addition, density variations between MPs made that the transfer of microplastics to the wellplate from the vials, and fluorescence measurements were more difficult to reproduce. Although an emulsifier agent was used (Tween 80), the commented problems could not be eliminated.

In contrast, good reproducibility was achieved in the solid-state. In a good approximation, the fluorescence intensity in the solid-state measurements is determined by the number (weight) of

fluorescent plastic particles introduced in the wellplate when there are no interferences. As it was experimentally confirmed, fluorescence intensity depends on the thickness of the plastic particles in the microplate. This approximation was experimentally confirmed thanks to a simple experiment using different masses of $PE_g$. The results obtained are represented below. As is shown, a linear correlation between fluorescence intensity and the plastic mass analyzed was demonstrated in the range studied. When the fluorescence intensity was measured in ≥ 0.1 g of $PE_g$, the linear correlation disappears. That fact could be explained by how the spectrofluorometer makes the fluorescence measurements.

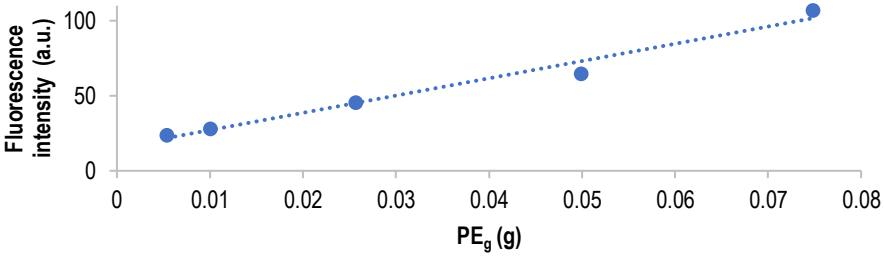



Figure 11. Average fluorescence intensity achieved at the analysis of diverse replicates of $PE_g$ weights. The $R^2$ obtained is 0.9784.

# 7. DEVELOPMENT OF THE STAINING METHOD

## 7.1 PRELIMINARY STUDIES

Preliminary studies were conducted comparing the "Aqueous" and the "Organic" methods. The "Organic" approach was evaluated using different types of solvent. The best results were obtained using hexane, chloroform and acetone, which matched with the results found in the literature[31]. Despite that, in the "Organic" approach, many experimental problems were found:

- Filtration step could not be performed successfully using acetone or water. NR adsorbed in the surface was removed using this experimental step.
- After using an $N_2$ current for the solvent evaporation, no fluorescence signal was observed.

- ❖ Solvent evaporation at room temperature was a very slow process (1 week was required to observe fluorescence signal).
- ❖ Drying microplastics at room temperature or heating the solution at 45 ºC, NR crystals were formed. These crystals make it difficult to obtain a clear fluorescence signal that belongs to stained microplastics.

On the other side, the "Aqueous" method only presents no successful results when acetone was used on the filtration step (as occurs in the "Organic" method, a discolor procedure takes place). Drying microplastics at room temperature, as happens on the "Organic" method, a week was approximately needed to obtain good fluorescence signals. When performing this step at 45 ºC, it is necessary to keep it overnight to obtain a satisfactory signal. That fact could be explained by the nonfluorescence behavior presented by Nile Red in hydrophobic environments. Therefore, only the "Aqueous" method using water on the filtration step and drying microplastics at 45 ºC was properly optimized.

## 7.2 AQUEOUS METHOD OPTIMIZATION

The results obtained from the different experiments performed allowed to optimize the "Aqueous" method. The factors optimized as it was explained in the Experimental Procedure section were the incubation and drying time. The results achieved from the optimization experiments performed are presented in Table 4.

Table 4. Results acquired from the optimization of the "Aqueous" method.

| **Incubation** | **Fluorescence intensity** [a.u.] | **Drying times** | **Fluorescence intensity** [a.u.] |
|---|---|---|---|
| 15 min | 44.8 | 1h | 24.9 |
| 30 min | 50.6 | 2h | 32.9 |
| 60 min | 122.5 | 3h | 39.7 |
| 120 min | 105.3 | 4h | 44.1 |
| 4h | 107.3 | 5h | 51.9 |
| 6h | 103.1 | overnight | 132.3 |
| overnight | 109.8 | | |

(a) The incubation times were performed using a drying time of overnight.

(b) The drying times were carried out using an incubation time of overnight.

Thereafter, the incubation time used was 1 hour. On the other hand, microplastics drying step was carried out overnight. The reached results match with the conclusions exposed by Thomas

Maes *et al.*, who suggested that incubation times longer than 30-60 minutes led to a gradual aggregation of the unadsorbed dye (which has low water solubility and precipitates)[28]. For this reason, fluorescence intensity remained constant after 60 minutes of incubation. The excitation wavelength was also optimized using $PE_s$. For Nile Red, an excitation wavelength of 470-510 nm produces higher emission intensities. That fact agrees with the previously realized studies. The fluorescence measurements obtained once the method conditions were optimized are depicted in Figure 12.

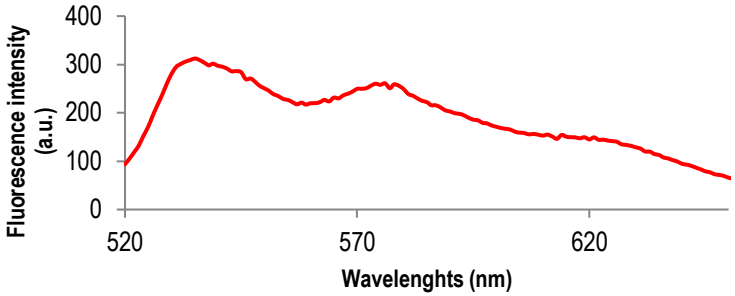



Figure 12. Fluorescence spectrum obtained by analysing stained $PE_s$ using the optimised conditions of the staining method. The excitation wavelength used was 500 nm.

After that, the reproducibility of the stained method was tested. To analyze it, the same experimental procedure for the analysis of the reproducibility in the solid-state measurements was performed. The results are shown below. As can be seen, the linear correlation between emission intensity and the number of particles in the wellplate was similar using $PE_g$, which are covalent-bonded to a green fluorescent dye and adsorbed stained PE.

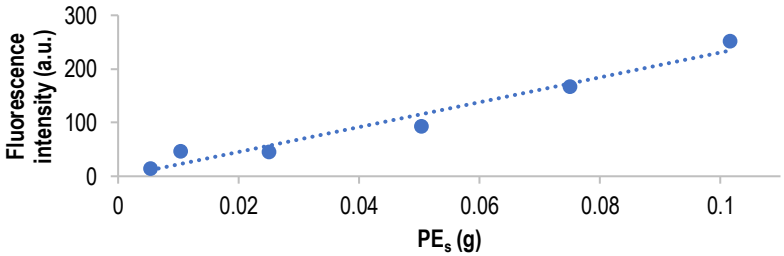



Figure 13. Average fluorescence intensity achieved for the peak at 535 nm when replicates of stained $PE_s$ weights were analyzed. The $R^2$ obtained is 0.9805.

# 8. MICROPLASTICS DETECTION

Once the fluorescence staining method was optimized with $PE_s$, other types of microplastics (MPs) were also tested (PS, PP and $PE_l$). The obtained results demonstrated that the efficiency and reproducibility of the "Aqueous" staining method did not decrease using other MPs types or sizes. That fact can be seen in Figure 14. In this figure, PS, PP and $PE_l$ after the staining procedure are illustrated. A comparison with the unexposed MPs can also be seen. Successful results were also acquired, matching with the results reported in the literature[28]. That fact demonstrates the utility of NR to detect and quantify small plastic particles. It could be visually observed that PS (violet) stained color was quite different compared with the stained color of PP and PE (red). Many factors could explain this experimental observation. As it was explained before, Nile Red has a strong solvatochromic behavior which absorption maximum undergoes a spectral shift that is roughly correlated with environmental polarity. Therefore, the monomer structure and their polarity could be responsible for that experimental difference. The monomers structures of the tested polymers are represented in the Appendix 4. Although all the polymers tested are non-polar, PP and PE have aliphatic hydrocarbon structures while PS has an aromatic radical. Other facts such as the crystalline structure, surface conditions or adsorption interaction may also influence in the absorption maximum shift. In Figure 15, the obtained fluorescence spectra from the measurements of the analyzed polymer types are disclosed. All the measurements were also performed using an excitation wavelength of 500 nm.

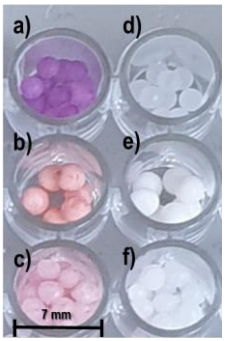


Figure 14. MPs visual appearance of PS (a), PP (b), $PE_l$ (c) using NR compared with unexposed MPs (d,e,f)

The different spectra achieved shown that PS is clearly different from PP and PE. It was experimentally confirmed that adsorbed NR in PP and PE have a similar response as dissolved NR in non-polar solvents such as hexane. As PS fluorescence spectra only present a broad maximum, it could be concluded that PS polarity is not low enough to exhibit two separate identifiable peaks, as happens in toluene (Figure 9). The monomer structure of PS could explain the similar fluorometric behavior between NR dissolved in toluene or adsorbed in PS. As it is

explained in Dalia G. Yablon work, many factors influence in the NR solvatochromic behaviors. Therefore, further studies of NR solvatochromic reaction in MPs environments are required[39].

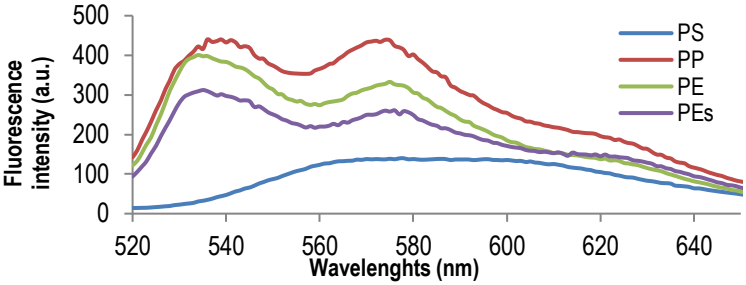



Figure 15. Fluorescence spectra obtained for different types of stained MPs (All the spectra were recorded using an excitation wavelength of 500 nm).

While only a selection of polymers were tested, given the mode of interaction of NR with polymer surfaces, there is no reason to suppose that NR would not be adsorbed to any other polymer types or surfaces. As it is explained on the bibliography, mainly Van der Waals interaction with additional dipole interactions can explain the adsorption of the dye to the polymer surface[28]. The plastic types used in this study are the more common plastics found on the marine environment but could be interesting to assess the selectivity of the method in more polar polymers such as PMMA, PVC or nylon.

## 8.1 EVALUATION OF ALTERNATIVE DYES

After optimized conditions were tested in various microplastics types using Nile Red, alternative colorants were analyzed using the same conditions. Nile Blue A (NB), Crystal Violet (CR) and Eosin Y were tested. In Figure 16, the results of the stained procedure can be seen. For Nile Blue A and Crystal Violet, the results were quite similar to Nile Red. In both cases, PS had a different behavior than PP and PE. In contrast, Eosin Y seems to have fewer efficiency to stain MPs compared to NB and CR. That contrast could be seen clearly on the image. MPs stained with Eosin Y were very similar to the unexposed MPs. The reasons that justify these experimental observations are not clearly understood.

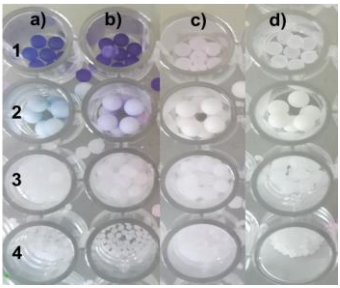



Figure 16. MPs visual appearance of PS (1), PP (2), $PE_l$ (3) and $PE_s$ (4) using different dyes (NB (a), CR (b), Eosin Y (c)) compared to unexposed MPs (d).

Further investigations about how the different used dyes adsorbed on the MPs surface are needed. Fluorescence studies could not be successfully analyzed with CR or Eosin Y stained MPs. Crystal Violet preliminary studies showed that this dye was nonfluorescent using water as a solvent at any excitation wavelength. It was also demonstrated experimentally that CR was not a useful dye to detect MPs by fluorescence approaches because no significant signal was detected. Similar to the CR case, microplastics stained with Eosin Y could not be detected using fluorescence methods. In contrast to CR preliminary studies, Eosin Y was strongly fluorescent when water was used as a solvent (Appendix 3).The main problem detected in Eosin Y probably was the staining method used. The results obtained for Nile Blue A are shown below.

For NB, it was acquired that the optimized excitation wavelength was between 450-500 nm, which provide a maximum fluorescent intensity for stained microplastics. The optimized excitation wavelengths, as well as the emission spectra shape for PP, $PE_s$ and $PE_l$, were very similar between Nile Red and Nile Blue. Contrary to that, stained PS fluorescence could not be recorded even though the staining process was successfully carried out. Differences between PS and the other types of microplastics tested could not be justified. Different hypothesis commented before would explain that experimental differences, but further studies are highly recommended to confirm it.

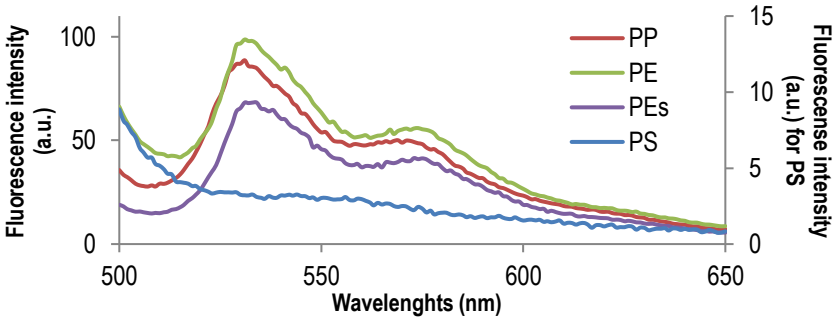



Figure 17. Fluorescence spectra of stained MPs using Nile Blue A.
(All the spectra were recorded using an excitation wavelength of 480 nm.)

An important fact to highlight is the similar experimental behavior between Nile Red and Nile Blue A. That fact could be reasoned based on the similar structure of both fluorescent dyes (Figure 10). As it was discussed above, the key feature influencing the Nile Red's fluorescence spectra would be the rotation around the C-N bond. Considering the similar structure, the same hypothesis could be used to explain the fluorescence spectra of Nile Blue A. This hypothesis is

supported with the similar experimental fluorescence spectra obtained for both dyes. To confirm that, a solvatochromic study of Nile Blue A was carried out. The obtained results allowed to conclude that Nile Blue A has a solvatochromic behavior too, even though not as strong as Nile Red. The fluorescence spectra of Nile Blue A dissolved in chloroform, acetone, methanol and water are compared in the Appendix 2.

# 9. APPLICATION OF THE NR STAINING METHOD TO SEDIMENT SAMPLES

## 9.1 DENSITY FLOTATION STEP ASSESSMENT

Finally, the staining method was tested in environmental samples showing a complex matrix. Firstly, spiked samples using reference plastics ($PE_g$ and $PE_s$) were analyzed. $PE_g$ could be recovered easily because of their green surface's color. Besides that, stained $PE_s$ recovery rates were worse. That fact could be reasoned for the microplastic color surface after adsorbed NR. As it is commented on the literature, the recovery rates of microplastics in visual counting strongly varies depending on their surface color. For blue, green and violet hues, 70-100% recovery rates were obtained. Despite that, yellow, pink and orange microplastics recovery values were 0-40%[7]. Recovery results of staining spiked particles of various polymer types in fine marine sediments are shown below.

Table 5. Recovery of NR stained microplastics from sediment samples.

| **Microplastic** | **Number/Weight** (g) | **Number/Weight** (g) | **Recovery rate** (%) |
|---|---|---|---|
| $PE_g$ | 0.04162 | 0.04049 | 97 |
| $PE_s$ | 0.06656 | 0.03555 | 53 |
| $PE_l$ | 0.07872 | 0.07624 | 97 |
| PP | 5 | 5 | 100 |
| PS | 8 | 8 | 100 |

(a) $PE_g$ and $PE_s$ recovery rates were calculated using the calibration study exposed in Figure 11 and Figure 13 respectively. Recovery rates of the other plastic types could not be determined this way because any calibration study was performed.

(b) $PE_l$ recovery rates were calculated by weight difference. PP and PS recovery rates were calculated by direct counting because of their bigger size scale.

## 9.2 INDUSTRIAL PLASTICS ANALYSIS

To evaluate the applicability of the fluorescence approach in real environmental samples, industrial plastics were also analyzed. Firstly, reference samples of industrial plastic types were measured. Nile Red and Nile Blue A were assessed. In Figure 18, the staining method was successfully performed in the industrial plastics tested. After that, sediment spiked samples of the different industrial plastic types were assessed using a combination of the density flotation step and the staining method. The followed experimental procedure was presented in section 5.4.2. The results demonstrated that the industrial microplastics could also be stained in complex sediment matrices. As it can be seen in the presented image, the method was also successfully applied using Nile Blue A. When Nile Blue A was used to stain industrial microplastics in sediment spiked samples, the color solution was violet. In reference samples, the color solution was blue. Furthermore, as happens with Nile Red, aggregations of the unadsorbed dye were formed in KCl saturated solution. That could be reasoned for the polarity increment presented by the KCl saturated solution in comparison with water. The increment of the solution's polarity produces a decrease in the NB solubility.

Figure 18. MPs visual appearance of PS (a), PP (b) and PET (c) using NR (7,9) and NB (8,10) after performing the staining method in reference industrial plastics samples (7,8) or in sediment spiked sample (9,10).

Then, the fluorescence spectra of the different industrial plastics using NR were measured. The fluorescence spectra of the reference industrial plastic samples and the sediment spiked samples were very similar. As it is shown, the fluorescence spectra shape of PP and PS industrial plastics were similar to the spectra obtained when standard microplastics were analyzed. However, PET was not tested with reference microplastics. Furthermore, looking at the monomer structure (which is represented in the Appendix 4) it could be predicted that the maximum emission wavelength should appear at longer wavelengths than PP and PS. In addition, only one peak should be seen. That prediction is corroborated with the experimental fluorescence spectra of the polymer. The fluorescence spectra acquired for PP, PS and PET using Nile Red are showed in Figure 19. Another important fact to highlight is the differences found in the intensities between hydrophobic polymer types (PP and PS) and hydrophilic ones analyzed (PET).

That point could be explained by the important influence of the plastics polarity in the intramolecular charge transfer state of the NR. Same results were observed on the literature[5,37]. The high-density of PET (>1.2 g/cm$^3$) could also influence in the decrement of the fluorescence intensity[5]. These limitations could be overcome by increasing the sensitivity of the method.

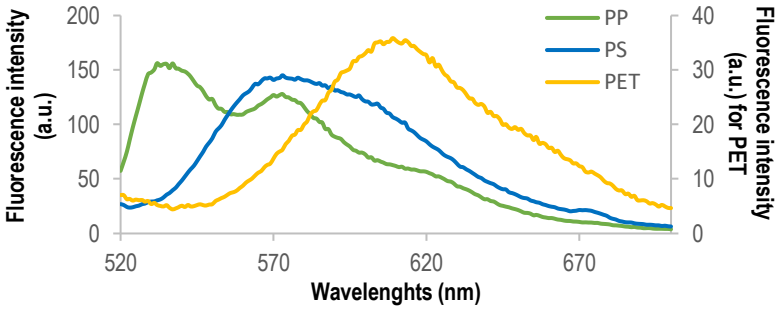



Figure 19. Comparison of the fluorescence spectra of the different industrial plastics analyzed using NR. Important maximum emission band and fluorescence intensity variations were observed between the different plastics analyzed. The excitation wavelength used was 500 nm.

The fluorescence spectra of the different industrial plastics using Nile Blue A are shown in Figure 20. As it happened when NR was tested, differences in the fluorescence spectra shape between reference samples and sediment spiked samples were minimum. A similar response was observed between standard PP and industrial PP. PET did not present important fluorescence intensity as happened in the NR study. The emission band was also presented at longer wavelengths than PS and PP. A significant difference between the standard PS and industrial PS could be observed. As shown in Figure 20, industrial PS gave an intense emission band.

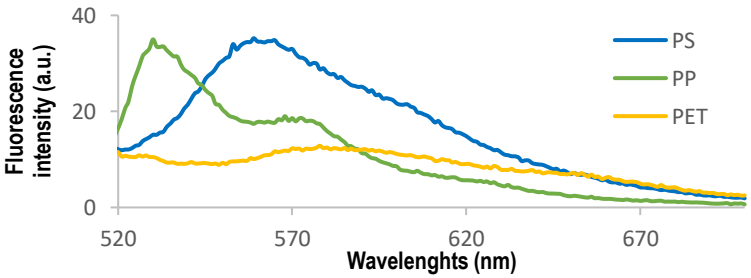



Figure 20. Comparison of the fluorescence spectra of the different industrial plastics analyzed using NB. As happened with NR, important differences in the emission band were observed between the different polymer types. The excitation wavelength used was 500 nm.

Further studies are needed to determine how the emission band varies depending on other factors like crystallinity, shape, color within the same polymer type. Thus, reference and industrial polystyrene plastics should be analyzed to confirm any general behavior.

## 9.3 STUDY OF PHOTODEGRADED PLASTICS

Spiked samples with reference microplastics were photodegraded with UV light. Then, they were analyzed to determine the accuracy of the fluorescence approach using Nile Red in degraded samples. The obtained recovery values did not vary in comparison to the non-photodegraded microplastics (see Table 5). The staining method was also successfully applied in all the samples tested. The fluorescence spectra presented by $PE_I$ after the different degradation processes were compared with the fluorescence spectra shape of reference $PE_I$ in Figure 21. As shown, the fluorescence spectra shape did no present important variations compared to the unexposed MPs in the time scale studied. This behavior was observed in all types of MPs analyzed (PP, PS and PE of both sizes). That fact allowed the correct identification of the polymer types even though an important degradation process had occurred. The most used identification approaches (FT-IR and Raman spectroscopy) are not able to realize that accurate identification. In addition, this fluorescence staining method combined with the density separation does not require important pre-treatment of the environmental sample or expensive instrumentation. To confirm this, extended studies about the behavior of the photodegraded plastics in the fluorescence approach using a fluorescence dye would be required. Therefore, it could be of great interest new investigations of other degradation procedures and how they affect the staining process and the fluorescence spectral shape.

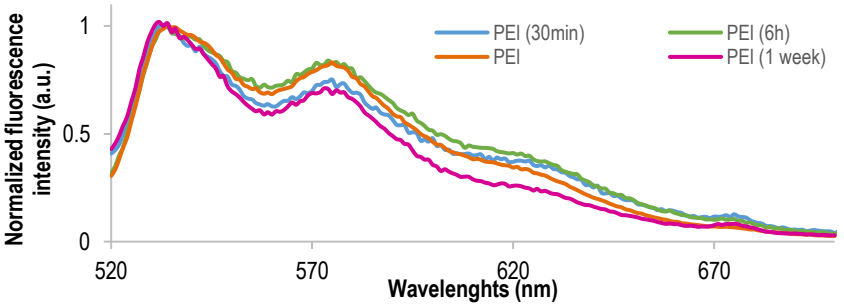



Figure 21. Comparison of the fluorescence spectra of $PE_I$ after different degradation processes occurs. The excitation wavelength used was 500 nm.

# 10. CONCLUSIONS

In the present study, the development and optimization of a staining method that allowed the microplastic determination using a fluorescence-based approach have successfully been designed. The achieved results led to the following conclusions:

- The "Aqueous" method with a drying step at 45 °C was chosen as the most appropriate approach for the MPs Nile Red staining. The optimized incubation time was set to 1h, whereas the drying time required was set to overnight.
- The optimized method was successfully applied to different polymer plastic types (PS, PP, PE, PET). Thus, the solvatochromic behavior of NR permitted microplastics to be grouped by polymer polarity.
- The optimized "Aqueous" approach was also successfully applied using Nile Blue A. Similar results were obtained using either NR or NB. The similar structure between NB and Nile Red could explain their similar response in the staining method. The only difference observed was that any fluorescence signal could be recorded by reference PS using NB.
- The suitability of this method to sediment samples was also tested using Nile Red and Nile Blue A. Promising results were obtained using both dyes in standard and industrial microplastics. Achieved results from the photodegraded plastics study were also satisfactory as the fluorescence spectral shape of the different polymers did not vary in the time scale studied.

All these results led to conclude that this method is a simple and sensitive approach to determine the most common plastic debris in the marine environment.

Although promising results were accomplished in this project, further studies are required to implement this analytical fluorescence-based approach as a routine method to the microplastics determination in environmental samples. The studies that should follow the present study are:

- Testing the optimized method with more polar polymers like PMMA, PVC or nylon. As discussed previously, low fluorescence intensity is achieved in the analysis of these plastic types. The limitations of the method in polar polymer types must be studied.
- Assessing the efficiency of the density flotation step with denser plastic polymers. A comparison of various saturated salt solutions (i.e. NaCl, $ZnCl_2$, or NaI) would also be of great interest.
- Analyzing how the fluorescence spectrum varies depending on other factors such as crystallinity, shape, and color for the same kind of polymer.
- More in-depth studies of how the fluorescence spectra vary when photodegradation processes occur must be made to evaluate the efficiency of the method. The different degradation procedures that plastic debris undergo in the marine environment should also be studied.
- Chemometric analysis tools could be helpful for the identification and quantification of the different plastic polymer types in plastic debris mixtures. The application of these chemometric techniques could also be useful in the study of the possible variations in the fluorescence spectra of the degraded plastics.

# 11. REFERENCES AND NOTES


1. Andrady, A. L. *Mar. Pollut. Bull.* **2011**, *62* (8), 1596–1605.
2. Hidalgo-Ruz, V.; Gutow, L.; Thompson, R. C.; Thiel, M. *Environ. Sci. Technol.* **2012**, *46* (6), 3060–3075.
3. Li, M.; Peng, J.; Qiu, Q.; Zhan, Z.; Wang, J.; Tan, Z. *Estuar. Coast. Shelf Sci.* **2016**, *176*, 102–109.
4. Silva, A. B.; Bastos, A. S.; Justino, C. I. L.; da Costa, J. P.; Duarte, A. C.; Rocha-Santos, T. A. P. *Anal. Chim. Acta* **2018**, *1017*, 1–19.
5. Erni-Cassola, G.; Gibson, M. I.; Thompson, R. C.; Christie-Oleza, J. A. *Environ. Sci. Technol.* 2017, 51 (23), 13641–13648.
6. Fundéu BBVA: "Microplastic" word of the year 2018 https://www.fundeu.es/recomendacion/microplastico-palabra-del-ano-2018/ (accessed May 20, 2019).
7. Hanvey, J. S.; Lewis, P. J.; Lavers, J. L.; Crosbie, N. D.; Pozo, K.; Clarke, B. O. *Anal. Methods* **2017**, *9* (9), 1369–1383.
8. GESAMP "Sources, fate and effects of microplastics in the marine environment: a global assessment" (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP, 2015; No. 90, 96 p.
9. Li, J.; Liu, H.; Paul Chen, J. *Water Res.* **2018**, *137*, 362–374.
10. Auta, H. S.; Emenike, C. U.; Fauziah, S. H. *Environ. Int.* **2017**, *102*, 165–176.
11. Boucher, J. and Friot D. Primary Microplastics in the Oceans: A Global Evaluation of Sources, IUCN: Gland, 2017; 43 pp.
12. SKYE-Finish Environmental Institute: Microplastic sources information https://www.syke.fi/en-US/Current/Microplastics__a_risk_to_inland_and_mari(42493) (accessed Mar 12, 2019).
13. Sun, X.; Li, Q.; Zhu, M.; Liang, J.; Zheng, S.; Zhao, Y. *Mar. Pollut. Bull.* **2017**, *115* (1–2), 217–224.
14. Gonçalves, C.; Martins, M.; Sobral, P.; Costa, P. M.; Costa, M. H. *Environ. Pollut.* **2019**, *245*, 600–606.
15. Horton, A. A.; Jürgens, M. D.; Lahive, E.; van Bodegom, P. M.; Vijver, M. G. *Environ. Pollut.* **2018**, *236*, 188–194.
16. Courtene-Jones, W.; Quinn, B.; Ewins, C.; Gary, S. F.; Narayanaswamy, B. E. *Environ. Pollut.* **2019**, *244*, 503–512.
17. Nuelle, M. T.; Dekiff, J. H.; Remy, D.; Fries, E. *Environ. Pollut.* **2014**, *184*, 161–169.
18. Mai, L.; Bao, L. J.; Shi, L.; Wong, C. S.; Zeng, E. Y. *Environ. Sci. Pollut. Res.* **2018**, *25* (12), 11319–11332.
19. Shim, W. J.; Hong, S. H.; Eo, S. E. *Anal. Methods* **2017**, *9* (9), 1384–1391.
20. Schymanski, D.; Goldbeck, C.; Humpf, H. U.; Fürst, P. *Water Res.* **2018**, *129*, 154–162.
21. Song, Y. K.; Hong, S. H.; Jang, M.; Han, G. M.; Rani, M.; Lee, J.; Shim, W. J. *Mar. Pollut. Bull.* **2015**, *93* (1–2), 202–209.
22. Falkenhagen, J.; Elert, A. M.; Sturm, H.; Eisentraut, P.; Braun, U.; Becker, R.; Duemichen, E. *Environ. Pollut.* **2017**, *231*, 1256–1264.
23. Fischer, M.; Scholz-Böttcher, B. M. *Environ. Sci. Technol.* **2017**, *51* (9), 5052–5060.
24. Peez, N.; Janiska, M. C.; Imhof, W. *Anal. Bioanal. Chem.* **2019**, *411* (4), 823–833.
25. Paul, A.; Wander, L.; Becker, R.; Goedecke, C.; Braun, U. *Environ. Sci. Pollut. Res.* **2018**, 1–11.
26. Zhang, J.; Tian, K.; Lei, C.; Min, S. *Anal. Methods* **2018**, *10* (24), 2881–2887.

27. David, J.; Steinmetz, Z.; Kučerík, J.; Schaumann, G. E. *Anal. Chem.* **2018**, *90* (15), 8793–8799.
28. Maes, T.; Jessop, R.; Wellner, N.; Haupt, K.; Mayes, A. G. *Sci. Rep.* **2017**, *7*, 44501.
29. GREENSPAN, P.; MAYER, E. P.; FOWLER, S. D. *J. Cell Biol.* **1985**, *100* (3), 965–973.
30. Seddon, K. R.; Carmichael, A. J. *J. Phys. Org. Chem.* **2000**, *13*, 591–595.
31. Tamminga, M. *SDRP J. Earth Sci. Environ. Stud.* **2017**, *2* (2).
32. Marionnet, C.; Tricaud, C.; Bernerd, F. *Int. J. Mol. Sci.* **2015**, *16* (1), 68–90.
33. Edimburgh Instruments: Jablonski's diagram information https://www.edinst.com/blog/jablonski-diagram/ (accessed May 8, 2019).
34. Lakowicz. J R. Introduction to Fluorescence. In *Principles of fluorescence spectroscopy*; Springer: New York, 2006; pp 1–26.
35. Jameson, D. M. Emission and Excitation Spectra. In Introduction to fluorescence; CRC Press: Boca Raton, 2014; pp 57–74
36. Molecular Devices: How fluorescence microplate measurements work http://www.moleculardevices.com/pages/instruments/gemini.html (accessed Apr 5, 2019).
37. Shim, W. J.; Song, Y. K.; Hong, S. H.; Jang, M. *Mar. Pollut. Bull.* **2016**, *113* (1–2), 469–476.
38. Zuehlsdorff, T. J.; Haynes, P. D.; Payne, M. C.; Hine, N. D. M. *J. Chem. Phys.* **2017**, *146* (12).
39. Yablon, D. G.; Schilowitz, A. M. *Appl. Spectrosc.* **2004**, *58* (7), 843–847.

# 12. ACRONYMS

CR: Crystal Violet

FT-IR: Fourier-Transform Infrared Spectroscopy

HPLC-SEC-RI: High-Performance Liquid Chromatography-Size Exclusion-Chromatography-Refractive Index

MPs: Microplastics

NB: Nile Blue A

NIR: Near-Infrared

NMR: Nuclear Magnetic Resonance

NOAA: National Oceanic and Atmospheric Administration

NR: Nile Red

PE: Polyethylene

PET: Poly (ethylene terephthalate)

POPs: Persistent Organic Pollutants

PP: Polypropylene

PS: Polystyrene

PUR: Polyurethane

PVC: Poly (vinyl chloride)

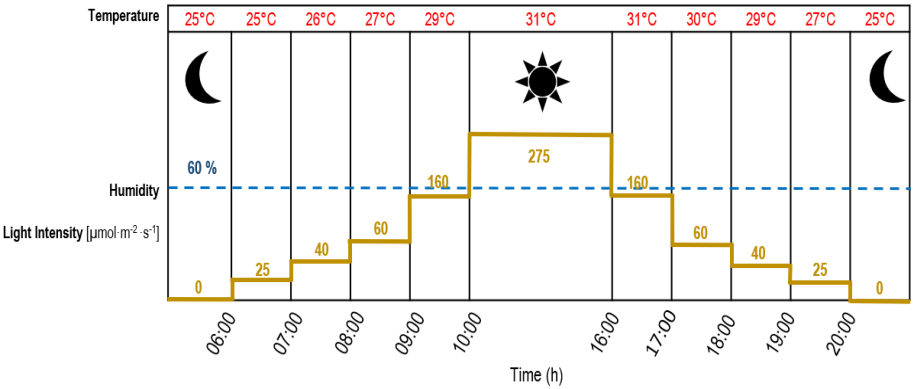
Pyro-GC/MS: Pyrolysis-Gas Chromatography-Mass Spectrometry

RAE: *Real Academia Española*

SEM-EDS: Scanning Electron Microscopy-Energy Dispersive Spectroscopy

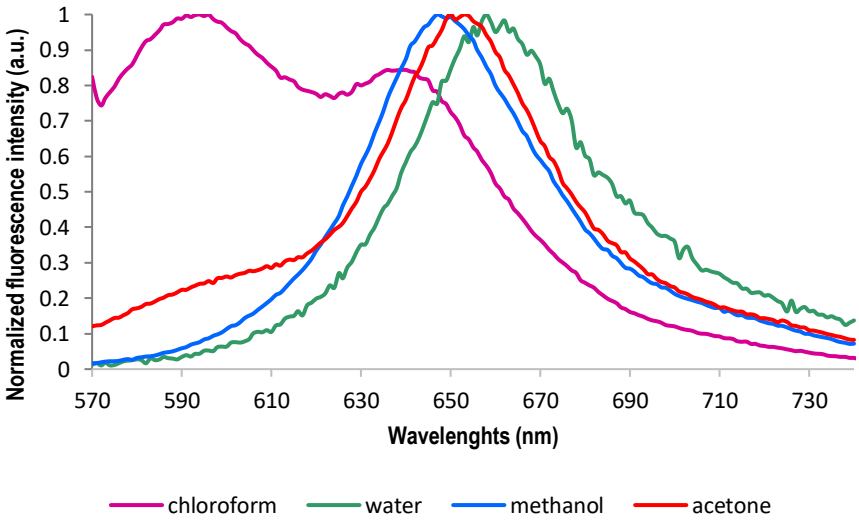
# APPENDICES

# APPENDIX 1: CONDITIONS USED IN THE ENVIRONMENTAL CHAMBER



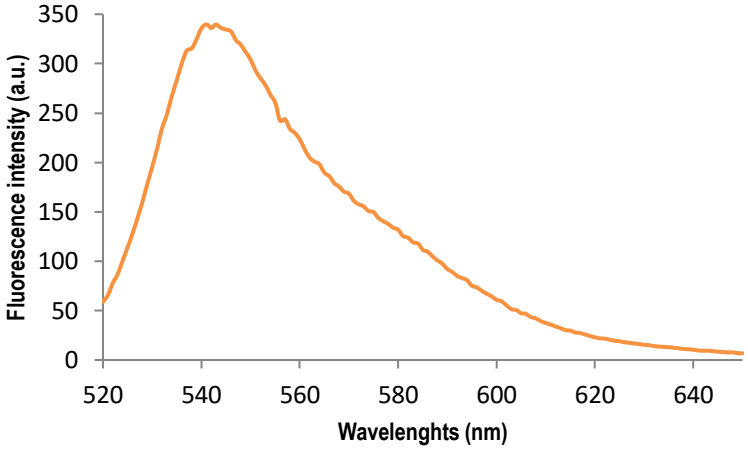

In this figure, the conditions used in the low-intensity photodegradation of the reference microplastics are depicted. As is shown, a real photodegradation process of the plastics in the sand's beach is simulated. The standard microplastics were subjected to these environmental conditions for one week. The light intensity was measured in Photosynthetic Photon Flux Density (PPFD) with units of µmol/s·m$^2$.

# APPENDIX 2: NILE BLUE A FLUORESCENCE SPECTRA IN DIFFERENT SOLVENTS



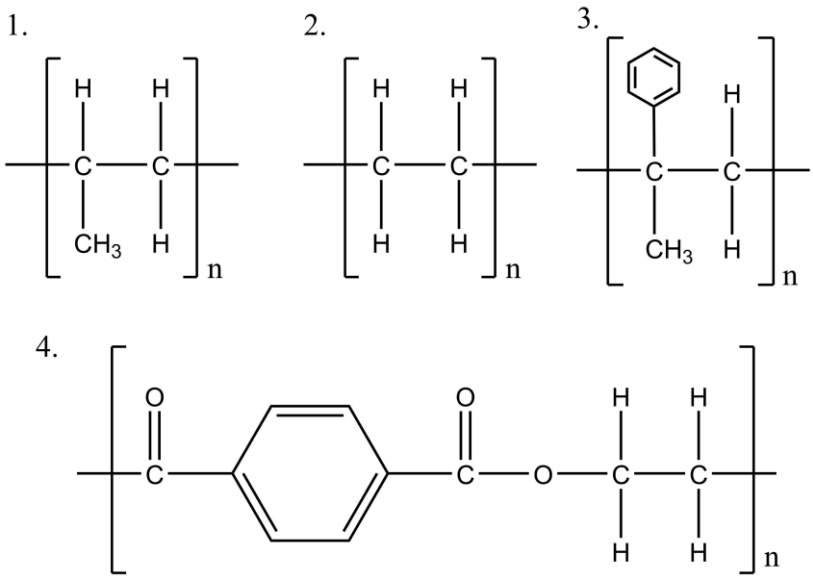

A comparison of the Nile Blue A fluorescence spectra in different solvents is illustrated. Nile Blue A presents also a solvatochromic behavior like NR. Acetone maximum emission wavelength appeared at longer emission wavelengths than expected. Apolar solvents such as hexane or toluene could not be tested because NB is insoluble in these solvents. The excitation wavelength used was 560 nm.

# APPENDIX 3: EOSIN Y FLUORESCENCE SPECTRUM IN WATER




Fluorescence spectrum of Eosin Y using water as a solvent. The excitation wavelength used was 500 nm.

# APPENDIX 4: MONOMER STRUCTURE OF THE DIFFERENT PLASTIC TYPES TESTED




The monomer structures of PP (1), PE (2), PS (3) and PET (4) are represented. The various polymers are ordered by increasing polarity: PE ~ PP < PS < PET.