A novel approach to optically detect microplastic in an environmental matrix with Rapid-FLIM

Ein innovativer Ansatz zum optischen Nachweis von Mikroplastik in einer Umweltmatrix mit Rapid-FLIM

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Kurzfassung

Plastik, insbesondere Mikroplastik, stellt eine zunehmende Gefahr für unser Ökosystem dar und für Tiere, die in kontaminierten Umweltbereichen leben. Die Forschung zeigt, dass der Nachweis von Mikroplastik in der Umwelt schwierig ist und mit einer zeitaufwendigen Probenentnahme einhergeht. Die Extraktion von Mikroplastik aus der Umwelt muss sorgfältig durchgeführt werden, da gängige Identifizierungstechniken wie die Nahinfrarot- oder Raman-Spektroskopie durch die Umweltmatrix behindert werden. Momentan fehlen optische Messmethoden, um Mikroplastik direkt in der vorliegenden Matrix zu identifizieren. Daher werden Untersuchungen zum Rapid-FLIM Verfahren durchgeführt, das Kunststoffe aufgrund ihrer Fluoreszenzeigenschaften quantitativ identifiziert. Die Ergebnisse der Untersuchungen an künstlich präparierten Matrizen aus Fichte, Gras und rotem Polyethylen hoher Dichte zeigen, dass eine quantitative Unterscheidung der drei Materialien mit Rapid-FLIM möglich ist.

Abstract

Plastic, especially microplastic, is an increasing thread for our ecosystem and animals living in contaminated environmental areas. Research shows that the detection of microplastic in the environment is difficult and goes hand in hand with a time consuming sample preparation. The extraction of microplastic from the environment has to be done carefully because common identification techniques like near infrared or Raman spectroscopy are hindered by environmental matrices. There is a lack of optical measurement methods to identify microplastics in the matrix at hand. Thus, investigations on Rapid-FLIM, which identifies plastics due to their fluorescence properties, are done. The results of the investigations on artificially prepared matrices using spruce, grass and red high density polyethylene show that a quantitative differentiation of the three materials is possible using Rapid-FLIM.

1 Introduction

The global plastic production has doubled from 234 million tons (mt) in 2000 to 460 mt in 2019. Furthermore, the global annual plastic waste has increased more than twofold from 150 mt in 2000 to 353 mt in 2019. Merely 15 % (55 mt) of 353 mt plastic waste are fed into the recycling process, whereby 22 mt cannot be recycled and are further disposed. In the end, only 9 % (33 mt) of the plastic waste that is forwarded to recycling can actually be recycled. 19 % of the 353 mt of plastic waste are incinerated and roughly 50 % are disposed in landfills. There are 22 % of plastic waste remaining, which are disposed in dumpsites leaking into the environment [1].
Pollution of the environment by plastic waste, especially microplastic (MP) waste is a well-known global issue that increases every year [2]. In the publication [3] it was shown that 4.8 to 12.7 mt of the plastic waste enter the environment and defragment to MP due to various environmental factors. The impact of ubiquitous microplastic pollution on the ecosystem and animals living in the contaminated environment is largely unclear. Additionally, the impact of microplastics in the environment on human health is still unknown.
While the effects of MP on the ecosystem and the health of animals and humans are largely unexplored, there are no appropriate optical measurement techniques to reliably determine the microplastic concentration in the environment [4]. At the moment, environmental samples are collected, the microplastic is extracted and the identification is done with Raman or micro Fourier transform infrared (FTIR) spectroscopy. Raman spectroscopy is used to identify particles of 500 nm in size. Using Micro-FTIR, particles in the range of 10 to 500 µm can be analyzed. However, Raman spectroscopy and Micro-FTIR methods are afflicted with disadvantages. Raman spectroscopy is unsuitable if the plastic is coated with organisms or if pigments are added to the plastic, since these overlay the Raman signal. Micro-FTIR is vulnerable to water because of the superimposition of the absorption band of water and the measured spectrum. The disadvantages imply that the MP samples have to be

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carefully prepared if analyzed using Raman or Micro-FTIR, making it very time-consuming [5]. The time consuming sample preparation may not be necessary if the particles could be identified directly in the matrix at hand.
The potential of the frequency-domain fluorescence lifetime imaging microscopy (FD-FLIM) technology for the identification of plastic types is shown in [6]. Moreover a differentiation of plastic and wood is possible with FD-FLIM [7]. The fluorescence excitation hereby happens with a sinusoidally modulated laser diode (488 nm wavelength) and the phase shifted and amplitude damped fluorescence signal is detected by a FD-FLIM camera. By integrating the fluorescence photons with a pixel tapping algorithm, the camera reconstructs the phase shifted sinusoidally signal from eight image pairs. Using the reconstructed sinusoidal fluorescence signal, the phase shift is measurable. By the measured phase shift, a calculation of the fluorescence lifetime and thus a qualitative identification and differentiation of different materials, like MP are possible.
Besides the FD-FLIM measurement, another possible method to detect MP quantitatively in an environmental matrix is the Rapid-FLIM method, which has not yet been investigated to identify MP in the environment. Rapid-FLIM relies on the measurement of one image pair and thus is eight times faster than a standard FD-FLIM measurement. Using Rapid-FLIM, the normalized difference (ND) at a defined phase shift is calculated by the two images. As the fluorescence lifetime of a FD-FLIM measurement and thus the phase shift is specific for each material, a detection of MP in an environmental sample seems possible.
To investigate the Rapid-FLIM technology two prepared samples were inspected: the first one containing red high density polyethylene (HDPE), spruce and grass at a larger scale and a second one containing a red HDPE and spruce particle in section 3.

2 Theory

In frequency domain fluorimetry, the sample is excited using a sinusoidally or rectangularly modulated light source having a defined modulation frequency ω [8]. The harmonic excitation causes a phase shifted Φ, equivalent shifted (B, b) and amplitude damped (A, a) fluorescence emission (see **Figure 1**). The phase dependent fluorescence lifetime can be calculated from the measured phase shift Φ and the defined modulation frequency ω using equation 1.

$$\tau = \tan(\Phi)\,/\omega \qquad (1)$$

Areal measurements of phase dependent fluorescence lifetimes are performed using a FD-FLIM camera [9]. Using the FD-FLIM camera, the fluorescence emission can be measured by a pixel tapping algorithm. In a single pixel of the FD-FLIM camera, a charge swing driven by a switch is integrated, whereby the pixel tapping algorithm toggles the switch. The charge swing is a construction of two cambers opened in opposite directions (C1 and C2). While the switch is activating C1 the photons are integrated in C1 causing an integrated intensity in the pixel of the first chamber. If the switch is pointing on C2, all incoming photons are collected in the C2 also causing an integrated intensity for the second chamber in the pixel.

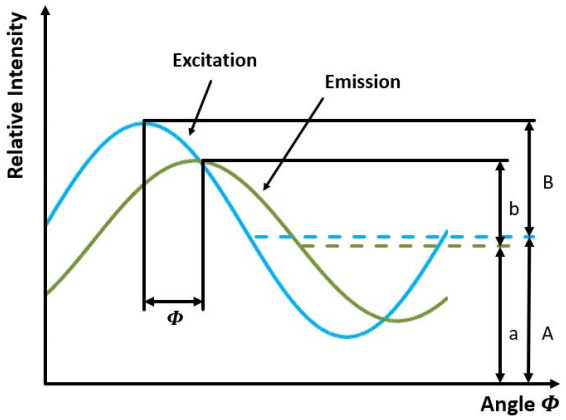

Figure 1 Laser excitation signal (blue) causing a phase shifted, amplitude damped and equivalent shifted fluorescence emission (green) signal.

The diagram in **Figure 2** shows an integration of the photons in C1 running over phase angle values from 0 to 180°, resulting in an intensity I_1. At 180°, the switch changes to allow photon integration in C2 over the second half period of the sine leading to an intensity I_9.

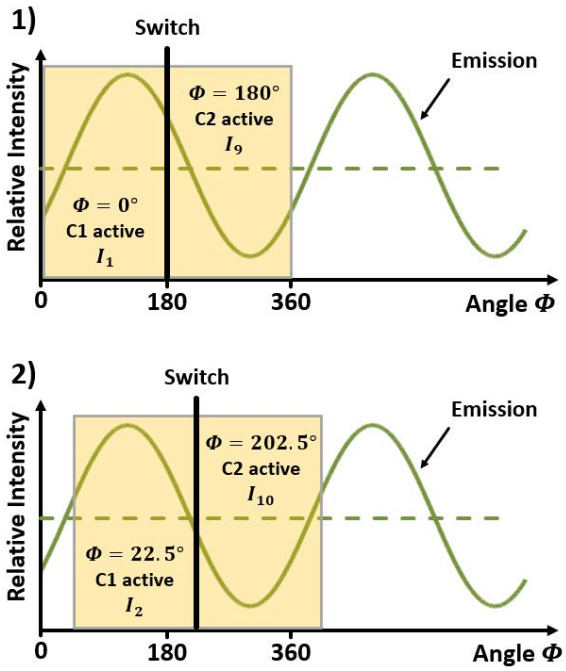

Figure 2 Schematic representation of the fluorescence emission (green) and the integration windows for C1 and C2, whereby: **1)** C1 is active for phase angle values between 0° and 180° resulting in an intensity I_1 and C2 is active from 180° to 360° giving the intensity I_9; and: **2)** C1 is active from 22.5° to 202.5° resulting in an intensity I_2 and C2 from 202.5° to 22.5° leading to the intensity I_{10}.

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A double image is created from these two photon integration steps, one image for phase angle value 0° and one for phase angle 180°. Next, the starting phase angle value is shifted and a second double image is taken using the different starting phase angle values. In order to reconstruct the sinusoidal fluorescence emission, the integration in C1 and C2 has to be done several times using shifted starting phase angle values. For example, the next integration step of C1 starts at 22.5° integrating photons until 202.5° resulting in an intensity I_2 as shown in the Figure 2. C2 starts at 202.5° integrating photons until 382.5° giving the intensity I_{10}. The repetition of the integration in 22.5° phase shifted steps leads to 16 intensities I_1 - I_{16} per full period of the sinusoidal fluorescence emission per pixel. The integration procedure is done for each pixel separately resulting in 16 intensity images. Using the 16 intensity images, the sinusoidal fluorescence signal can be reconstructed and the calculation of the phase dependent fluorescence lifetime is possible by means of the obtained phase shift between excitation signal and emission signal. Using the FD-FLIM technique, a fast and robust determination of fluorescence lifetimes is possible. In addition, a qualitative differentiation of several materials in a single fluorescence lifetime image can be done.

An even faster way to quantitativly differentiate materials is the Rapid-FLIM method [10]. Compared to the 16 images taken from an FD-FLIM measurement, using Rapid-FLIM only 2 images have to be captured, which implies that Rapid-FLIM is eight times faster. As it is illustrated in **Figure 3**, Rapid-FLIM uses two integration windows having a length of 180° phase angle value at a defined phase start angle, a predefined value between 0° and 180°. The example in Figure 3 shows the set starting phase angle of 60° causing a integration in chamber C1 between 60° and 240°. After 240° are reached the switch is toggled and the second integration in chamber C2 starts until the starting angle of 360° + 60° is reached. The integrations give two measured intensity values I_1 and I_2 of C1 and C2 for each pixel, resulting in two intensity images. Using these resulting intensity images, a Rapid-FLIM image X_{RF} can be calculated by taking the normalized difference (ND) using equation 2.

$$X_{RF} = (I_1 - I_2)/(I_1 + I_2) \qquad (2)$$

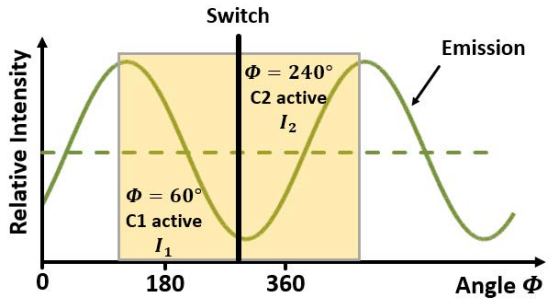

Figure 3 Schematic representation of the fluorescence emission (green) and the integration windows for C1 and C2 at an example phase angle start value of 0°. The integration results are two intensities I_1 and I_2.

Since the intensity differs with the predefined starting point of the phase angle, the phase angle starting point has to be adjusted if no differences are detected in the image of the X_{RF} (i.e. if the X_{RF} is a zero matrix). Once the phase angle starting point causes a non-zero X_{RF}, the evaluation can be executed.

3 Experimental

Two different prepared samples were used for the investigations. The first sample contains red HDPE, spruce and grass (Ex I.) and the second one contains HDPE and spruce particles (Ex II.). The sample preparation, experimental procedure and data evaluation are explained in this chapter.

3.1 Sample preparation

The red HDPE sample is used to investigate whether MP can be quantitatively detected in a simple environmental matrix and has been cut from a Euronorm E2 box. The spruce sample has been cut from a dry wooden board at the TH Rosenheim. Grass has been collected from the lawn on TH Rosenheim campus. According to the literature [11], grass has a maximum fluorescence intensity in the range of 660 nm to 750 nm when excited in the blue/near ultraviolet wavelength region. Thus, the fluorescence signal emitted by grass can be completely blocked if the optical filters are chosen appropriate.

The investigations using Rapid-FLIM were done using the sample constellation prepared like shown in **Figure 4**. Spruce and HDPE were placed next to each other and grass was positioned on top (see Figure 4, left). For the investigations using the large samples, the sizes do not matter, as only the magnified image (see Figure 4, right) was under investigation.

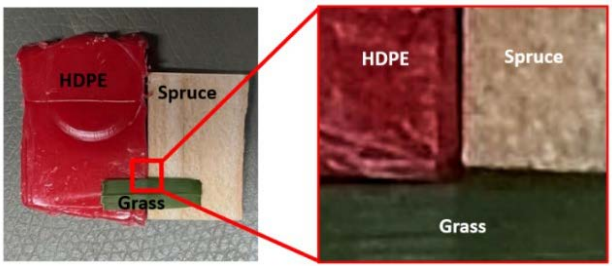

Figure 4 Prepared sample of red HDPE, spruce and grass (left) and the magnified image in which the Rapid-FLIM measurement was performed (right).

To investigate if Rapid-FLIM is a suitable measurement method to detect MP and differentiate it from environmental materials, a second sample was prepared like shown in **Figure 5**. Small particles of HDPE and spruce were scuffed of the big HDPE and spruce specimen. The particles of HDPE and spruce were placed next to each other in a petri dish (see Figure 5, left). Figure 5 on the right shows the magnified image of the measurement area containing HDPE and spruce.

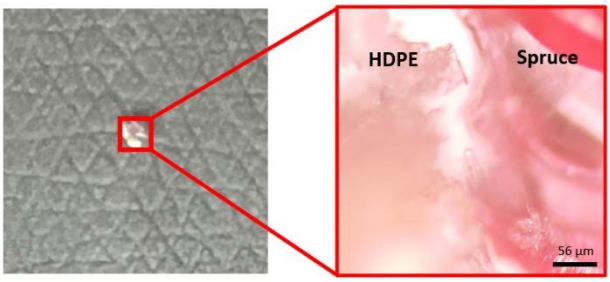

Figure 5 Prepared sample of red HDPE and spruce (left), whereby the sample size is approximately 1 mm, and the magnified image in which the Rapid-FLIM measurement was performed (right).

3.2 Experimental setup

The experimental setup presented in [7] was used for the investigations and a schematic of the setup is shown in **Figure 6**. The setup consists of a 445 nm laser diode from Omicron to excite the sample and a pco.flim camera from Excellitas PCO AG to capture the ND image X_{RF}. The excitation light of the laser diode is guided through a liquid light guide into the microscope, a PSM 1000 from Motic. The light transmits through an optical notch filter (Exciter) to narrow the wavelength bandwidth of the laser diode. After the light passes through the magnifying objective, the sample present on the sample stage is excited. The occurring reflectance, stray light and fluorescence radiates back into the microscope, whereby the band pass filter (Emitter) blocks the unwanted stray light and reflection. The emitter band pass filter has a cut on wavelength of 460 nm and a cut off wavelength of 520 nm, which is chosen to block the fluorescence intensity of grass completely [11]. At the light exit of the microscope, the pco.flim camera is mounted, which detects the fluorescence intensity.

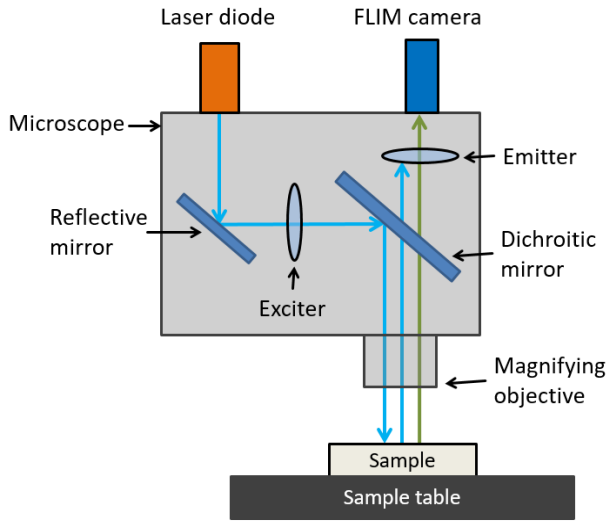

Figure 6 Schematic representation of the experimental setup containing a laser diode, a FLIM camera and the optical filters (Exciter, Emitter) assembled on a microscope.

3.3 Experimental procedure

To investigate if it is possible to detect MP and differentiate it from spruce using Rapid FLIM, the optical output power of the 445 nm laser diode was set to 500 mW. In order to search for an optimal value of the phase angle start point (Ex I.), a first measurement was performed at a phase angle value of 0 degree using the large samples (Figure 4). In addition, the exposure time was adjusted to 1200 ms and a magnification of x10 was chosen. After the measurement at a phase angle of 0°, the phase angle value was increased in 15° steps and the Rapid-FLIM measurement was repeated at every iteration, whereby the last phase angle start value was 180°. The result of the measurements were thirteen images of ND. The initial value of the phase angle causing the maximum difference of the calculated XRF's calculated for Grass, HDPE and Spruce in the ND image was used in Ex II. where Spruce and HDPE particles were used for the measurements (Figure 5).

3.4 Evaluation algorithm

The evaluation was done in NIS Elements from NIKON, which was also used to aquire the ND images. After taking the ND image, a lookup table (LUT) was applied. Using the LUT, the maximum difference can be determined by shifting the boundaries of the LUT to higher or lower values of XRF.
Additionally, NIS Elements provide a denoise AI function, which processes the ND image using different image processing filters to filter the noise. Using the denoise AI function on each ND image and shifting the LUT boundaries, the result is a false color ND image. Using the false color ND image, the materials a differentiation of materials present and the optimal phase angle start value can be defined.

4 Results

4.1 Results of Rapid FLIM Ex I.

In Ex I. the materials HDPE, spruce and grass were inspected using the sample in Figure 4. The fluorescence intensity of the measruement using the larger material samples are shown in **Figure 7**. Using the fluorescence intensity, a differentiation of spruce from HDPE and grass is possible, but HDPE and grass cannot be clearly distinguished. Moreover, the fluorescence intensity of grass is nearly blocked completely due to the optical band pass filter.
In **Figure 8** the ND image using a start phase angle of 75° is presented. All ND images taken at other phase angle starting values were inspected, whereby 75° showed the best contrast in the ND image between the used materials. Using the ND image at 75° phase angle, HDPE, spruce and grass are clearly distinguishable. The ND results show that grass has a ND of 0.01 to 0.04, which indicates that nearly no fluorescence intensity is present. The ND of HDPE is in the region of 0.1 to 0.25, while the ND of grass is inbetween 0.4 to 0.5. This ND value regions allow a

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quantitative differentiation of the three materials HDPE, spruce and grass. Additionally, the grass can be omitted in Ex. II since the fluorescence intensity of grass is completely blocked. The light blue areas in Figure 8 also show that the fluorescence intensities of spruce, grass and HDPE are convoluting, which is useful to determine the material edges.

Figure 7 Fluorescence intensity image of HDPE, spruce and grass.

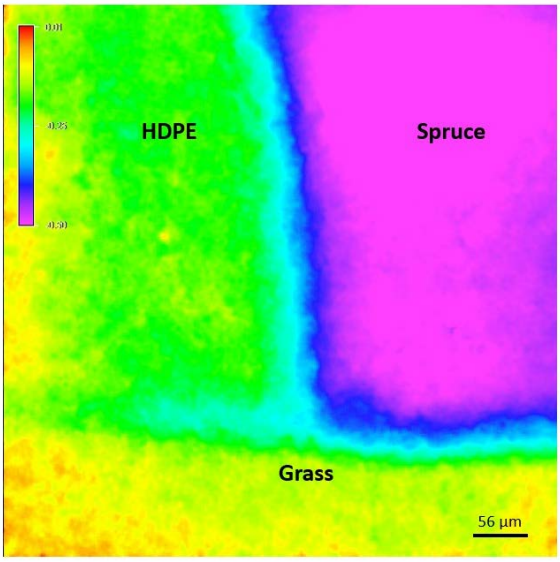

Figure 8 Denoised image of normalized difference from HDPE, spruce and grass.

4.2 Results of Rapid FLIM Ex II.

Knowing that grass has no influence on the ND measurements, it was omitted in Ex. II and only spruce and HDPE were investigated. The fluorescence intensity of Ex. II can be seen in **Figure 9**. Using the fluorescence intensity image a differentiation of spruce and HDPE can be made by the contrast, but the fluorescence intensity of HDPE is very low. Thus, it is pretty hard to argue that HDPE is present in the image or not.

As HDPE cannot be clearly seen in the fluorescence intensity image, the ND image is used to differentiate spruce and HDPE (see **Figure 10**). In the ND image, spruce has a ND value range between 0.36 and 0.47, which is consistent to the results of the investigations on the large HDPE, spruce and grass samples (Ex. I). Furthermore, the ND of HDPE ranges from approximately 0.1 to 0.28, which is also consistent to the observations in Ex. I.

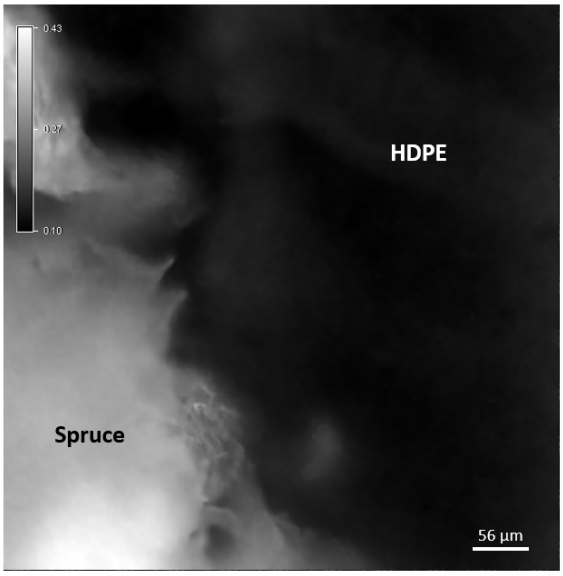

Figure 9 Fluorescence intensity image of HDPE and spruce particles.

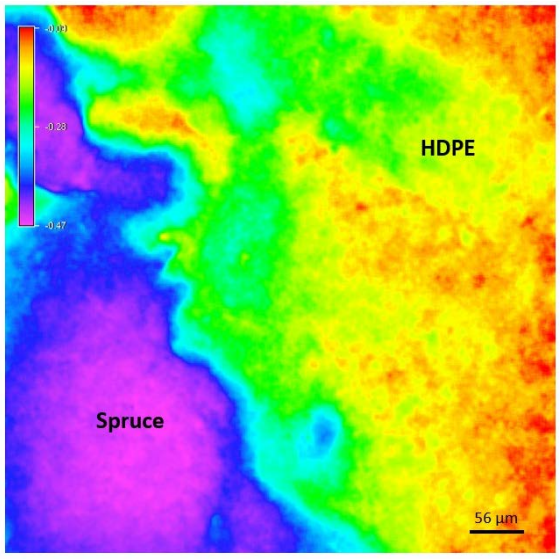

Figure 10 Denoised image of normalized difference from HDPE and spruce.

Unfortunately, the fluorescence intensity of HDPE is so low that ND value of 0.03 occur at the right border of the image (red false color in Figure 9), which can be counteracted by increasing the exposure time or laser power. Nevertheless a differentiation of spruce and HDPE is possible using the ND image.

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5 Conclusion

The FD-FLIM is a promising technique to identify MP in an environmental matrix. The FD-FLIM technique is a phase dependent measurement technique, which uses 8 double images to measure the phase shift and to calculate the fluorescence lifetime. Rapid-FLIM could be an even faster identification method for MP in an environmental matrix, as it requires only one image pair, whereby the phase starting angle is predefined by the measurand. In order to get a fast determination of the starting phase angle, a search algorithm has to be applied, which can be handled in a teaching procedure for an automated solution. The result of a Rapid-FLIM measurement is an image of normalized intensities, integrated over two half periods of the sinusoidal fluorescence emission.

In this contribution, Rapid-FLIM was used to investigate two simple and artificially prepared environmental samples, containing spruce, HDPE and grass: one containing large parts of HDPE, spruce and grass (Ex. I) and the second on consisting of a HDPE and spruce particle having approximately 1 mm in size (Ex. II).

The results of Ex. I show a clear differentiation of HDPE and spruce using the ND image data. Additionally, the optical band pass filter in the fluorescence emission path of the measurement system completely blocks the fluorescence signal of grass. Thus, the fluorescence emission of grass has no influence on the measured results. Therefore, only spruce and HDPE have to be differentiated in Ex. II. In Ex. II, HDPE and spruce can be distinguished, although the fluorescence intensity of HDPE has been low. However, the low fluorescence intensity of HDPE can be counteracted by increasing the exposure time or laser power.

In conclusion, the measured results of the ND images show the potential of the Rapid-FLIM method to quantitatively detect MPs and differentiate MPs from given environmental materials. Further research has to be done in order to exploit the full potential of the Rapid-FLIM measurement technique. More plastic types have to be investigated in different sizes, in order to determine the detection limits of the Rapid-FLIM method.

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