water

MDPI

Article

River-groundwater Interaction and Recharge Effects on Microplastics Contamination of Groundwater in Confined Alluvial Aquifers

Edoardo Severini [1,*], Laura Ducci [1], Alessandra Sutti [2], Stuart Robottom [3], Sandro Sutti [4], and Fulvio Celico [1]

[1] Department of Chemistry, Life Science and Environmental Sustainability, University of Parma - Parco Area delle Scienze 11/a, Parma 43124, PR (Italy); edoardo.severini@unipr.it (E.S.), laura.ducci@unipr.it (L.D.), fulvio.celico@unipr.it (F.C.)
[2] Institute for Frontier Materials, Deakin University - 75 Pigdons Rd, Waurn Ponds, 3228, VIC (Australia); alessandra.sutti@deakin.edu.au (A.S.)
[3] Institute for Intelligent Systems Research and Innovation, Deakin University - 75 Pigdons Rd, Waurn Ponds, 3228, VIC (Australia); stuart.robottom@deakin.edu.au (S.R.)
[4] GLOBE ITALY - GLOBE Program: Mantova (Italy); sandro.sutti@gmail.com (S.S.)
* Correspondence: edoardo.severini@unipr.it; Parco Area delle Scienze, 157/A, 43124 Parma, PR (Italy).

Sampling protocol, microplastics extraction, and analyses

This protocol was formulated for the preparation of environmental samples to detect and quantify small microplastics using fluorescence microscopy. It was derived from already published methods.

Before starting

- All the laboratory material and instruments must be washed with filtered water (*e.g.* Milli-Q to avoid contamination) before and after every sample handling event. Laboratory members should wear white cotton laboratory coats.
- Microplastics extraction should be performed in a dedicated space, to avoid contamination during other laboratory activities.

Microplastics extraction

1. Sample 5 L using a glass beaker or a metallic Bailer sampler and place in a glass bottle. This large volume is recommended to maximize microplastics identification in low concentrated environmental samples. If sampling is performed in wells or piezometers, a metallic bailer sampler and a metallic wire must be used.
2. Due to possibly large amounts of suspended solids, the sample must be left to sediment for at least 24h.
3. The sample is filtered on PCTE filters (47 mm diameter and 10 µm pore diameter). Start with the liquid-rich fraction and proceed to the sedimented material as the last fraction. The bottle and vacuuming tools must be carefully rinsed with Milli-Q water, as microplastics tend to attach to the walls.
4. The filters are placed in a 250 ml glass beaker, capped with aluminium foil, and left dry in an oven at 60° C for 24 hours.
5. Perform the digestion of organic material by adding 20 ml of 30%H_2O_2 for every filter, to a maximum of 100 ml.
6. In the same beaker, filters are placed in oven for 1h at 60° C and then to 100° C for 7h.
7. Rinse the filters carefully with milli-Q water and collect the rinse water in the same beaker. Check backlight for possible residuals.

8. The beaker must be capped with aluminium foil and left drying in an oven at 60° C.
9. Add a solution of K_2CO_3 with a density of 1.54 g/cm^3 to the dry matter in the beaker.
10. Leave the high-density floation solution for 15 minutes over a magnetic stirrer to encourage the redispersion of the dried sample, which may have become stuck to the glass walls during desiccation.
11. Pour the solution into glass density-separation funnels, cover the mouth of the funnels using aluminium foil, and let rest for 12 h, promoting the separation of sediments (denser) from microplastics (less dense).
12. Remove the lower part of the solution from the funnel, filter it and recycle the K_2CO_3 solution.
13. The upper part of the solution is filtered on a new PCTE filter and let dry at normal temperature in a covered Petri dish.
14. The filtration unit must be carefully rinsed with further K_2CO_3 solution to retrieve all the microplastics attached to the walls.

Microscopic analysis

15. Arrange the microscope set-up for fluorescence imaging, using an external light source and orange photographic filters. If the light source is working with batteries as power source, continuously check the light intensity, as a lower light can affect the microplastics fluorescence with Nile red.
16. Prepare a 1 mg/L solution of Nile Red and methanol before the analysis.
17. Apply 3-4 drops of solution and cut the filter membrane in half while still wet.
18. Put the half filter on a standard microscope slide (previously washed with Milli-Q), cover with a coverslip, and fix it with tape.
19. Store in the dark at 60° C for 10 min.
20. Perform the image acquisition, using the same light intensity and exposure time of the camera for all the photos, since the subsequent ImageJ characterization is based on pixel brightness.
21. Acquire images according to the Autostitch requirements, *e.g.* 30% overlapping of the photos

Microplastics identification and characterization

22. Import images in Autostitch and set the program as follows:
    - Width 4000 px.
    - Rendering options: Multi-Band. If the reconstruction of the panoramic image can't be completed, a Linear blending method could resolve this issue.
    - System Memory: 2.00.
    - JPEG Quality: 100.
    - Ransac parameters: 5000 Max iterations.
23. Import the output image in a raster graphics editor and correct filter defects using a blue brush.
24. Import the image in ImageJ and set the image scale according to your microscope and modify the script below for every image (*i.e.* use a different scale).
25. Perform the following script for detection and quantification of microplastics:

```
filename = getTitle();

run("Split Channels");
selectWindow(filename + " (green)");
close();
```

```
selectWindow(filename + " (blue)");
close();
selectWindow(filename + " (red)");

run("Subtract Background...", "rolling=50");

run("Window/Level...");
setMinAndMax(20, 220);
run("Apply LUT");
run("Close");


run("Auto Threshold...", "method=RenyiEntropy ignore_white white");

setOption("BlackBackground", false);
run("Convert to Mask");


run("Set Scale...", "distance= Ø Ø Ø known= Ø Ø Ø unit=unit");
\\insert in the above row the values of the settled scale before running the
script

run("Analyze Particles...", " size=2430-Infinity circularity=0.00-0.952
show=Outlines display add");
```

26. Save the Results table and close all the windows except the ROI Manager (necessary for the next script).
27. Open again the image and perform the results verification using the following script:

```
filename = getTitle();

run("Split Channels");
selectWindow(filename + " (green)");
close();
selectWindow(filename + " (blue)");
close();
selectWindow(filename + " (red)");
roiManager("Show All without labels");
```

28. Save the verification image in the same folder with all the other documents.
29. Aggregate the .csv files from all the filters in a single file to perform statistical analyses.

Among the above-mentioned parameters in the ImageJ macro, the fixed parameters were selected according to other published works and adapted to the filter images in this work. In detail, the rolling value was lowered from 1500 [40] to 50, producing better results with the final images, probably due to the different resolution of the elaborated images. A linear enhancement of brightness and contrast was performed using the Window/Level tool, modifying the values as reported above. In the Autotreshold, the RenyiEntropy method was preferred to others, according to Prata *et al.* [45] who reported better results in presence of impurities, such as the suspended solids. Finally, during the particle analyses, a minimum object size of 1000 μm^2 was preferred to the lower limit of 400 μm^2 set by Erni-Cassola *et al.* [40]. Together with field blanks and the final check for false positives, the ImageJ script was also tested using artificially made filter images

(Figure S4), resulting in good recovery rates, with an overestimation of +0.46% for microplastics of large dimensions (Figure S4a) and +0.23% for microplastics of small dimensions (Figure S4b).

QA/QC of the methodology

- **Sampling method**:
  Glass bottles (surface waters and springs) and a metallic bailer with a metallic cable (groundwater). Sample location and date are reported respectively in Fig. 1 and Fig. 4. All the sampling material was carefully washed using Milli-Q water before every sampling event.
- **Sampling size***:*
  The volume of samples was set to 5 L for both sample and field blanks. The volume was selected as a compromise among a minimum representative volume, the expected microplastics concentration according to Koelmans *et al.* [46] and a reasonable volume of sample to store and manipulate, taking into account that the same volume has been used for the field blanks. The concentrations of microplastics in surface waters and groundwater are higher than those reported in Koelmans *et al.* [46], partially justifying the relatively small sampling volume.
- **Sample processing and storage:**
  Samples were stored in the same glass bottle with which they were collected.
- **Laboratory preparation**:
  - *Instruments*: All the instruments and lab surfaces were carefully cleaned using Milli-Q water before every use. Metallic or glass instruments were always preferred over plastic ones (*e.g.* beakers).
  - *Laboratory conditions*: A special room was designated for microplastics analysis. Here, the air conditioning unit was turned off and access was restricted to the authors wearing white cotton laboratory coats. All authors were trained on QA/QC measures and contamination management measures before sample and instrument handling.
- **Negative and positive controls**:
  - *Negative controls:* three field blanks were used to characterise baseline microplastics contamination, which was characterised by microplastics with statistically different geometric characteristics. Three further field blanks were analysed to assess contamination from reagents (filtered reagents vs unfiltered reagents). The contamination was numerically lower, but the observed microplastics had geometric characteristics similar to those of microplastics found in the other field blanks. A further negative control was performed on the ImageJ script, with which some artificial image samples (Figure S4) were analysed with the ImageJ macro to check for microplastics overestimation of bigger (418.3 μm diameter, scale = 0.0765 px/μm) and smaller (432 microplastics with 40 μm diameter, scale = 0.0765 px/μm) microplastics.
  - *Positive controls*: the positive controls set was prepared at two critical points in the analysis procedure. The first during the removal of microplastics from the filters before their rinsing with the high-density K_2CO_3 solution. Here, filters were backlit to

check for any remaining material and carefully washed. The second positive control was performed over the ImageJ script, with which some artificial image samples (Figure S4) were analysed to check for the loss of bigger and smaller microplastics. According to Koelmans *et al.* [46], only three studies out of 55 have performed analyses on positive controls during the whole process, while others have done it only in partial steps of the analyses as us.

- **Sample treatment**:
  For microplastics smaller than 300 mm, the quality of visual inspection requires sample digestion, at least for surface waters [46], as these microplastics have dimensions comparable with microplankton and other organic (and inorganic) debris often present. The digestion was performed using 30% H_2O_2, which is widely used for the digestion of organic matter and was reported to affect minimally the polymers if used with an exposure time lower than 48 h. As a further precaution, to ensure greater reliability of microplastics analysis by visual inspection and fluorescence analysis, the inorganic sediments were removed by density separation using a high-density solution of K_2CO_3. This allowed the elimination of most of the sediment, which otherwise could have compromised the microplastics identification (obscuration).
- **Data analysis and reporting**:
  - *Blank correction*: our approach for the characterisation of the contamination is based on the geometric properties of microplastics rather than their abundance in the field blanks. As such, the problem of numerical accounting for contamination can be bypassed and microplastics contamination affects only the limits of detection.
  - *Limit of detection*: the geometric characteristics of microplastics on the field blanks were statistically different from those in the surface water and groundwater samples. As such, these values were used as the threshold for the identification of microplastics in the environmental samples. Although this constitutes a great asset of the method, since it bypasses the issue of (possible) numerically high contaminations in field blanks, it is also a disadvantage, due to the inability to characterise microplastics with geometric properties similar to those in the field blanks. However, we believe that this approach provides a functionally acceptable way to assess baseline contamination and can be used in combination with more traditional methods.

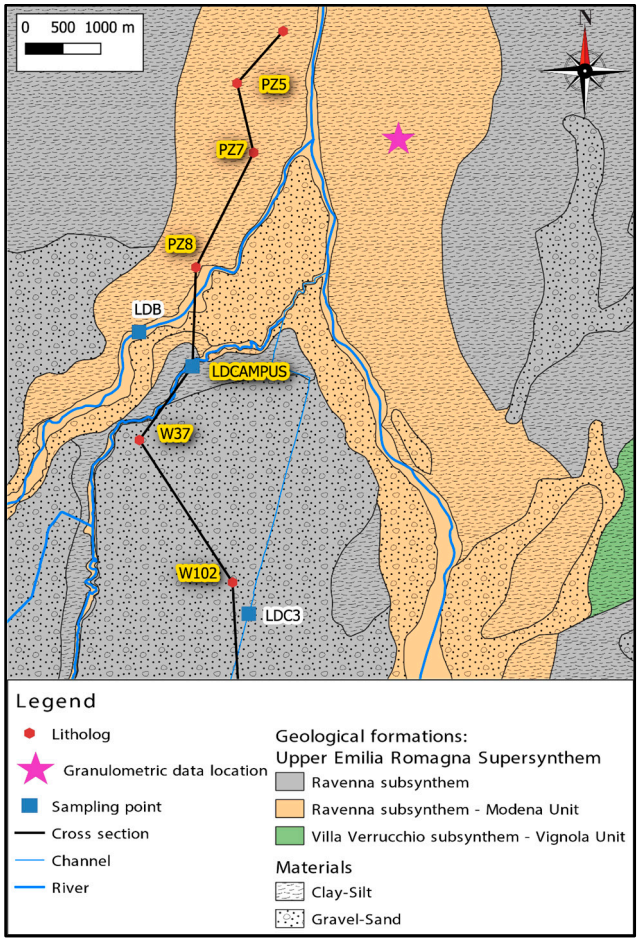

Figure S1. Location of granulometric data.

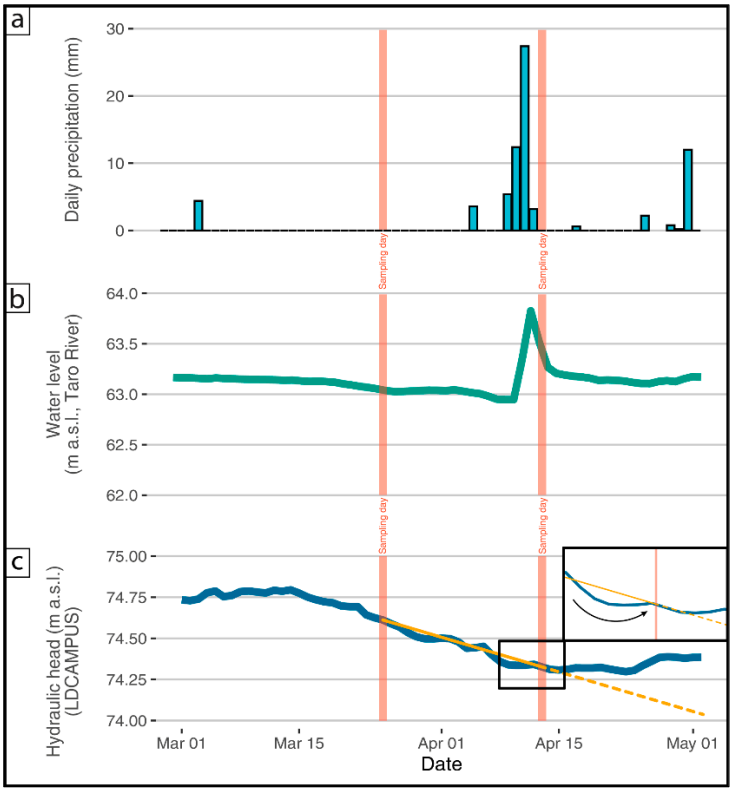

Figure S2. Hydrological data measured in surface and groundwater. (**a**) Precipitation data were measured in the Parma meteorological station. (**b**) Water level measured in the Taro River. (**c**) Hydraulic heads measured in the LDCAMPUS piezometer using a pressure transducer with a datalogger; the orange line represents the measured (continuous) and projected (dashed) hydraulic head, with a regression coefficient of 0.015 d^{-1}. A zoomed frame of the recharge event is also presented. Red bars indicate the sampling day.

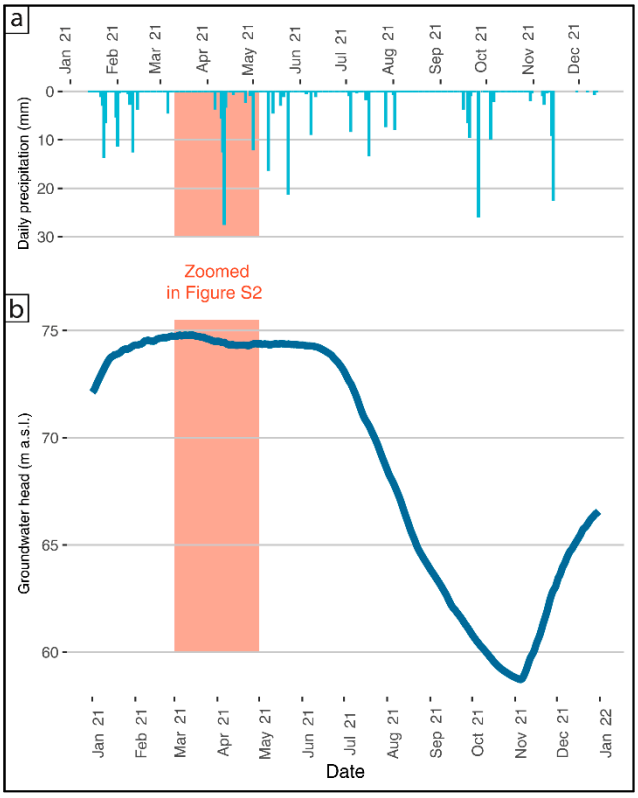

Figure S3. Hydraulic head and precipitation during 2021 in LDCAMPUS. (**a**) Cumulated daily precipitation data. (**b**) Groundwater head. The red area is presented more detailly in Figure S2.

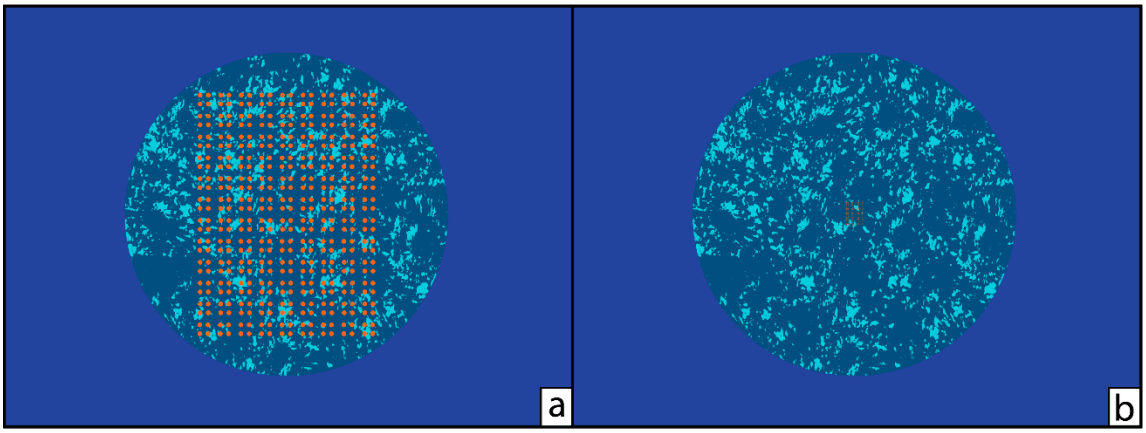

Figure S4. Artificial filter images. (**a**) Artificial filter image with round microplastics (432 microplastics with 418.3 µm diameter, scale = 0.0765 px/µm) (**b**) Artificial filter image with round microplastics (432 microplastics with 40 µm diameter, scale = 0.0765 px/µm). Both images were reconstructed using colours from real filter images to be as realistic as possible, especially with sediments, which could result in false positives.

Table S1. Granulometric composition (ASTM D422 – 63) between 1-1.60 (m bgl). Sample mass: 235.93 (g); final composition: sand (13.33%), silt and clay (86.67%).

Sieve size (mm)	Weight (g)	Percentage passing (%)
4.75	0	100
2	0.26	99.89
1	1.78	99.14
0.425	6.89	96.21
0.15	15.01	89.85
0.075	7.51	86.67

Table S2. Granulometric composition (ASTM D422 – 63) between 5-5.7 (m bgl). Sample mass: 226.81 (g); final composition: sand (12.64%), silt and clay (87.36%).

Sieve size (mm)	Weight (g)	Percentage passing (%)
4.75	0	100
2	0.06	99.97
1	0.46	99.77
0.425	1.79	98.98
0.15	8.19	95.37
0.075	18.16	87.36

Table S3. Microplastics abundance and statistical analysis results. When the Kruskal-Wallis test is significant, the p values reported are those of the Dunn's post hoc test, adjusted using the Benjamini–Hochberg method.

Counting			**Analysis**				
Date	Sample	Concentration (microplastics/L)	Parameter	Comparison	Difference tested	Test	p *value*
13-Apr-21	LDC3	17.8	Area	Ctrl-SW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	2.82E-29
13-Apr-21	LDCAMPUS	17.5	Area	Ctrl-GW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	2.58E-27
13-Apr-21	LDS1	63	Area	SW-GW	Sample origin	Mann Whitney U test	1.20E-01
13-Apr-21	LDT	76.6	Area	GW	Sampling time	Mann Whitney U test	1.31E-01
13-Apr-21	LDB	66.6	Area	SW	Sampling time	Mann Whitney U test	1.27E-01
25-Mar-21	LDC3	12.2	Circularity	Ctrl-SW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	6.19E-11
25-Mar-21	LDCAMPUS	17.4	Circularity	Ctrl-GW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	1.90E-17
25-Mar-21	LDS1	9.6	Circularity	SW-GW	Sample origin	Mann Whitney U test	5.84E-03

25-Mar-21	LDT	96.6		GW	Sampling time	Mann Whitney U test	4.27E-01
25-Mar-21	LDB	5.4		SW	Sampling time	Mann Whitney U test	1.17E-02
25-Mar-21	Average	17.2	Feret diameter	Ctrl-SW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	7.11E-31
13-Apr-21	Average	61.1		Ctrl-GW	Sample contamination	(Kruskal-Wallis test) Dunn's post hoc test	1.06E-30
				SW-GW	Sample origin	Mann Whitney U test	2.76E-02
				GW	Sampling time	Mann Whitney U test	8.35E-01
				SW	Sampling time	Mann Whitney U test	4.33E-01

Table S4. Microplastics geometrical characteristics. The median values for environmental samples are those calculated after the exclusion of the contamination by ImageJ.

Value	Sample	Median	Min	Max	IQR
Area (μm^2)	Ctrl	2430	1033	39267	2861
	GW	9110	2483	680180	13245
	SW	8304	2500	1057296	12867
	Before recharge SW	8496	2515	856808	16019
	After recharge SW	8304	2500	1057296	11990
	Before recharge GW	10411	2494	178726	18753
	After recharge GW	8692	2483	680180	12886
Circularity	Ctrl	0.952	0.276	1.000	0.143
	GW	0.823	0.189	0.949	0.208
	SW	0.786	0.152	0.951	0.254
	Before recharge SW	0.821	0.152	0.951	0.223
	After recharge SW	0.776	0.173	0.951	0.259
	Before recharge GW	0.830	0.243	0.949	0.178
	After recharge GW	0.811	0.189	0.948	0.215
Feret diameter (μm)	Ctrl	74.5	45.1	481.0	47.3
	GW	153.0	80.6	1534.0	96.6
	SW	145.0	73.4	1474.0	99.6
	Before recharge SW	145.0	78.5	1384.0	114.0

After recharge SW	146.0	73.4	1474.0	100.0
Before recharge GW	150.0	80.6	747.0	102.0
After recharge GW	153.0	83.1	1534.0	95.1