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Laboratory Experiment

Lighting Up for Learning—Fluorescence Analysis of Microplastic Particles by Secondary School Students Using Nile Red

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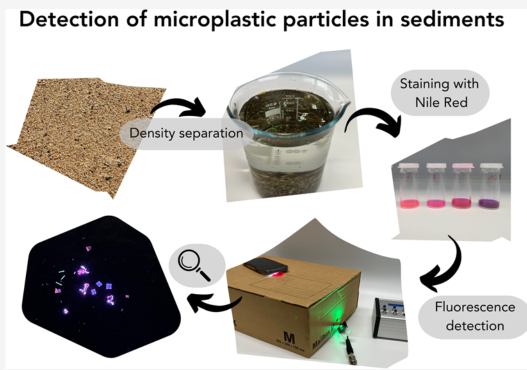
ABSTRACT: Microplastics have been detected in most ecosystems around the world. They affect the environment and organisms in it, including humans and possibly their health. Hence, the analysis of microplastic occurrence in the environment is highly relevant. However, there are only a few practical and easy-to-implement methods published for school use, and therefore, microplastics still receive little attention in the classroom. This review presents an approach for separation and detection of microplastic particles in sediment with secondary school students based on methods used in current research. After sieving and density separation, the fluorescence marker Nile Red is used to selectively stain microplastic particles. Subsequently, the particles can be detected using a DIY low-cost fluorescence photobox. It offers an opportunity to address the problems associated with microplastics in a school context and can be used as an example for further discussion.



KEYWORDS: *High School, First-Year Undergraduate, Analytical Chemistry, Hands-On Learning, Fluorescence Spectroscopy, Dyes, Microplastics, Low-Cost Method, Nile Red*

■ INTRODUCTION

Microplastic particles are ubiquitous in our environment. It is not surprising that attention of media and the public around this topic has increased over the past few years. Thus, a major part of the public knows that microplastics are small plastic particles with potentially negative effects. However, there are knowledge gaps regarding sources and occurrence of microplastic particles and their effects and risks on human health.[1−3]

It is usually known that in marine ecosystems this pollution is rather abundant,[1] but the public often underestimates the heavy contamination of terrestrial ecosystems with plastic articles.[1,2,4] Microplastics have also been detected in the human body, and recently it was shown that they have some concerning effects on human health.[5] As a consequence, research on the analysis of microplastic particles is rapidly growing, steadily increasing our knowledge of the pollution issue. In state-of-the-art analytical approaches, sediment samples are usually separated by sieving to selectively analyze particles of a distinct size. However, they also contain loads of matrix that are often removed by density separation to remove more dense sediment from the lower-density plastic particles. Some try to remove biological materials with digestion using H_2O_2 (30%), Fenton's reagent, HCl, or enzymes.[6] Identification is then often done visually or by IR or Raman spectroscopy or GC-MS.[6−8]

Recently, fluorescent dyes have been used extensively to selectively dye microplastic particles. Nile Red has become a standard for these staining procedures, as it has been reported to yield the best staining results.[9,10] Due to its nonpolar nature, the dye can adhere to the polymer surfaces by van der Waals interactions as well as dipole interactions for more polar plastic types.[10] This staining process is rather specific, with only a few natural polymers like chitin being stained by it as well. Thus, prior digestion of the samples is often recommended.[11]

While most protocols are primarily focused on identifying the number of microplastic particles in a sample, there already are some approaches to group microplastic particles using Nile Red staining. The intensity and color of the fluorescence of the microplastic particles is reported to vary between plastic types due to their surface polarity and the use of filters but also due to their shape, color, and thickness.[8] There are protocols optimized for coloring the nonpolar plastic types like PP and PE[11,12] and others that yield better results for the more polar plastic types like PVC.[13] This results in reports showing differences in fluorescence intensity and color between the more polar plastic types such as PA and PET and nonpolar plastic types such as PE, PP, and PS.[10,12,14,15] There is also a recognizable red shift of the fluorescence emission spectra between the nonpolar and more polar plastic types.[16,17]

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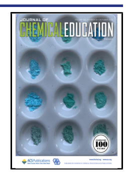

Although these protocols are suitable for research, most of them use staining solutions that contain harmful chemicals such as chloroform or hexane. Ethanol is also used as an alternative but yields inferior results.[8,9] The subsequent identification requires a fluorescence microscope or an adapted stereo microscope, which not many schools possess.[8]

It is therefore not surprising that only a few publications describe the analysis of microplastics in a school or education context in a teaching-focused and laboratory-based way. Most of the methods that focus on the investigation of samples from the environment are designed for college undergraduate students.[18−20] There is a model experiment[21] proposed for secondary schools, but microplastic particles are only extracted from sediment samples and not stained for detection in any way.

Experiments proposed there[21] and that of Rowe et al.[18] analyze sediment samples via density separation to separate microplastics from the matrix. In this process they use media of lower density like a solution of sodium chloride with sucrose (1.38 g/cm^3),[21] which cannot separate the denser plastics such as PET and PVC, for the school context or environmentally harmful chemicals like a solution of zinc chloride[18] at the university level. The subsequent identification is missing in school[21] or only done visually with a stereo microscope.[18] Labbe et al.[19] and Scircle and Cizdziel[20] analyze plankton and water samples. Therefore, they do not use density separation but have implemented staining with Nile Red dye for better identification and opted for using a professional fluorescence microscope or an adapted stereo microscope for detection. To sum up, there is still potential for a more extensive set of experiments in a school context. Challenges are (i) suitable media for density separation and staining that both work better and are less harmful and (ii) an affordable detection setup for fluorescence analysis.

RATIONALE

In this article we present an experimental setting for secondary schools that is not only able to extract microplastic particles from sediment but also allows the detection of those particles. With this experiment, we can demonstrate in a simple form both the extent of pollution in the environment and the problem of proper analysis methods. To further strengthen the connection to real-world problems, the methods used are simplified state-of-the-art environmental analytics methodologies. Our main aim was to develop an easy-to-use, nonhazardous, and low-cost method that allows students to analyze sediment samples for microplastic particles. Therefore, density separation is done with a new, nontoxic, highly dense separation medium, a saturated solution of potassium carbonate. This solution allows the extraction of even the most dense types of particles (PET and PVC) from a sediment sample up to a density of 1.54 g/cm^3.[22]

Furthermore, we present a modified, highly efficient, and quick staining protocol using Nile Red with acetone and water as solvents. Therefore, the staining can be easily done by high school students. For detection we use a low-cost fluorescence photobox where materials have been reduced and optimized, as has been done in a similar way very recently,[23] improving on already published concepts.[24−26] With these measures, the most simple and cheapest variant of our analysis protocol can be done for about 30 €, a budget achievable for most secondary schools. The method can be applied in laboratory units, in curricular activities, and in interdisciplinary projects, seminars, or other prescientific theses.

MATERIALS AND METHODS

When used as a model experiment for secondary schools, the sediment sample can be made by adding self-made microplastic particles to a sediment sample. Those particles can be produced by cutting, chopping, or grating of most types of plastics, namely, PVC, PET, PS, PU, PA, PC, PLA, PCL, ABS, PMMA, and polyester resins.

Figure 1 shows an overview of all of the steps and time recommendations for the analysis of microplastic particles in sediments. If you only want to stain and detect the microplastics in class due to lack of time, just the last three steps can be carried out in a typical school lesson.

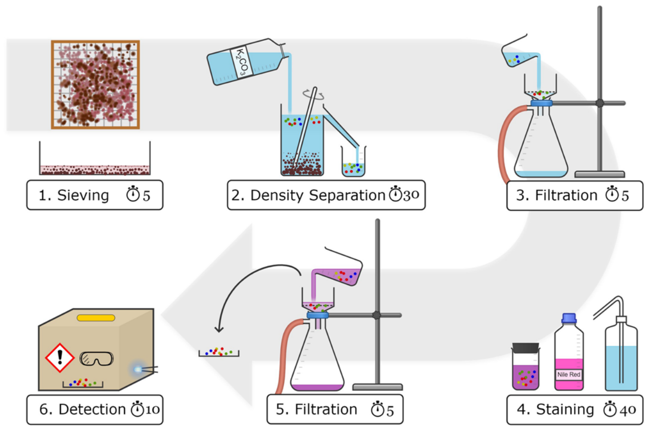

Figure 1. Overview of all steps for fluorescence analysis of microplastic particles in sediments.

Sample Preparation

Sieving the dry sample as a first step is recommended to achieve the best results during fluorescence analysis (see Figure 1). For this one should use sieves with mesh sizes of 5 and 0.25 mm. By removing the larger particles, the sample amount can be reduced. This step is particularly important if real-world samples are examined. By removing particles smaller than 0.25 mm, the subsequent density separation is heavily accelerated, as small particles would need a longer time to settle to the bottom.

Density Separation

For density separation, an overflow beaker is filled to three-fourths with saturated potassium carbonate solution. If expensive lab glassware is not available, a measuring cup is a very good low-cost alternative, as shown in Figure 2. The sediment sample is added under permanent light stirring. The heavier sediment is allowed to settle for 10 min. Particles that sunk to the ground are retained, while particles on the surface are transferred out of the overflow beaker by carefully adding saturated potassium carbonate solution. The yielded suspension of particles in the solution is filtered and washed with distilled water.

Staining with Nile Red

The solid particles are covered with a 100 mg/L solution of Nile Red in acetone. The same amount of distilled water is added, and the samples are left for 30 min for staining. In most cases, 15 min proved to be enough to stain all plastic particles,

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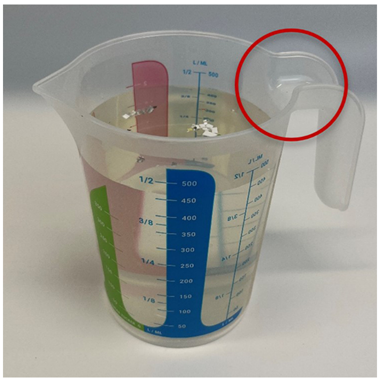

Figure 2. A measuring cup with a lowered handle is a perfect low-cost alternative to an overflow beaker.

which can further accelerate the lesson. The stained sample is then filtered, washed with acetone and water, and transferred to a Petri dish for fluorescence analysis.

Crafting of a Low-Cost Fluorescence Photobox

Undoubtedly most schools will lack the funds to buy a fluorescence microscope. However, a very simplified version focused only on the most relevant parts of the apparatus can be built with just a paper box and a cutter knife by simply cutting one hole on top of and one hole on one side of the box. As can be seen in Figure 3, the setup does not need a beam splitter and an excitation filter. As a substitute, monochromatic light sources are used for excitation. In a simple setup, this can be a UV flashlight, but any (nearly) monochromatic light source is suitable. As an emission filter, one can use either very low cost filter films, where lighting gel filters usually give better results than color films, or more expensive but still affordable filter glasses. The principle is similar to that reported by Schaefer et al., except that the box allows fluorescence analysis to be done in a laboratory in daylight instead of darkness.[23] They also prove that use of cheap parts achieves great results. Furthermore, they use a modern smartphone as the detector, which our setup incorporates as well. The built-in zoom serves as the lens and ocular. It can also be advantageous to glue black foil or black paper to the floor of the box to minimize stray light during detection.

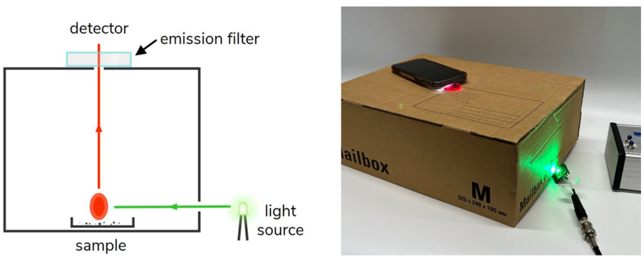

Figure 3. Setup of a low-cost fluorescence photobox.

The fluorescence of particles stained with Nile Red can be induced with UV light (blue fluorescence, λ_{ex} = 300–405 nm), blue light (λ_{ex} = 405–500 nm), or green light (λ_{ex} = 500–600 nm) and is detected as blue fluorescence, green fluorescence, or red fluorescence.[8] For each of the wavelengths, suitable emission filters have to be used, depending on the light source applied. Depending on individual needs and school budget, there are options available for both light sources as well as emission filters (see Table 1).

Table 1. Setups Used for the Fluorescence Analysis of Microplastic Particles

Setup	Version	Excitation	Emission
1	Blue fluorescence	Nichia UV LED 375 nm (2 €)	SCHOTT long-pass filter GG-435 (44 €)
2	Green fluorescence	Nichia blue LED 470 nm (1 €)	SCHOTT long-pass filter OG-530 (34 €)
3	Orange fluorescence	Nichia green LED 520 nm (1 €)	SCHOTT long-pass filter RG-610 (44 €)
4	Low-cost version	UV flashlight (10 €)	Lighting gel filters (e.g., Selens) (20 €)

Analysis in the Low-Cost Fluorescence Photobox

The stained sample is analyzed in the low-cost fluorescence photobox. Excitation is achieved with monochromatic LEDs or a UV flashlight by illumination from the side of the sample. A suitable long-pass filter is used to cover the detection opening of the photobox, and the smartphone is used for detection. The camera app on the phone can be used to zoom in and take photos for further analysis. It is recommended to turn off the night mode and lock the focus of the camera for best results. Photos can be used to count microplastic particles in the sample.

Workshop and Learning Goals

Using our analysis protocol, we developed a 3 hour lab workshop on microplastics taking place at the university in a learning lab. The target audience was students from higher secondary education (ages 16–18). In order to make this method accessible for a diverse group of students, the focus of the workshop was mainly on environmental education and raising awareness rather than pure chemistry-oriented goals.

A successful transition to sustainability requires an informed society.[1] It is well-known that microplastics are small plastic particles, but there are still knowledge gaps regarding their sources, occurrence, and impacts.[1−3] We aimed to address this gap with input designed on this topic. There is also a difference in how media communicate the risks induced by microplastics and how science communicates those facts.[27] Therefore, we wanted a critical discussion of all of the results presented to be part of the learning experience as well.

With all of these factors in mind, the goals of our workshop and the experimental setting were the following:

- Comprehend and discuss density separation
- Use fluorescence and fluorescence spectroscopy for detection
- Use simple laboratory techniques (filtration, pipetting, etc.)
- Get to know definition and sources of microplastics
- Increase awareness of microplastic pollution
- Highlight impacts of microplastics on the environment and human health
- Critical discussion of scientific findings
- Sensitize students to and cause them to reflect on their consumption and environmental behavior

Depending on the individual focus of the instructors, other learning goals can also be accomplished.

To achieve our goals, the workshop consists of theoretical and practical parts. A detailed time schedule and details of the

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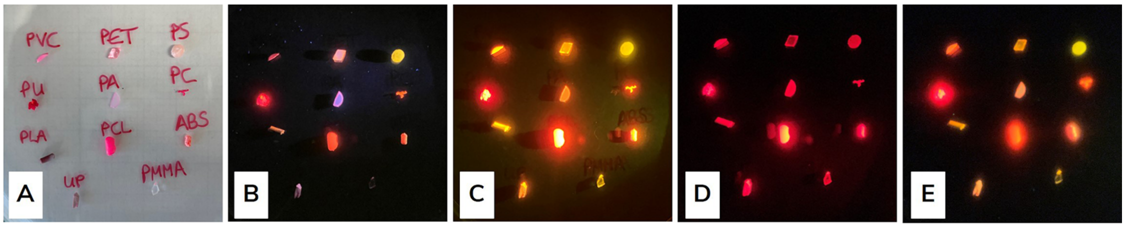

Figure 4. Detection of stained (A) microplastic particles with (B) setup 1, (C) setup 2, (D) setup 3, and (E) low-cost version 4.

discussed topics are found in the Supporting Information for Instructors.

All 49 students who participated in the workshop completed a pre- and postintervention questionnaire. In the preintervention survey the knowledge regarding microplastics (definition, sources, impacts) was assessed. The postintervention survey was designed to gather student feedback on the workshop in general using selected items from Bogner[28] as well as more specific questions on the methods. In a pre/post design we measured change in selected behavior-based environmental attitudes.[29]

■ HAZARDS

Students should always use appropriate personal protective equipment. When disposing after density separation, students should be reminded to decant the solution first and scoop out the solids from the containers second. Then the sediments should be deposited in the regular garbage can and not down the sink, as large quantities of sediments down drains can cause clogged pipes in the laboratory. The Nile Red safety data sheet indicates no specific hazards, but the Nile Red solution is a powerful dye and will stain skin and clothing. The excitation LEDs are quite bright, and students should be instructed not to look directly at them while they are turned on. UV safety glasses should be worn when using an open excitation source, such as the UV flashlight or UV LED to prevent the eyes of the students from being exposed to the harmful UV light.

■ RESULTS AND DISCUSSION

Using a saturated potassium carbonate solution for density separation, almost all types of microplastics can be separated from more dense sediment, allowing for easier staining and detection of particles. The staining protocol is then able to make most types of polymers (PVC, PET, PS, PU, PA, PC, PLA, PCL, ABS, and PMMA as well as UP resins) detectable by fluorescence analysis (see Figure 4). We even achieved staining of PVC with this protocol, where other protocols struggle to yield reliable results.[11] Of all the plastic types tested, only PE and PP were not dyed sufficiently for detection with our fluorescence photobox.

For optical detection, LEDs and a UV flashlight were used in conjunction with suitable filters (see Table 1). Figure 4 shows a comparison of the setups used. Results are good for all three types of fluorescence. In accordance with the literature,[12,15] green fluorescence generally yields the best results for detection (setup 2). However, the lowest-cost variant also yields very usable and attractive results.

Our protocol yields an almost uniform intensity of fluorescence for all tested excitation wavelengths. This enables detection of the particles with only one setup and thus simplifies the analysis.

However, we are not able to report any difference in fluorescence regarding polarity of plastic types, in contrast to the literature.[10,12,14,15]

Workshop and Student Feedback

A total of 49 students aged 16−18 participated in the workshop in groups of two or three. They were able to perform all of the experimental steps in the recommended time frame of 90 minutes. The materials were prepared, including the fluorescence photobox. The students did not require any specific help from the instructors (the authors) other than basic lab techniques such as pipetting and filtration.

Figure 5 shows an example photo of a model sample taken by students. They worked through the whole protocol and are

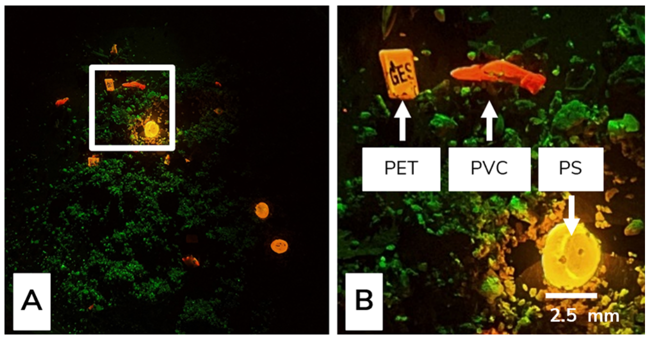

Figure 5. Photo from the fluorescence photobox, taken by students after working through the whole protocol. (A) The students used setup 2 for fluorescence detection. (B) The cropped picture shows fluorescence for PET, PVC, and PS. Pictures were taken by an iPhone 13. The identification of the plastic type was done visually by the author.

now able to count the fluorescent microplastics particles in the sample from the picture. The zoom of the smartphone, the fairly high resolution, and the ability to zoom in on the picture taken allow for detection of extremely small particles that can hardly be noticed without magnification.

In the presurvey, students defined microplastics as small plastic particles. The knowledge gaps regarding the sources and environmental and health impacts we found were the same as reported in the literature.[1−3] In the feedback form, the identification of microplastics using this protocol received positive feedback from the students (see Figure 6).

They highlighted that they especially appreciated working self-directed and in small groups as well as getting to know a range of new methods. Furthermore, they particularly liked the fluorescence analysis. Students remarked that their most important learning was on the topic of microplastics and environmental problems themselves as well as getting to know the process of analysis and the difficulties thereof.

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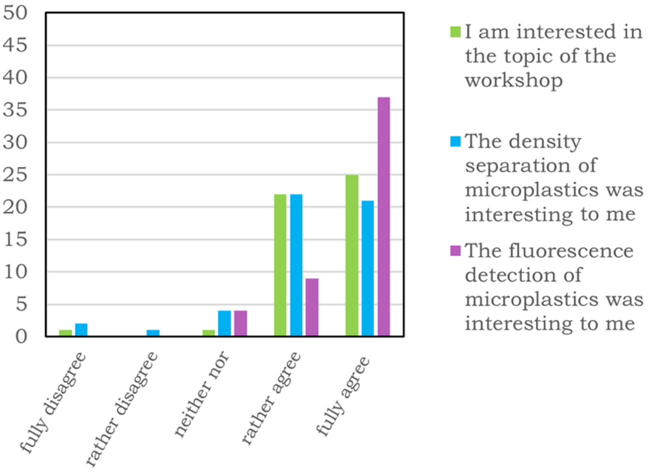

Figure 6. Student feedback after microplastic workshop (n = 49).

Overall, many students mentioned that the workshop made them aware that microplastics are not only ubiquitous in their environment but are also highly relevant for themselves and that the workshop increased their awareness. We were able to show this by comparing our pre- and postintervention surveys. There we found a significant increase in the behavior-based environmental attitudes (1: fully agree; 5: fully disagree): "I buy products in refillable packages" (M_{pre} = 3.37, SE_{pre} = 0.15; M_{post} = 3.80, SE_{post} = 0.16; p = 0.014); "If I am offered a plastic bag in a store, I take it" (M_{pre} = 2.06, SE_{pre} = 0.18; M_{post} = 1.77, SE_{post} = 0.17; p = 0.001).

CONCLUSION AND OUTLOOK

The model experiment we developed is designed to give students a comprehensive overview of state-of-the-art research methods for isolation and quantification of microplastic particles in sediment samples. Where harmful chemicals or expensive instruments are used in environmental analyses, we developed protocols with harmless chemicals, low-cost equipment, and easy-to-do steps that can be performed by secondary school students. Furthermore, we reduced costs to a minimum, making the methods affordable for schools. We also analyzed real-world sediment samples from Austrian riverbeds and Adriatic sand beaches, and the first results are intriguing. Further development of the method for better suitability for real-world samples will be the focus of future work, achieving even higher levels of relevance and stronger connections to the students' everyday lives.

In addition to getting to know scientific processes and some selected principles of environmental analysis, with this approach students can be made aware of the ubiquitous microplastic pollution, a very current problem of our society. Experiments can be used as an impulse for critical reflection of consumerism, especially for consumption of plastics, and is an important step to educate students for sustainable development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.3c00370.

SI for Instructors: instructor notes, materials and costs, tips, and suggestions for a 3 hour course (PDF, DOCX)

SI for Students: Student handouts and lab instructions (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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