Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supplementary information: Fate and biological uptake of polystyrene nanoparticles in freshwater wetland ecosystems

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1 Tracking the gold core of the polystyrene nanoparticles in environmental samples

1.1 Digestion of water samples

Water samples were analysed at the end of every experimental week. Microwave-assisted acid digestion was performed as the standard procedure to assess the gold concentrations, and MilliQ water was used as a field blank. This procedure showed recoveries of 75% of the gold-doped polystyrene nanoparticles (Au-doped PS nanoparticles) compared to the expected nominal concentration (49.49 mg/L stock solution).

Aliquots of 1.5 mL of each sample were transferred to 6 mL TFM inserts for three position rack (Milestone Srl, Italy) where 0.5 mL of H_2O_2 (30 %, ultratrace Sigma Aldrich), 0.5 mL HCl (Scharlau, 37% ultratrace) and 0.5 mL HNO_3 (Scharlau, 69% ultratrace) were added. Samples were then transferred to the digestion vessels.

An ETHOS advanced microwave lab-station (Milestone Srl, Italy) was used with aid of the easy-CONTROL software with a 1-hour temperature program (15 minutes 0 - 140 °C; 15 minutes 140 - 200 °C; 30 minutes at 200 °C).

After cooling to less than 50°C, digested samples were quantitatively transferred to 15 mL Falcon tubes in a clean room and made up to 15 mL with MilliQ water (conductivity 18 MOhm cm^{-1}, Milipore Advantage A10) after 2-3 times MilliQ rinsing of caps and microsampling tubes. These 10-times diluted samples were stored at 6 ˚C until ICP-MS analysis.

Analytical blanks and recoveries were carried out in every digestion batch to assure quality control. These were analysed after each batch and corrective measurements were taken when deviations were present. Recoveries of Au nanoparticles and dissolved gold (0.1 ppb and 1 ppb) were generally at 75% and 95-105% levels, respectively. Repeatability of the digestion procedure was tested on selected samples obtaining 100% repeatability between different digestion batches.

1.2 Inductively coupled plasma mass spectrometry (ICP-MS) of digested water samples

Acidified samples were measured with an ICP-MS Perkin Elmer Nexion 350 D. The method for gold (194 atomic mass units (amu)) analysis consisted of 100 sweeps, 1 reading and 3 replicates, for a total of 15 seconds analysis per sample. Samples were transferred to the nebulizer using a CETAC autosampler (ACS 260 with and Ultem probe), measured three times using the aforementioned method and the results presented as the average.

The details of the materials used in the ICP- MS are as follows: Nebulizer Meinhard (TR-50-C0.5) with Ar gas flow normally around 1.02 L/min, Cyclonic spray chamber, ICP and auxiliary Argon gas flow of 14 and 1 L/min, respectively, cones and skimmer made of platinum, plasma RF voltage 1600 V and sample inlet flow at 0.3 mL /min.

Calibration was done using solutions of Au diluted in the same matrix as the one obtained from the digestion protocol (ca. 1.17% HCl and 2,32% HNO_3). The stock solution was 10 ug/mL gold in 10% HCl from Inorganic Ventures (USA) and the calibration curve ranged between 0.001, 0.01, 0.1, and 1 ug/L. Five blanks were generally measured and the limit of detection (LOD) was calculated as the average of the blanks plus eight times the standard deviation between them. LOD in the ICP-MS was typically below 1 ng/L.

1.3 Digestion of solid samples: sediment

To prepare the digestion procedure, mass balance calculations were performed. In sediment samples, the maximum concentration using the theoretical added Au (without considering the outlet and water column concentrations) would be 15 ug/g. Calculations for the digestion method for sediments were done based on this value.

The digestion method was optimized to avoid frothing and to keep the acid concentrations at the desired levels during digestion while at the same time reduce the ionic strength of the samples before injecting into the ICP-MS.

First, approximately 50 mg of the sample was weighed into a 3 mL-microinsert (Milestone) tube using an analytical balance (Mettler Toledo), addition of 1mL 1% HNO_3 (69% suprapure), and allowed to stand overnight in an airtight plastic bag inside a metal free fume hood, for at least 12 hours. Then, 2 mL HCl (37%, suprapure) were added stepwise (0.5 mL).

An ETHOS advanced microwave lab-station (Milestone Srl, Italy) was used with aid of the easy-CONTROL software with a 1-hour temperature program (15 minutes 0 - 140 °C; 15 minutes 140 - 200 °C; 30 minutes at 200 °C). After cooling down to less than 50 °C, digested samples were quantitatively transferred into labelled 50 mL falcon tubes in a clean room after 2-3 times matrix rinsing of caps and micro sampling tubes and made up to 50 mL with mixture of 5% HNO_3 and 5% HCl. The 50 mL containing the sample was allowed to stand for at least 2 hours to allow for undigested/ particle sedimentation (small amounts were observed presumably aluminosilicates). 1 mL aliquot sample from 50 mL tube was taken into a 15 mL falcon tube, internal standard (0.1 ug/L bismuth) was added at this point and the mixture was

topped with a matrix to get approximately 2% HNO_3 and 1% HCl. These 241-times diluted samples were stored at 6 °C until ICP-MS analysis.

During the entire procedure, there were runs of method blanks and recoveries of Au (dissolved and nanoparticles) either only in the acid mixture or mixed with 50 mg of samples from treated and untreated mesocosms.

1.4 Digestion of solid samples: biota

The digestion included weighing approximately 50 mg of the sample (depending on sample availability) into a 3 mL-microinsert (Milestone Srl) tube using an analytical balance (Mettler Toledo), addition of 1 mL 1% HNO_3 (69% suprapure), and allowed to stay overnight in an airtight plastic bag inside a metal free fume hood, for at least 12 hours. Then, 2mL HCl (37%, suprapure) were added stepwise (0.5 mL).

An ETHOS advanced microwave lab-station (Milestone Srl, Italy) was used with aid of the easy-CONTROL software with a 1-hour temperature program (15 minutes 0 – 140 °C; 15 minutes 140 – 200 °C; 30 minutes at 200 °C).

After cooling down, most samples were diluted after this step as follows: digested samples were quantitatively transferred into labelled 50 mL falcon tubes in a clean room after 2-3 times matrix rinsing of caps and micro-sampling tubes and made up to 50 mL with a mixture of HNO_3 and HCl that resulted in concentrations of 2 and 1 %, respectively. Samples did not show particulates by visual inspection after the digestion. 14.85 mL of this sample were transferred to separate tubes where internal standard (0.1 ug/L bismuth) was added. These diluted samples were stored at 6 °C until ICP-MS analysis.

During the entire procedure, there were runs of method blanks and recoveries of Au (dissolved and nanoparticles) either only in the acid mixture or mixed with 50 mg of samples from treated and untreated mesocosms. 92% of the blanks measured showed concentrations below limit of detection and the ones that showed values (2 out of 26 samples) above were very close to the LOD.

Recoveries were around 97 % for dissolved samples (dissolved spike on the sample or acids before digestion) and 75% for nanoparticles with respect to nominal concentrations. In general, spiked solid samples presented lower recoveries than spikes without sample.

1.5 ICP-MS analysis of digested solid samples

Samples obtained from the digestion procedure were measured with an ICP-MS Perkin Elmer Nexion 350 D. The method for gold (197 amu) and the mix of internal standards (indium 115, bismuth 209 and yttrium 89) analysis consisted of 20 sweeps, 1 reading and 3 replicates, for a total of 30 seconds analysis per sample. Samples were transferred to the nebulizer using a CETAC autosampler (ACS 260 with and Ultem probe), measured three times using the aforementioned method and the results presented as the average. After analysis of standards and several samples, it was decided to use bismuth as the internal standard.

The details of the materials used in the ICP-MS are as follows: Nebulizer Meinhard (TR-50-C0.5) with Ar gas flow normally around 1.02 L/min, Cyclonic spray chamber, ICP and auxiliary Argon gas flow of 14 and 1 L/min, respectively, cones and skimmer made of platinum, plasma RF voltage 1600 V and sample inlet flow at 0.3 mL /min.

Calibration was done using solutions of Au diluted in the same matrix as the one obtained from the digestion protocol (ca. 1 % HCl and 2 % HNO_3) including the internal standard mixture. The stock solution was 10 ug/mL gold in 10% HCl from Inorganic Ventures (USA) and the calibration curve ranged between 0.001, 0.01, 0.1, and 1 ug/L. Five blanks were generally measured and the limit of detection (LOD) was calculated as the average of the blanks plus eight times the standard deviation between them. LOD in the ICP-MS was typically around 1 ng/L.

2 Limit of detection of gold for ICP-MS analysis

The limit of detection of gold for ICP-MS was 0.007 µg/L for water samples and 0.02 µg/g for sediment samples. The limit of detection for biological samples is shown in Table S1.

Table S1. Limits of detection of gold (for ICP-MS, in µg/g) in the different biological samples for each of the 12 wetland mesocosms. In mesocosm 3, there were fewer *Carex* sp., therefore not as many samples as for the other mesocosms were possible to analyse, which is indicated as NA. Although macrophytes were identified and studied at the genus level, samples for analysis were separated considering morphological differences between the same genus, noted as numbers after the taxa names.

Sample	Mesocosm											
	M01	**M02**	**M03**	**M04**	**M05**	**M06**	**M07**	**M08**	**M09**	**M10**	**M11**	**M12**
Carex sp. above ground 1	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.0181	0.002	0.002
Carex sp. root 1	0.001	0.0161	0.001	0.001	0.0121	0.001	0.0121	0.0181	0.001	0.0181	0.001	0.0161
Carex sp. above ground 2	0.001	0.0161	NA	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Carex sp. root 2	0.002	0.0151	NA	0.001	0.001	0.001	0.0121	0.0181	0.001	0.0181	0.001	0.0141
Carex sp. above ground 3	0.002	0.0171	NA	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.0141
Carex sp. root 3	0.001	0.0171	NA	0.001	0.0121	0.001	0.001	0.0181	0.001	0.0181	0.001	0.0151
Juncus sp. above ground 1	0.001	0.0151	0.001	0.001	0.0121	0.001	0.0181	0.001	0.001	0.0131	0.001	0.0151
Juncus sp. root 1	0.024	0.0161	0.0251	0.0241	0.0121	0.001	0.0181	0.0181	0.001	0.0151	0.001	0.0141
Daphnia magna	0.146	0.077	0.138	0.091	0.046	0.004	0.141	0.072	0.004	0.224	0.003	0.059
Asellus aquaticus	0.047	0.012	0.023	0.163	0.012	0.001	0.018	0.018	0.001	0.013	0.001	0.014

3 Calculations

3.1 Particle density and number of particles added in each addition

The mass per gold-doped polystyrene nanoparticle (kg) was calculated following Eq. 1.

Eq. 1. *Mass (M) = density (D) x volume (V)*

The density and diameter for each particle component (gold, silica and polystyrene) are shown in Table S2. Volume was calculated estimating the volume of a sphere, following: $V = 4/3\pi r^3$, being r, radius. After summing the mass of each particle component, particle density was estimated, following eq. 1, this time as: D = M / V.

Table S2. The density for the gold-doped polystyrene nanoparticles was estimated using the density and volume for the gold core, and each shell: the Silica (SiO2) and Polystyrene (PS) one. The mass for each gold-doped polystyrene nanoparticle is estimated as the sum of the masses of each particle component.

Material	Diameter (nm)	Radio (m)	Density (kg/m^3)	Volume spheres (m^3)	Volume, core-shell-shell (m^3)	Mass (kg/part)
Au	13.44	6.7E-09	19320	1.27115E-24	1.27115E-24	2.456E-20
SiO_2	38.62	1.9E-08	2650	3.01604E-23	2.88892E-23	7.656E-20
PS	87.73	4.4E-08	1060	3.53544E-22	3.23384E-22	3.428E-19
PS-SiO_2-Au particles	87.73		1256			4.439E-19

To estimate the mass of gold added to each mesocosm in each nanoplastic addition, we followed Eq. 2. As explained in Materials and methods section, 2.283 mL of the NPs solution at a concentration of 49.5 mg/L were added in each addition.

Eq. 2. *Mass (M) = volume (V) * concentration (C)*

The number of particles added in each nanoplastic addition was calculated as shown in Eq. 3.

Eq. 3. *Number of Au-doped PS nanoparticles = added Au mass / Au mass per nanoparticle*

Therefore, after each nanoplastic addition, the theoretical number of particles was 6.60×10^{11} Au-doped PS nanoparticles per liter of lake water.

Having the number of nanoparticles per nanoplastic addition allowed us to estimate the theoretical concentration of polystyrene in lake water after each nanoplastic addition (Eq. 4) and also in all the wetland mesocosm (Eq. 5).

Eq. 4. *PS concentration in lake water after each NP addition = Number of Au-doped PS nanoparticles * PS mass per nanoparticle / average lake volume*

Eq. 5. *PS concentration in the wetland after each NP addition = Number of Au-doped PS nanoparticles * PS mass per nanoparticle / average wetland volume*

3.2 Sedimentation rate

The sedimentation rate for the gold-doped polystyrene nanoparticles was calculated following Eq. 6, and the parameters used in the equation are described in Table S3.

Eq. 6. $T = (9l\eta)/(2r2(\rho_p - \rho_s)g)$

Table S3. Data used for estimating the sedimentation rate for the Au-doped PS nanoparticles.

Abbreviation		Value
T (s)	Time for all particles to sediment	
l (m)	Depth of solution	The depth of the "lake" section: 0.087 m
η	Viscosity of solution	Water at 20° C = 1.002×10^{-3} Pa.s
r (m)	Radius of particle	Average particle diameter: 87.73 nm, radius: 43.87 nm
ρ_p (kg/m^3)	Density of particle	Estimated to be 1256 kg/m^3
ρ_s (kg/m^3)	Density of solution	1000 kg/m^3 for water
g (m/s^2)	The acceleration due to gravity	9.81 m/s^2

3.3 Mass balance calculations with water samples

As described in Materials and Methods section, water samples were taken throughout the experiment. Mass balance calculations were performed using the concentrations of gold measured on water samples. In this description, average values for the 6 nanoplastic´s exposed wetlands are the ones reported. Mass balance calculations were also performed for each nanoplastic´ exposed wetland separately.

The theoretical mass of gold introduced into the system in each nanoplastic addition was 0.113 mg, which in 6.97 L of lake water (on average), represent 16.21 µg of gold per liter of lake water after each nanoplastic addition.

For the mass balance calculations, the Eq. 7 was followed.

Eq. 7. *Proportion of nanoplastics that left the system = (accumulated mass of Au measured in outlet water) / (accumulated mass of Au added to the system)*

The accumulated mass of Au added to the system was considered as:

a. The theoretical accumulated mass of gold added to the wetlands, which was 1.13 mg of gold.

b. The accumulated mass of gold added to the wetlands considering the concentration of gold measured at the lake immediately after each nanoplastic addition. Following this approach, the accumulated mass of gold measured at the lake of the wetlands was, on average, 1.28 mg of gold.

Following these two approaches, the percentage of nanoplastics retained by the wetlands was 96.30% and 96.73 %, calculated with one approach or the other, respectively. We decided to report the second value since it is based on the actual measured Au concentrations at the lake after nanoplastic additions.

3.4 Calculations with solid samples

Solid samples were taken at the end of the experiment. The percentage of the total mass of NPs added, accumulated by the population of *D. magna* and *A. aquaticus* (i.e.: the NPs recovery in each invertebrate specie studied), was estimated following Eq. 8. This calculation was performed considering the data from the 6 nanoplastic´ exposed wetlands.

Eq. 8. *Percentage of the total mass of NPs added accumulated by X invertebrate specie = (Measured Au concentration in X invertebrate matrix * final biomass of specie X at the end of the experiment (g of dry weight) / accumulated mass of Au added to each wetland) * 100*

3.5 Note for all calculations

When measured Au concentrations were used for calculations, the Au concentration was estimated considering the recovery of the method, using Eq. 9. This apply both for water and solid samples (i.e., sediment and biological samples).

Eq. 9. *Estimated Au concentration = Measured Au concentration / recovery of the method.*

4 Supplementary results

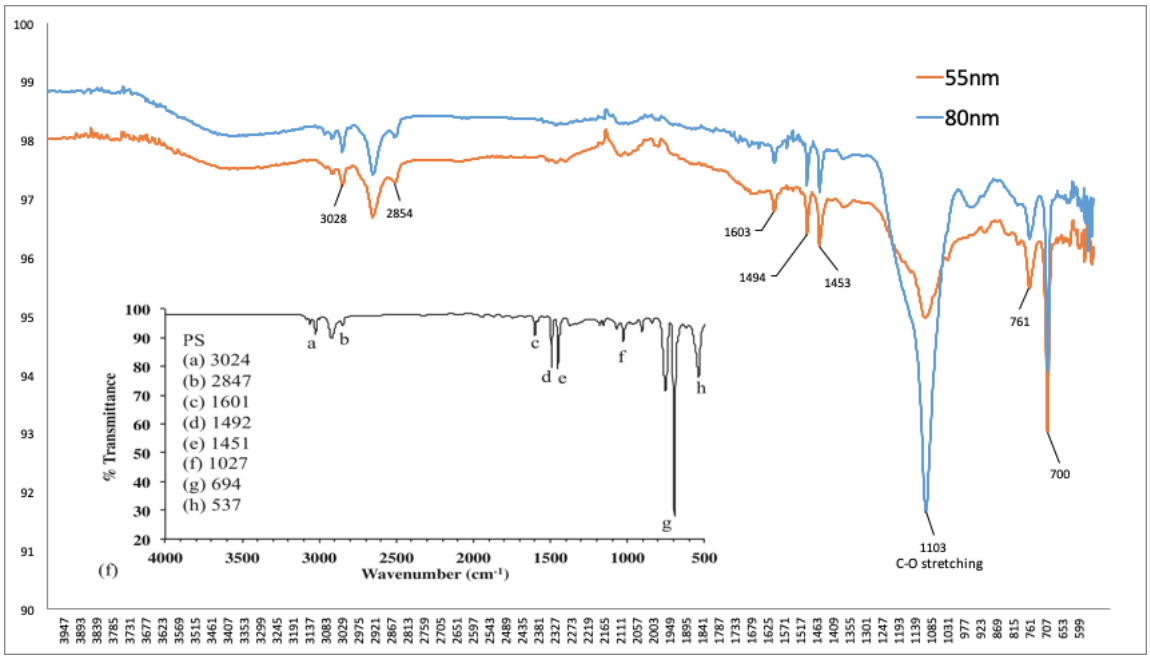

Figure S1. Results from the Fourier-transform infrared spectroscopy (FTIR) performed on the 88 nm gold-doped polystyrene nanoparticles used in this study (blue line). Polystyrene (PS) FTIR spectra, used as a reference, is shown as a small plot.

Table S4. Number (No.) of water samples analysed by inductively coupled plasma mass spectrometry (ICP-MS). Specifically, samples from control (C) and nanoplastic (NP) exposed wetlands are shown, and also the number of samples per treatment which values were above the limit of detection (LOD) for the ICP-MS per matrix. The counts are shown for each water label (before and after NP addition and outlet water) and for the total.

	No. of samples analysed			**No. of samples reported above LOD**	
	Total	From control wetlands	From NP exposed wetlands	From control wetlands	From NP exposed wetlands
Lake water before NP addition	120	60	60	4	60
Lake water after NP addition	120	60	60	3	60
Outlet water	372	186	186	23	177
Total N of water samples	612	306	306	30	297

Table S5. Number (No.) of solid samples (i.e., sediment and biological samples) analysed per matrix by inductively coupled plasma mass spectrometry (ICP-MS). Specifically, samples from control and nanoplastic (NP) exposed wetlands are shown, and also the number of samples per treatment which values were above the limit of detection (LOD) for the ICP-MS per matrix.

	No. of samples analysed			**No. of samples reported above LOD**	
	Total	From control wetlands	From NP exposed wetlands	From control wetlands	From NP exposed wetlands
D. magna	12	6	6	1	6
A. aquaticus	12	6	6	0	5
Juncus sp.	24	12	12	1	12
Carex sp.	68	36	32	10	32
Sediment	120	60	60	0	52
Total N of solid samples	236	120	116	12	107

Table S6. Results from the Linear Models (i.e., anova tests) performed for the number of individuals of *Daphnia magna*, *Daphnia magna* biomass (g of dry weight) and *Asellus aquaticus* biomass (g of dry weight) between treatments (control and NP exposed wetlands) at the end of the experiment. F value (F), degrees of freedom (d.f.) and p value for each test are shown.

Specie	**Response variable**	**Explanatory variable**	**F**	**d.f.**	**p value**
D. magna	Number of individuals	Treatment	1.1396	1	0.3135
D. magna	Biomass (g dry weight)	Treatment	1.735	1	0.2172
A. aquaticus	Biomass (g dry weight)	Treatment	0.0478	1	0.8313

Table S7. Results from the Linear Mixed Models (LMM) performed for pH, oxygen concentration (mg/L) and turbidity measurements that were taken throughout the experimental time. Turbidity measurements include data only from lake water. Chi-squared tests ($\chi 2$), degrees of freedom (d.f.) and p value for each explanatory variable are shown. All LMM models had the identity of the mesocosm modelled as a random effect.

Model	Model type	Dependent variable	Explanatory variable (fixed effects)	$\chi 2$	d.f.	p value
1	*LMM*	pH	Time	65.68	1	<0.001
			Treatment	1.26	1	0.2619
			Water sampling site[*1]	303.73	1	<0.001
2	*LMM*	Oxygen concentration	Time	25.60	1	<0.001
			Treatment	2.46	1	0.1167
			Water sampling site[*1]	31.64	1	<0.001
3	*LMM*	Turbidity	Time	12.26	1	<0.001
			Treatment	1.29	1	0.2568
			Time * Treatment	5.14	1	<0.05

[*1] *Water sampling site: at the lake or the outlet.*

Table S8. Nanoplastic concentrations measured (expressed as µg of Au/g and µg of PS/g) in the different solid samples (i.e., sediment and biological samples) that were collected at the end of the experimental time (70 days). Results show the number of samples (n), the mean and standard deviation (Std. Dev.) for gold and polystyrene concentrations, per matrix analysed. These results refer only to samples collected in NP exposed wetlands.

Matrix	Where	n	Mean Au Conc. (µg/g)	Std. Dev. Au Conc. (µg/g)	Mean PS Conc. (µg/g)	Std. Dev. PS Conc. (µg/g)
D. magna		6	1.0893	0.7330	15.2048	10.2311
A. aquaticus		6	0.2244	0.1043	3.1328	1.4563
Carex sp.	Leaves	16	0.0129	0.0137	0.1803	0.1910
Carex sp.	Roots	16	0.0849	0.0869	1.1853	1.2133
Juncus sp.	Leaves	6	0.0258	0.0205	0.3598	0.2858
Juncus sp.	Roots	6	0.4073	0.3181	5.6855	4.4403
Sediment	Lake´s sediment	6	6.7176	2.5694	93.7630	35.8634
Sediment	On the area with macrophytes	54	0.3370	0.4768	4.7036	6.6552

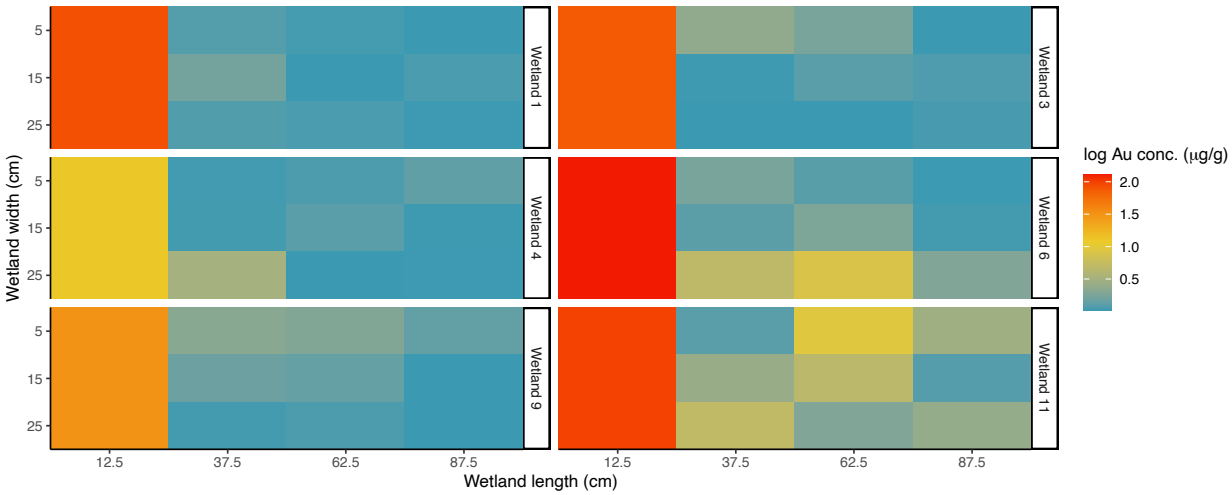

Figure S2. Distribution of nanoplastics (NPs) in surface sediment (µg Au/g dry weight) along the six nanoplastic exposed wetlands, sampled at the end of the experiment (day 70). Wetland´s identity is noted at the right of each heatmap. Au concentration (our proxy for NP concentration) is shown as $\log_{10}$.

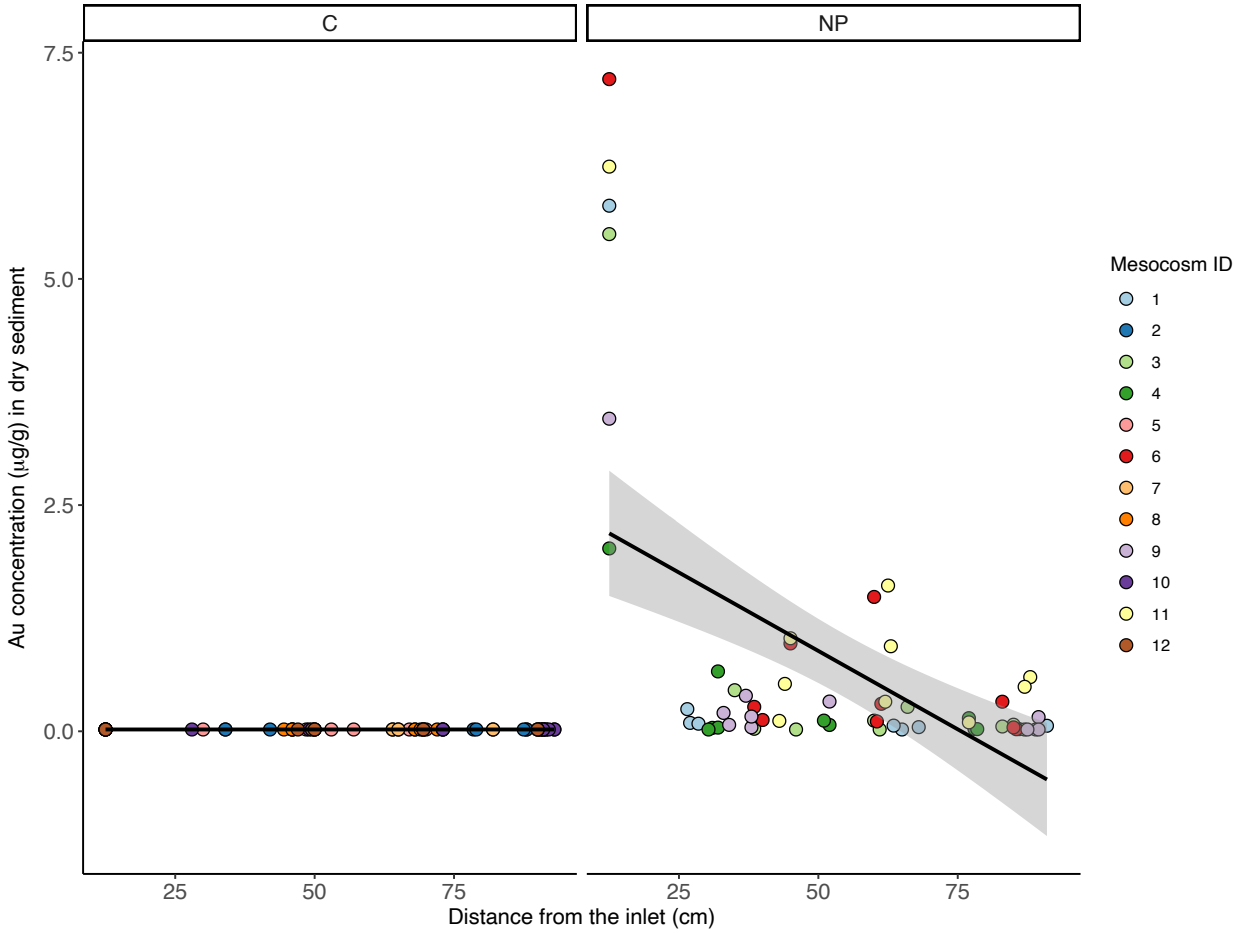

Figure S3. Au concentration (µg/g dry weight) in sediments in relation to the distance from the inlet, where nanoplastics (NPs) were added. Samples from control (C) and nanoplastic exposed (NP) wetlands are shown and the different colours of the points indicate different mesocosms identity (ID). The black line represents a linear model adjusted to data, and the grey shading, the confidence interval (95%).

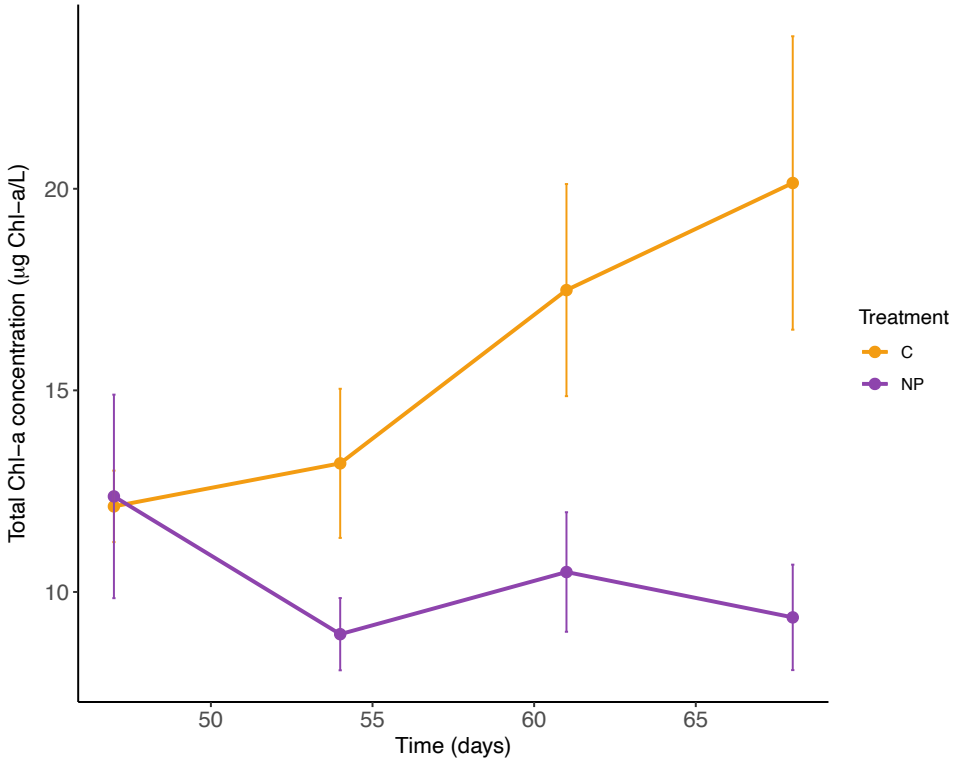

Figure S4. Total algae in lake water (measured as total concentration of Chlorophyll-a, µg Chl-a /L) measured weekly during the last 4 weeks of the experiment. Significant differences between control non-exposed (C, in orange) and nanoplastic exposed wetlands (NP, in violet) were found (Linear Mixed Model, significant interaction between Treatment and Time, $\chi 2=11.072$, d.f.: 45, $p<0.001$, slopes: 0.41 and -0.11 for control and NP exposed wetlands respectively).