# 8. Automated microplastic analysis: Nile red staining and random forest modelling
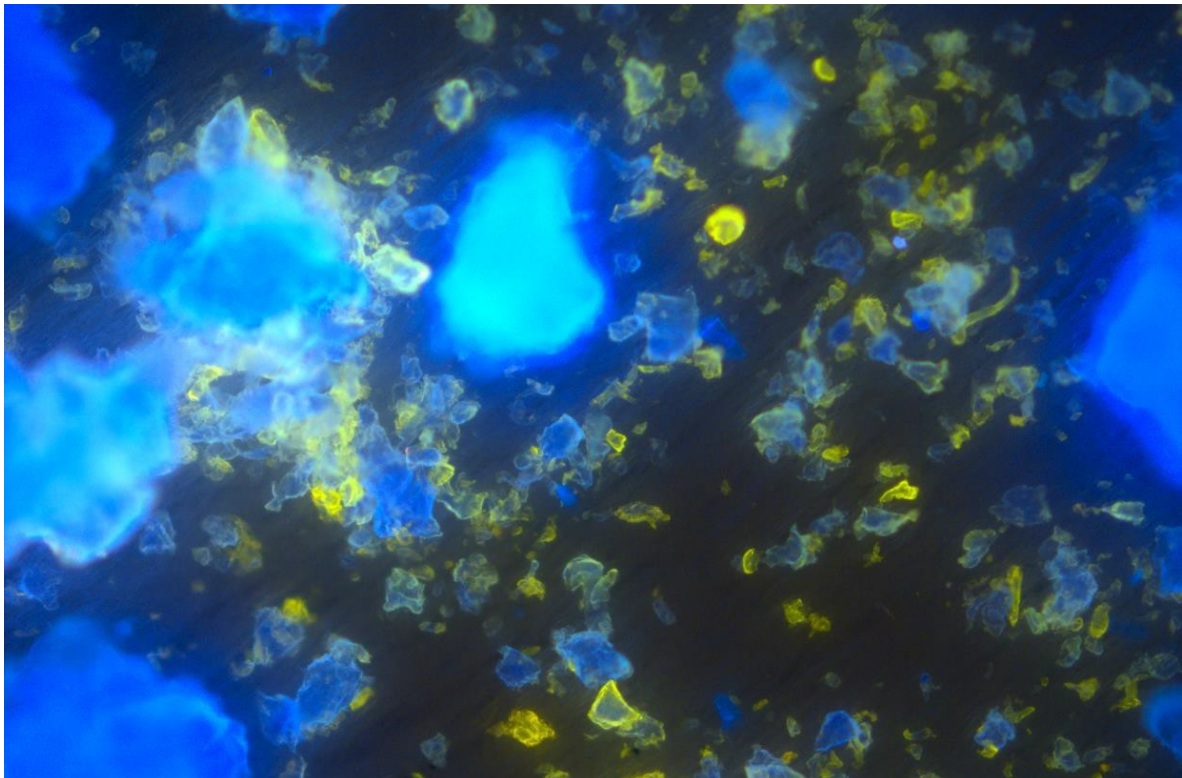
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# 8.1 Scope of Protocol

MPs, defined as plastic particles sized 0.1 µm – 5 mm (Hartmann *et al.*, 2019), are an issue of concern because of their ubiquity in the marine environment, caused by worldwide plastic manufacturing and inadequate waste management. The broad distribution of these global contaminants (2008/56/EC Marine Strategy Framework Directive, Descriptor 10; United Nations Sustainable Development Goal 14 target 14.1.1 [Gigliotti *et al.*, 2019]) has been confirmed in numerous research studies, going from surface waters to the bottom of the ocean and covering all depths, and from Arctic waters to Antarctic waters, including coastal, open and deep sea waters (Zhang, 2017; Pakhomova *et al.*, 2022). Moreover, growing empirical data point to potential adverse effects on organisms after ingestion (Franzellitti *et al.*, 2019). Risk assessment evaluations are needed to comprehend the consequences of MPs on an individual, population, and ecosystem level as well as on human health, and for this, credible MP monitoring data is crucial. Despite recent advancement and diversification of MP analysis techniques, which can provide information on biota exposure to MPs, many approaches are seen as being costly and time-consuming. Furthermore, the multitude of unsolved concerns regarding MP abundance, composition, distribution, fate, and effects in the marine environment highlight the need for more accessible, standardised and reliable analysis techniques.

The JPI Oceans-funded ANDROMEDA project is a multidisciplinary collaboration of 15 international partners focused on improving the quantification of NPs and MPs in our oceans and seas. Within the project, new sampling and advanced analysis methodologies that focus on smaller MP (< 10 µm) and NP (< 0.2µm) particles have been developed, which will enable a more accurate assessment of risks associated with plastic pollution. Novel sampling techniques as well as cost- effective MPs measurements methods have been developed for a more efficient and effective MP monitoring. As a result, a series of protocols related to MPs extraction, analysis and degradation were developed and optimized, and used by project partners. They are now made available to the plastic research community as SOPs.

This particular protocol focuses on the semi-automated, cost-effective MP analysis based on machine learning and RGB colour quantification of Nile red stained fluorescent particles. The developed, optimized (Meyers *et al.*, 2022) random forest (RF) models were tested for MPs down to 3 µm and proved accurate for their detection and the identification of their polymer types. The models demonstrated their ability to analyse pristine MPs in various marine matrices, artificially deep sea- and surface-weathered MPs, and ultimately MPs in real marine environmental samples.

## 8.2 Materials and Equipment

A.  Computer with sufficient storage capacity or access to a cloud storage service, and the open-source programmes ImageJ Fiji, R and R studio installed:
    o  Download ImageJ Fiji (https://imagej.net/software/fiji/downloads)
    o  Download R or R Studio (https://cran.rstudio.com)

B.  ImageJ scripts and R codes to create random forest models:
    https://github.com/NelleMeyers/RGB_models

C.  Unprocessed PTFE membrane filter (10 µm, ⌀ 47 mm)

D.  Fluorescence microscope (FM) or fluorescence stereomicroscope (FSM)
    o  Fluorescence stereomicroscope: e.g. Leica M205 FA Fluorescence stereomicroscope
    o  Software: LAS X software

**Table 1:** Setting specifications LAS X software for a Leica M205 FA Fluorescence stereomicroscope

| Filter | Exposure | Gain | Colour Controls | | | IL Light (%) | Zoom | Iris Control (100%) |
|---|---|---|---|---|---|---|---|---|
| | | | R | G | B | | | |
| • Blue filter - 10447407 Filter set ET GFP LP  M205FA/M165FC | 8.87 s | 1.8 | 2.4 | 0 | 7.9 | 100 | 1.00 | **100%** |
| • Green filter - 10450198 Filter set ET CY3 M205FA/M165FC ET545 | 78.01ms | 1.0 | 2.4 | 0 | 7.9 | 100 | 1.00 | **100%** |
| • UV filter - 10450609 Filter set ET UV LP M205FA/M165FC Climate control | 8.87 s | 1.8 | 0 | 21 | 100 | 100 | 1.00 | **100%** |

E.  Fluorescence microscope: e.g. LEICA DM 1000 fluorescence microscope
    o  Equipped with a camera and a LED for fluorescence
    o  Software: LAS Core software (Leica Application Suite version 4.13.0)

**Table 2:** Setting specifications LAS Core software for a LEICA DM 1000 fluorescence microscope

| Filter | Exposure | Gain | Saturation | Gamma | Hue/ Saturation |
|---|---|---|---|---|---|
| • Blue filter: filter System I3 S, BP 450–490 nm | 2.91 ms | 1.0 | 1.50 | 0.6 | H0 / S100 |
| • Green filter: filter system N2.1 S, BP 515–560 nm | 1.01 ms | 1.0 | 1.50 | 0.6 | Auto Colour |
| • UV filter: filter System A S, BP) 340–380 nm | 26 ms | 1.0 | 1.50 | 0.6 | H70 / S100 |

## 8.3 Protocol

For the sake of clarity, all folders mentioned in this protocol will be in green, all codes in red, all images in blue, all generated Excel files in purple and all working directories or lines of code in grey.

### 8.3.1 Particle Selection

To create the predictive models, two separate datasets are needed: one to train the Plastic Detection Model (PDM) to allow for plastic detection, and one to train the Polymer Identification Model (PIM) to allow for polymer identification of plastics. Before image acquisition and analysis, determine the type of particles you want to incorporate in your dataset, and perform a power analysis to know the required size of your training dataset. The size of the dataset will depend on the required statistical power, which depends on the chosen significance level α; the effect size; and the number of different categories (plastic/non-plastic for the PDM; different polymer types for the PIM).

To create a PDM dataset, photos of fluorescently dyed organic particles representative of the marine environment should be acquired. For example, chitin, cotton, flax, hemp, silk, wood and wool can be used to do so (Meyers *et al*., 2022). Furthermore, particle size should be representative of MP sizes observed in the marine environment. To obtain small particle sizes, plastics can e.g., be cryo‑milled using a cryogenic grinder.

For the plastic particles within both datasets (PDM and PIM), polymer types commonly observed in marine environmental samples should be selected (Suaria *et al*., 2020). Moreover, ensure a broad range of densities is covered. For example, seven of the most abundant produced plastic polymers worldwide could be included (Geyer *et al.,* 2017): nylon, polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyurethane (PUR) and polyvinyl chloride (PVC). It is strongly advised during the model training phase to use one polymer type per PTFE filter, to avoid additional µ-FTIR analyses of particles, and to allow for rapid image analysis as well as RGB dataset construction. The best practice to ensure model robustness is to use plastics from different manufacturers, as well as multiple PTFE filters per polymer type, to incorporate potential associated variation in fluorescence colouration.

To determine the dataset size needed to train a RF model, the required significance level α, the effect size d (Cohen, 1988) and the number of categories should be considered.

To create the models, particles should be stained as described in Meyers *et al.,* 2024 (a, b, and c). They can be spiked in and recovered from a matrix (seawater, sediment or biota) using the SOPs, with subsequent staining with the fluorescent dye Nile red, or they can be spiked in Milli-Q water and stained with Nile red directly after without performing a sample processing step. For subsequent analysis, particles should be extracted from marine samples as described in Meyers *et al*., 2024 (a, b and c).

## 8.3.2 Image Acquisition

A. Always take photos of the same particle(s) in the same frame in all the settings, making sure the stage does not move and you do not switch to a different magnification. For setting specifications for a Leica M205 FA fluorescence stereomicroscope and a LEICA DM 1000 fluorescence microscope, see Table 1 and 2, respectively.

B. Each photo series of a particle/number of particles consists of three photos: a photo acquired under a blue filter, under a green filter and under a UV filter. If possible, a bright field photo (no filter) should be acquired as well. For further photo acquisition details, see Section 8.2 'Materials and equipment'.

C. All photos should be saved in "TIFF" format. Store them under the 'Photos' folder. If taken, bright field photos should be stored in the sample folder (and not under 'Photos'). Generated CSV files will be saved under the 'RGB' folder after photo analysis in ImageJ.

**D.** Using the LAS software to acquire photos, label each photo with a unique code. For each photographed filter area, a series of three images is required, taken under the blue, green and UV filter. Generated CSV files of each photo will be saved under 'RGB' after photo analysis in ImageJ. For the code to run correctly, Photos should be named as followed, in capital letters:

> *'name of the filter'* **+_+** *'number'* **+_+** *'colour'*
> **For example:** *'PET_1_BLUE'; 'PET_1_GREEN', and 'PET_1_UV'*

In the following example, the construction of an RGB dataset to create a PDM is described, using the script where batch mode is not used. To create a dataset for a PIM, the same procedure should be followed, with the exception that photographs of different plastic polymer particles will be analysed, rather than plastic and organic particles.

## 8.3.3 Creating Machine Learning Models

### 8.3.3.1 File Organisation

Create a folder called 'Automated_MP_analysis', and four subfolders called 'Coding', 'Model_constructions', 'Training_datasets', and 'Model_visualisations'. Save the ImageJ code ('RGB_extraction_batchmode' or 'RGB_extraction_nobatchmode') and the two R scripts ('RGB_dataset' and 'PDM_PIM') under 'Coding'. Next, classify your sample images:

1. <u>Batch mode (FM - multiple photos/PTFE filter)</u>
   Create three subfolders within *'Model_constructions'*: *'Photos'*, *'Metadata'* and *'RGB'*. Store all images (blue, green, uv) within *'Photos'*.

2. <u>No batch mode (FSM - 1 photo/PTFE filter)</u>
   Create subfolders per photographed PTFE filter within *'Model_constructions'*. Within these filter-specific folders, each time create three distinct folders: *'Photos'*, *'Metadata'* and *'RGB'*. Store all filter-specific images (blue, green, UV) within *'Photos'*.

### 8.3.3.2 ImageJ

- Open ImageJ Fiji.
- Open a new macro through 'Plugins' > 'New' > 'Macro'.
- Paste the ImageJ script *'RGB_extraction_batchmode'* or *'RGB_extraction_nobatchmode'* in the new window
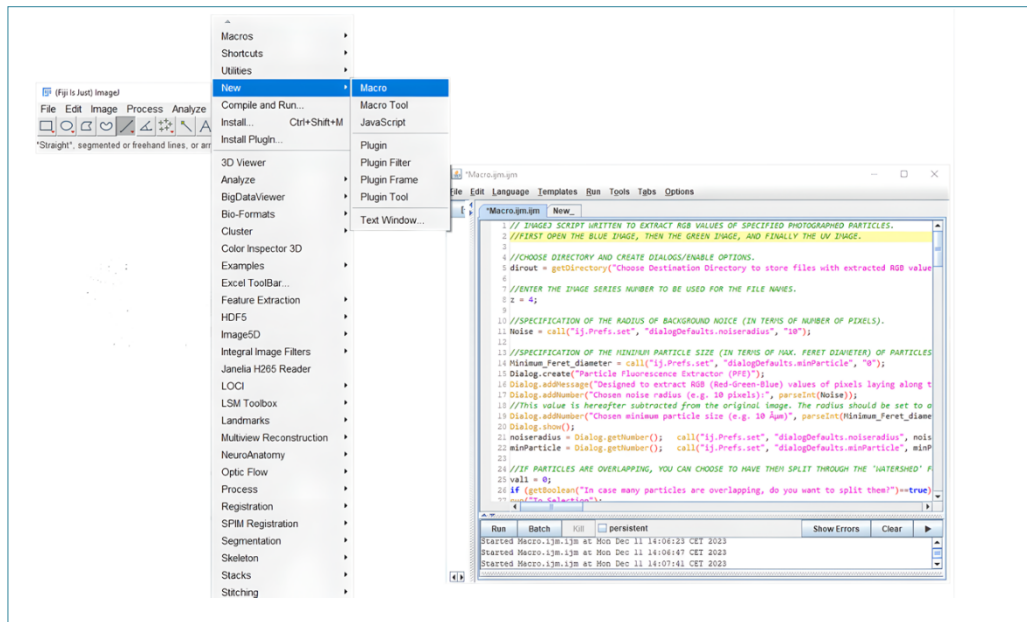
**Figure 1:** Open script in imageJ

## Setting the Correct Scale

**A.** Start by setting the correct scale. To do so, use a bright field photo of an unprocessed PTFE filter, acquired with the exact same microscope specifications used for the bright field photo of the actual sample. Open the photo ('File' > 'Open') and use the line-selection tool to draw the diameter of the PTFE filter (see Figure 2). Next, set the scale ('Analyse' > 'Set scale'), enter the known length of the filter diameter under 'Known distance' in μm (47000), enter 'μm' as unit of length, and make sure 'Global' is checked so that the same scaling factor is used for all photos while the program is opened. Once scaled, it is best to note down the corresponding number of pixels per μm, in case rescaling would be necessary in the future.
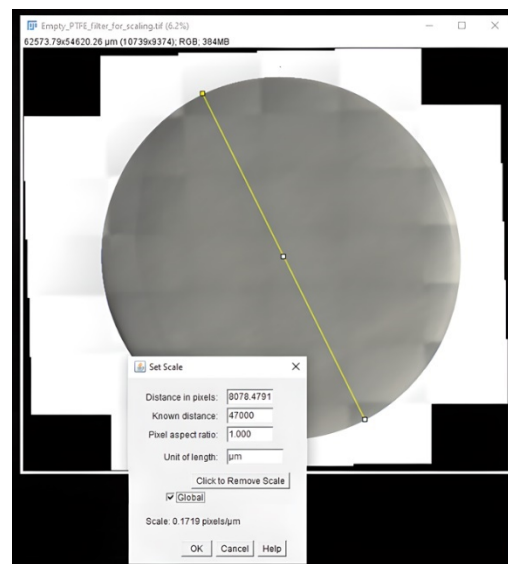
Once this is done, press 'OK', and close the photo.



**Figure 2:** Setting the correct scale

## B. Extraction of RGB value

Open the blue filter photo ('File' > 'Open') in ImageJ followed by the green filter and lastly the UV filter (in this specific order) (see Figure 3). Run the macro 'Run' > 'Run'. Uncheck the 'Disable Global calibration' box (and check 'Disable these Messages') to make sure the scaling from the unprocessed PTFE filter is used to scale the photos. Select the 'RGB' folder you just created as a destination directory to store the CSV files with extracted RGB values (dialog box).
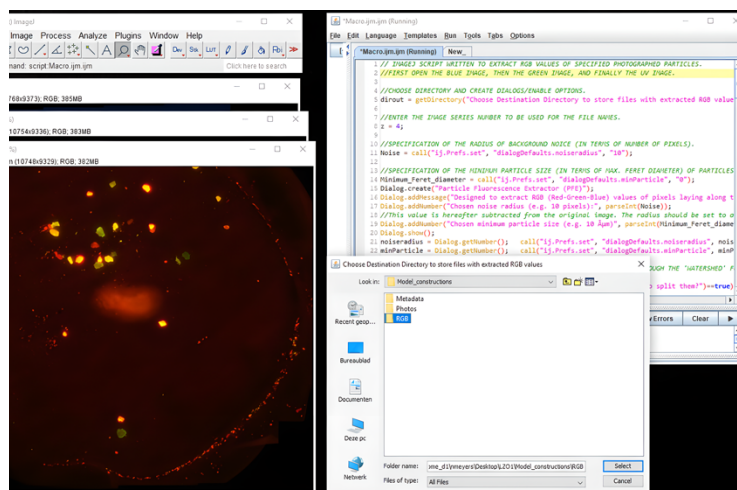


**Figure 3:** Open the three pictures (blue, green and UV filter) in imageJ, and run the macro

## C. A new dialog box pops up. Specify the noise radius the program should use (i.e. from what number of pixels onward a selected area should be considered a particle rather than noise) (see Figure 4) Next, specify the minimum size of the recognised particles for which the RGB values of the pixels on the maximum Feret diameter should be extracted. Enter for example '10' in both fields, then press 'OK'.
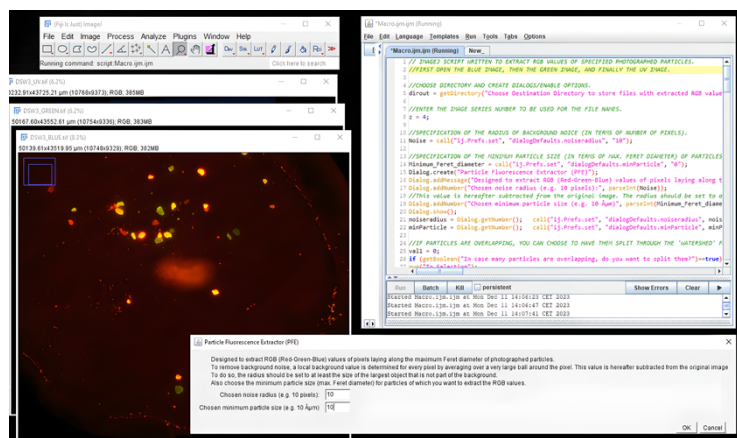


**Figure 4:** Specify the noise radius and min. particle size for which RGB values should be extracted

D. For the next step, an option is for the program to recognize overlapping particles and split them into separate particles.

**Note:** If the exact number of particles present is important and the number of overlapping particles is high, it is advised to choose 'no', as unique particles are sometimes mistakenly split into multiple particles.

E. The program asks for the scale to be set. The scale was already specified prior to this step, but to verify the accuracy, the diameter of the filter can be drawn using the line-selection tool (e.g. on the UV photo as borders are clearly visible).
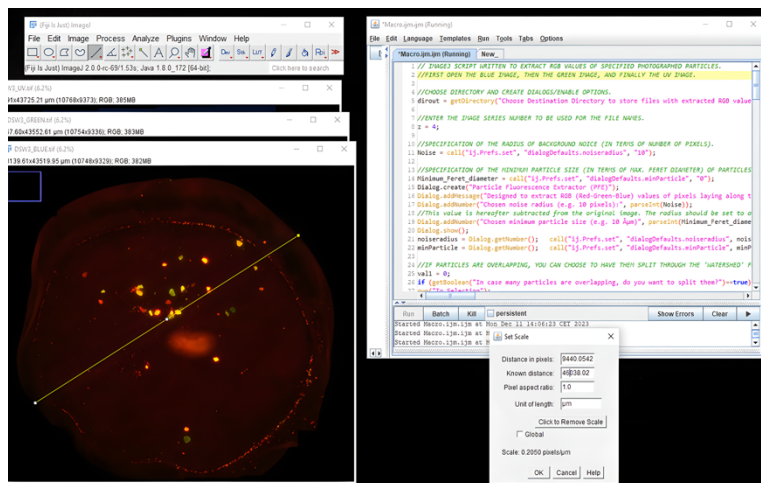
**Figure 5:** Test whether the scale has been set

Next, press 'OK'. The length shown under 'known distance' should now approximate the diameter of the reference PTFE filter, which is 47000 µm. As circularity of the current filters is not a 100%, the value will deviate slightly from the set value. Don't make any changes, but make sure the 'Global' box is still checked (see Figure 5).

**F.** The blue filter photo will be processed (the first opened photo). First, a threshold needs to be set to identify all particles present (see Figure 6). To do so, move the upper slider bar until all particles are red. Make sure this specific window is selected, otherwise no changes will be observed when adjusting the slider bar. Next, press 'Apply'. Now, all selected red particles turn white. Following this, press 'Ok'. It is important to respect the order of pressing 'Apply' first followed by 'ok', and not the other way around.
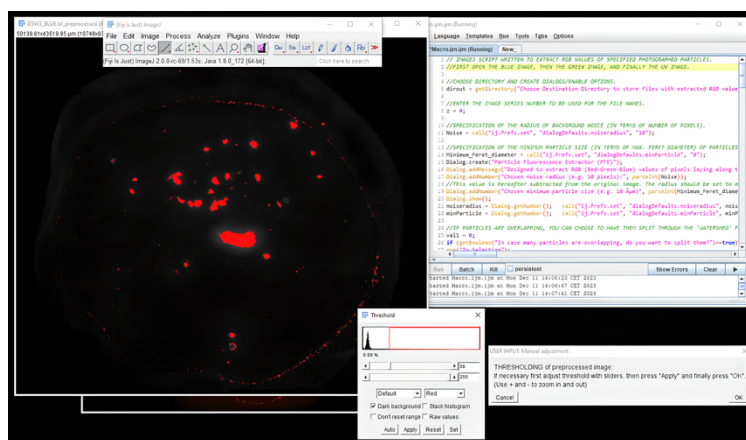


**Figure 6:** Set the correct threshold

**G.** Subsequently, all particles will be selected in yellow on the original photos. The progress here can be observed in the menu bar. 'Do you want to move the ROIs (Regions of Interest) for the next picture?' pops up, choose 'yes' for this question (see Figure 7).
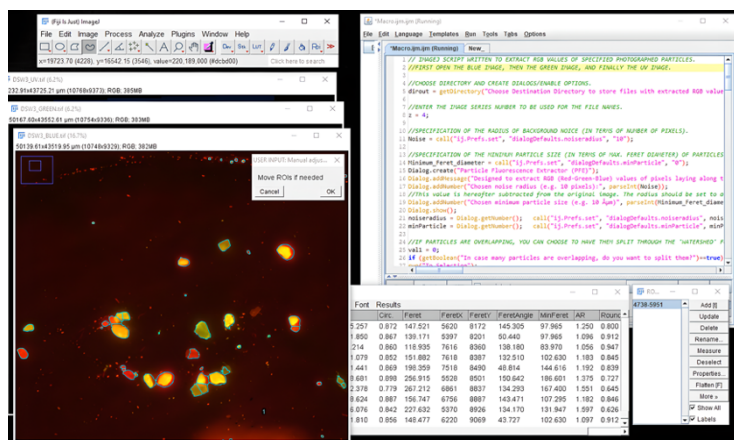


**Figure 7:** The ROIs are now visible on the original photos

**H.** Once the program is ready, all particles will be selected in blue. Select the line selection tool if not selected, put the cursor on a particle, click right and keep it pressed down to move the whole particle selection until the particle outlines match with the particles (see Figure 8).
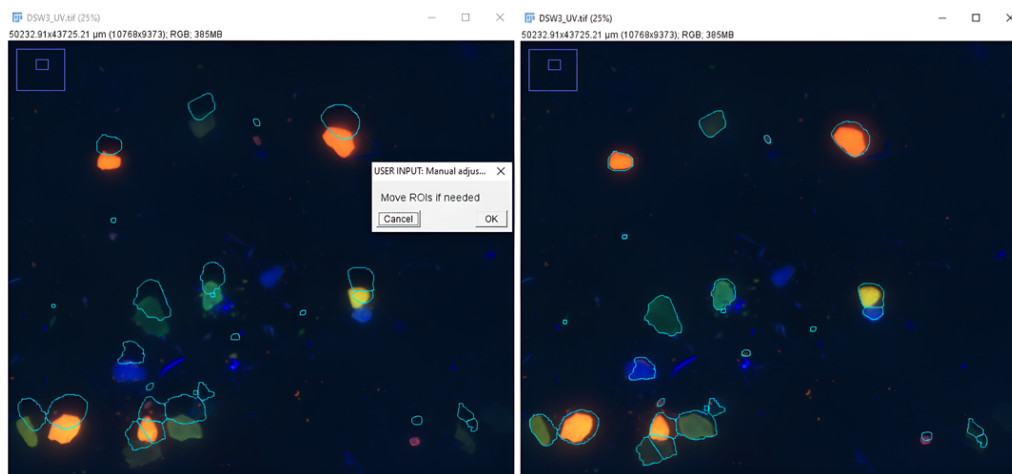


**Figure 8:** Move to ROIs to the correct position if necessary

It is advised to zoom in while doing so. Next, press 'Ok'. Following this, RGB values files will be generated for all particles on the blue filter.

**Note:** If the selection would turn yellow, it can no longer be moved. If this happens, go to the 'ROI manager' window and click on the number, which represent the selection. Following this, the selection should turn blue again. Next, choose 'Yes' when 'Do you want to move the ROIs for the next picture?' pops up again, this time for the green filter photo. Move the selection if needed, so that all particles are correctly outlined, then press 'Ok' for 'Move ROIs if needed'. Repeat the same process for the UV filter.

When using the batch mode script, the next series of three photos of a sample (photographed under a blue, green and UV filter) will open, after which the threshold process and extraction of RGB values will be repeated. This process will be repeated until all photos stored in that folder are processed.

### 8.3.3.4 RStudio

### Calculation of RGB-Statistics

**A.** Go to the folder 'RGB' of a specific sample a check whether files have been generated.

- o *'Numbered_particles_1.tif'*, *'numbered_particles_2.tif'*, and *'numbered_particles_3.tif'* are the blue, green and UV filter photos with numbered particles (see Figures 9a, b and c),

respectively. '*PET_4_numbered_particles.tif*' is black-white outline of all analyzed particles, here particles are also numbered (see Figure 9d). When opening the metadata file '*particle_measurements_4_details.xl*', In column 'A' the particles numbers can be found which correspond with the numbered particles in the '*PET_4_numbered_particles.tif*' photo. Notice however that this numbering does <u>not</u> correspond with the numbering on the '*numbered_particles*' photos. In column 'E', the size (maximum Feret diameter) of the corresponding particles can be found. More particle characteristics can be found in other columns.

o  For every particle on the three '*numbered_particles*' photos', a CSV file per colour filter can be found: e.g. for particle **<u>1</u>**, a file '*PET_BLUE_p**1**.tif*', '*PET_GREEN_p**1**.tif*' and '*PET_UV_p**1**.tif*' can be found.

o  These files contain RGB data for each pixel located on the maximum Feret diameter of a selected particle. In the file, each row presents a pixel, where 'X,Y, Red, Green, Blue' stand for the x-coordinate, the y-coordinate as well as the red (R), green (G) and blue (B) value (all ranging between 0-255) of that pixel, respectively.

**Note:** For the script with batch mode, all numbered colour filters photos as well as all CSV files can be found in the same RGB folder.
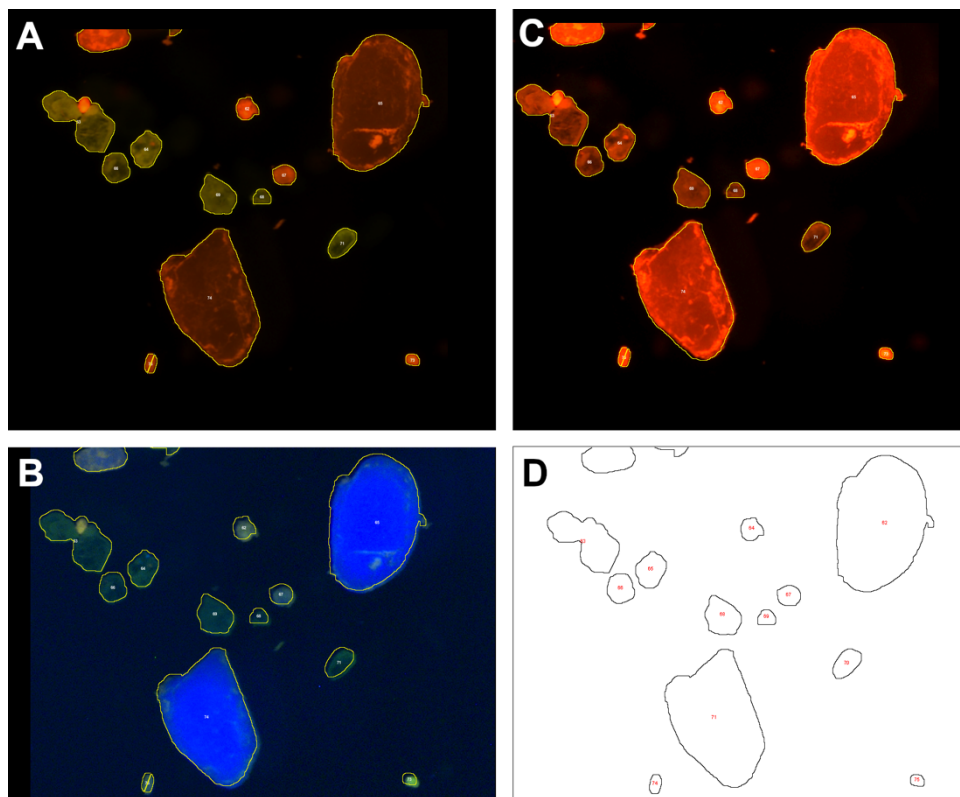


**Figure 9:** LRTB: 'numbered_particles' for the blue (a), green (b) and (c) UV filter, with all particles numbered, as well as a (d) black-white outline.

B. Open the 'RGB_dataset' script in R. In this step, the mean, median, 10th and 90 percentiles of the RGB value of all analysed pixels belonging to a particle (= per CSV file) will be calculated in R. As a first step, create three new folders within your RGB folder: 'BLUE', 'GREEN' and 'UV'. Next, copy all '_BLUE'-CSV files into the BLUE folder, all '_GREEN'-CSV files into the GREEN folder, and all '_UV'-CSV files into the UV folder.

C. Once the R script is opened, adapt the working directory to the directory where the 'BLUE'-CSV files are stored:

*setwd("…/Automated_MP_analysis/Model_constructions/PET/RGB/BLUE")*

- Change the working directory again for the Green CSV files:
  *setwd("…/Model_constructions /PET/RGB/GREEN")*

- and do this once more for the UV CSV files further on in the script:
  *setwd("…/Model_constructions /PET/RGB/UV")*

- Next, change the working directory to the location where the UV CSV files are stored after 'Export': Export<-setwd(*"…/Model_constructions /PET/RGB/UV")*

- Lastly, choose a name for the generated dataset, e.g. '*PDM_RGBdataset*':
  *Filename<- paste(Date," _PDM_RGBdataset'.xlsx",sep= "")*

→ After this, run the whole script. A dataset containing all particle statistics will be generated in the UV folder.

D. Open the generated dataset and delete the first row '*Fulldataset_bluefilter*'. Following this, select all statistics (column D until AM, row 3 until the end of the dataset), right click on the selected data and select 'Convert to number'. Lastly, adapt all cells so that the values have 0 decimals. Right now, the dataset with RGB statistics of photographed particles is ready. Each line in the Excel file now contains RGB statistics of an analyzed particle which can be traced back on the analysed photos through its number. As a last step, save the dataset with RGB statistics (e.g. as '*PDM_RGBdataset.xlsx*') under the folder '*training datasets*'.

E. If desired, a RGB dataset for a PIM can be constructed as a next step, containing RGB statistics of different plastic polymers.

### 8.3.3.5 ML Model Construction

A. Open the code '*PDM_PIM*' in R.

o Adapt the working directory to where the newly generated datasets are stored:

*setwd("…/Model_constructions /Training_datasets")*

- o Choose a name for the decision tree plot of the generated RF model, e.g. '*PDM_setseed7*': *jpeg(file = "Model_visualisations/PDM_setseed7.jpg")*

**B.** Run the script under '#Generate model'. A random forest model will be generated following randomization of the training dataset. A total of 1000 bootstrap decision trees will be generated (ntree = 1000) (see Figure 10), where all 36 variables are randomly sampled as candidates at each split (mtry = 36). The confusion matrix, a performance measurement technique, associated with the RF model is also generated, as well as the out-of- bag (OOB) error rate (%), which is a measure for prediction error. In the folder '*Model_visualisations*', a JPG-file is generated with a plotted decision tree representative of the random forest output. However, be aware that these models are visually complex and often uninterpretable. To generate a more readable friendly model for the purpose of visualisation, a depth parameter can be specified.

```
tree.RGB <- randomForest(
    Material ~ .,
    data = RGB_dataset[, -1],
    ntree=1000,
    mtry = 36,
    replace=F,
    importance = T,
    keep.forest=T,
    keep.inbag=T,
    do.trace=50

)

print(tree.RGB)

jpeg(file = "PDM_visualisation/PDM_setseed7.jpg")
plot.getTree(tree.RGB)
dev.off()
```

**Ntree = 1000** → Number of bootstrap replicates (= number of trees to grow). The performance of a RF model will plateau beyond a certain number of trees. For more information, run the script under '#*Model information*'.
**Mtry = 36** → Number of variables randomly sampled as candidates for splitting at each node.

**Figure 10:** RF model specifications in the script

**C.** A 5-fold cross-validation can be performed to validate the performance of the generated model under '#K-fold cross validation'.

**D.** To determine the required number of bootstrap trees for accurate results (= as a function of model prediction error), as well as the required minimum training panel size and the required minimum number of parameters, run script under '#Model information'.

### 8.3.4. Microplastic Analysis using Generated Models

To analyse particles of unknown origin within a sample, a RGB dataset of the particles is needed. To create this new dataset, follow the same steps as done to build the model training dataset(s).

**A.** First, create a new subfolder within '*Automated_MP_analysis*' called '*MP analyses*'. Create subfolders in the same way as done for the model construction ('*Photos*', '*Metadata*', and '*RGB*'). Use PTFE filter-specific folders if batch mode is not used.

**B.** Create a RGB dataset in the same manner as done before, this time of unknown particles photographed after sample extraction and staining with Nile red. In the dataset, replace the content in the column '*Material*' by '*Unknown*'. Give the dataset an appropriate name, e.g. '*sample1_RGBdataset.xlsx*'.

**C.** Open the code '*PDM_PIM*' in R.

- Under #Generate model', make sure the first working directory is again set to where the model training datasets are stored: *setwd("…/Model_constructions /Training_datasets")*, Create a read_excel()-function for this dataset: *RGB_dataset <- read_excel("PDM_RGBdataset.xlsx")*.

- Under #Model_prediction, adapt the working directory to the directory where the RGB dataset created from the analysed sample is stored, e.g. *setwd("…/Analyses/sample1/RGB/UV")*. Create another read_excel()- function for the generated dataset of unknown particles:
  *Unknown_dataset <- read_excel("sample1_RGBdataset.xlsx")*.

- Chose a name for the Excel file with particle predictions which will be generated when running this code. It is advised to use the name of the dataset, and to add '*_MP_predictions*', followed by the model used to perform the predictions, e.g. "*sample1_RGBdataset_ MP_predictions_PDM'.xlsx*"
  *Filename<- paste(Date," sample1_RGBdataset_ MP_predictions_PDM'.xlsx.",sep= "")*

**D.** Run the complete code. Following this, an Excel file will be generated with model predictions on the plastic or non- plastic identity of the unknown particles, in case the PDM dataset is being used. If the PIM dataset is used, predictions of polymer types will be generated for these particles (see Figure 11).

| Particle_number | Material | Predicted Material |
|---|---|---|
| 1 | UNKNOWN | PLASTIC |
| 2 | UNKNOWN | PLASTIC |
| 3 | UNKNOWN | PLASTIC |
| 4 | UNKNOWN | ORGANIC |
| 5 | UNKNOWN | ORGANIC |
| 6 | UNKNOWN | ORGANIC |
| 7 | UNKNOWN | ORGANIC |
| 8 | UNKNOWN | ORGANIC |
| 9 | UNKNOWN | PLASTIC |
| 10 | UNKNOWN | ORGANIC |
| 11 | UNKNOWN | ORGANIC |
| 12 | UNKNOWN | ORGANIC |

| Particle_number | Material | Predicted Material |
|---|---|---|
| 1 | UNKNOWN | PET |
| 2 | UNKNOWN | PET |
| 3 | UNKNOWN | PVC |
| 9 | UNKNOWN | PS |

**Figure 11:** Particle identity predictions from PDM (left) and PIM (right) of an unknown sample dataset.

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