2. Chemical marker identification to study exposure of biota to tyre wear particles

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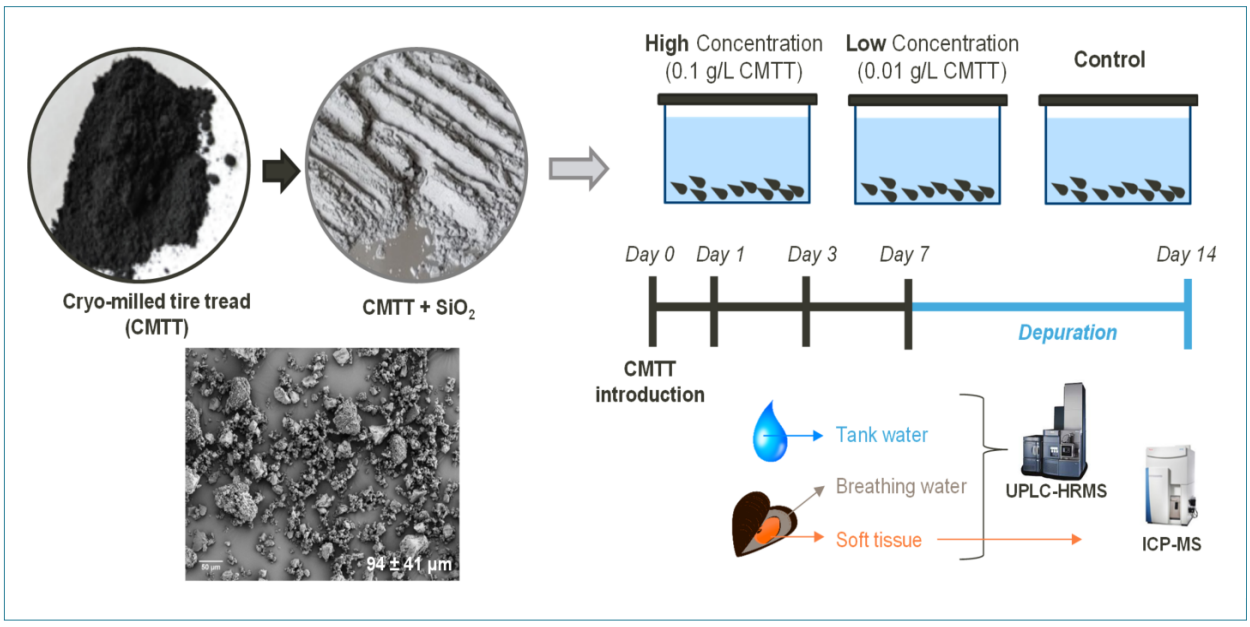
Deliverable 2.5 Analysis techniques for quantifying nano-and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023.



2.1 Scope of Protocol

The knowledge regarding suitable chemical indicators to assess the exposure of aquatic organisms to tire wear particles (TWPs) is still very scarce; this is particularly true when it comes to marine organisms. The combination of possible different scenarios, such as the uptake of TWPs and/or leached chemicals, makes the determination of TWPs in biota very challenging. The detection of organic additives used during the tire production (such as vulcanization accelerators and antioxidants) and the zinc (Zn) content (known to be almost 1% in the tire) might be useful for this purpose. The aim of this protocol was to investigate suitable chemical tracers to assess to which extent the exposure of mussels to TWP is reflected by internal concentrations of tire-related chemicals. To do so mussels (*Mytilus edulis*) have been exposed to a mixture of 20 types of cryo-milled tire tread (CMTT) particles (< 100 µm) .Mussels were exposed to CMTT particles for 7 days, followed by additional 7 days of depuration. At day 1, 3, 7 and 14, both water and tissues samples were collected and analysed by UPLC-HRMS and ICP-MS for the detection of organic tire-related chemicals and Zn content, respectively.

2.2 Materials and Equipment

Table 1: Equipment Used

Equipment	Name	Manufacturer
• Analytical balance	• Excellence Plus • BL 1500S	• Mettler Toledo • Sartorius
• Ultrasonic bath	• BANDELN SONOREX	• BANDELIN Electronics
• Microwave digestion	• MW PRO	• Anton Paar Multiwave PRO
• Mini spin plus	• Eppendorf	• Eppendorf AG
• Shaker	• Grant - bio shaker	
• Centrifuge	• Eppendorf Centrifuge 5804	• Eppendorf AG

• Mini centrifuge	• Galaxy 14D	• VWR INTERNATIONAL
• Balance	• METTLER PM4800 DeltaRannae	• Mewes Wagalneunik
• Freeze Dryer	• Christ Alpha 1-4 LSC plus	• VACUBRAND
• Turbovap Evaporator	• Turbovap	• Biotage GB
• Mass Spectrometer (Q-OF)	• Xevo G2-XS	• Waters Corp., Milford, US
• UPLC	• ACQUITY I-class UPLC system (FTN-sample manager, column manager and binary solvent manager)	• Waters Corp., Milford, US

Table 2: Solvents and Substances Used

Substance	Manufacturer
• Methanol	• Biosolve
• Acetonitrile	• Biosolve
• Milli Q-Water	• Barnsted GenPure water purification system
• Formic acid	• Biosolve
• Pure Sand (SiO_2)	• Th.Geyer GmbH&Co.KG
• Nitric acid	• Chemsolute
• HCL 37%	• Merck KGaA
• H_2O_2 30%	• Merck KGaA

2.3 Protocol

2.3.1 Tire additives by RPLC-HRMS

To evaluate the internal and external concentration of tire related chemicals in mussel samples, the tank water, breathing water, and soft tissue were analysed. The tank water was

sampled together with the mussel samples. Once the breathing water was released by the mussels, the soft tissue was collected afterwards.

2.3.1.1 Sample Preparation

Soft Tissue

1. **Wet weighing:** Weigh and measure the whole mussel shell. Thereafter, remove the soft tissue and weigh it separately.
2. **Freeze-drying:** Insert the soft tissue into an Eppendorf tube (5 ml) and freeze dry it for 48 hours, ensuring that all the sample is dried afterwards.
3. **Ball-milling:** Insert inside the ball milling a suitable number of milling stainless-steel balls (between 10 and 15) according to their size (we adopted a combination between 2- and 3-mm diameter stainless-steel balls). Transfer the content of the Eppendorf tubes into the ball-mill and run it for 2 min at a frequency of 30 s^{-1}. Pour the contents of the stainless ball-milling boxes in a weighing paper and remove the stainless balls (the use of a magnet can be helpful during this procedure).
4. **Dry weighing:** Before pouring the content of the weighing paper into a falcon tube (15 ml), weigh the empty tubes and then weigh them again with the sample inside. The subtraction of both weights allows to obtain the dry weight of the sample.
5. **Sample Extraction:** Insert a defined volume of solvent (methanol) ensuring that all the sample is completely submerged. Place the samples in the ultrasonic bath for a total of 45 min, and vortex the samples at least 2 times in between ensuring that no larger agglomerates are formed.
6. **Centrifugation:** Centrifuge the samples for 10 min at 4193 x *g*. Afterwards, pour the supernatant into a separate vial, and store the falcon containing the precipitate for further analysis. Pipet out a certain volume of extract (e.g., 500 µl) and place it into Eppendorf tubes (1.5 ml), and centrifuge them again at 7558 x *g* for 10 min. These two centrifugation steps will ensure the removal of most of the suspended particulate matter from the extracts.
7. **Dilution:** Dilute the obtained extract 1:1 with ultrapure water and place the sample in the LC system for analysis.

Tank and Breathing Water

Filtration: the water samples were defrosted and filtrated by using a regenerate cellulose (RC) syringe filters (0.45 µm) into LC glass vials. Afterwards, place the sample in the LC system for analysis.

2.3.1.2 Analysis by UPLC-HRMS

The analysis was performed an ACQUITY UPLC system coupled to a Xevo G2-XS mass spectrometer equipped with an electrospray ionization source (Waters Corp., Milford, USA). Chromatographic separation was performed using the ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm) with a flow rate of 0.45 mL / min and a column temperature of 45 °C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The gradient was as follows: 0 min 2% B, 12.25 min 99% B, 15.00 min 99% B; 15.10 min. 2% B, 17.00 min 2% B. The volume of injection was 10 µL. Full-scan spectra were collected from m/z 50-1200 in both positive and negative (centroid) modes.

Source conditions were set as follows: temperature 140°C, capillary voltage −0.8 kV, dissolving temperature 600°C, sampling cone voltage 20 V, and source offset 80 V. Nitrogen was used as a cone gas and argon as a collision gas. The flow of the cone gas was 50 L / h. The desolation gas flow is 1000 L/h. In order to ensure accurate precision mass during the MS analysis, leucine encephalin was infused as lock spray via the reference probe and a two-point calibration was applied.

Two MS data sets were collected in parallel using low collision energy (6 eV, effectively the accurate mass of the parent ions) and high collision energy (15–40 eV, fragment ions) in order to obtain the greatest extent of structural information on each suspect.

2.3.2 Zinc Content of Mussel Samples by ICP-MS

For this analysis, the stored precipitate samples obtained from the previous RPLC-HRMS sample preparation (at step 6) will be used.

1. **Solvent evaporation:** Perform a nitrogen evaporation on the stored precipitates if the sample looks completely dry.
2. **Freeze-drying:** Freeze-dry the obtained samples for an additional 24 hours to ensure that all the water content is removed.
3. **Dry weighing:** Weigh the dry samples and subtract the weights of the empty falcon tubes previously taken to calculate the amount of sample obtained.
4. **Acid digestion:** perform an acid digestion of the samples by adding a suitable volume of acids (NHO_3 and H_2O_2) according to the weight and placing them inside a microwave oven.

   The corresponding parameters are reported in the table below:

Parameter	Condition
• P rate limit	• 0.5
• performance limit (Watt)	• 1500
• pressure limit (Bar)	• 40.0
• IR limit (°C)	• 210
• Inside temperature (°C)	• 240
• Number of vessels	• 16
• Weight of the sample (g)	• 0.300

5. **ICP-MS:** Zn analysis of the digestate was conducted by inductively coupled plasma mass spectrometry (ICP-MS) with the following instrument and operational conditions: Thermo Scientific iCAP Q S, nebulizer: PFA-ST MicroFlow nebulizer, spray chamber: MicroMist quartz cyclonic, RF power: 1548 W, nebulizer gas flow: 0.95 L/min, sample flow rate: 0.395 ml/min, collision gas flow: 5ml/min, cooling gas flow: 14 L/min, auxiliary gas flow: 0.8 L/min) and inductively coupled plasma optical emission spectroscopy (ICP-OES; Spectro ARCOS, nebulizer: cross flow nebulizer, spray chamber: Scott type glass chamber, RF power: 1400 W, nebulizer gas flow: 1.2 L/min, sample flow rate: 2 mL/min, cooling gas flow 14 L/min, auxiliary gas flow: 1.2 L/min).

2.4 Quality Control Measures

2.4.1 Recovery Experiment Performed for UPLC-HRMS

In order to assess the efficiency of the overall sample preparation procedure, a recovery test experiment was performed by using commercial mussels. The recovery test should include a proper number of samples in order to assess the recovery of the extraction procedure (RE), the matric effect (ME) and the process efficiency (PE) (also known as apparent recovery).

1. **Standard mixture preparation:** Two spike solutions were prepared by mixing the standard compounds in order to obtain the standard mixtures with a concentration of 50 (Spike 1) and 15 ng/ml (Spike 2).
2. **Weighing, freeze-drying and milling:** The commercial mussels' samples were prepared by following the steps from 1 to 4 in Section 2.3.1.1 'Sample Preparation'.

In order to create a unique powder mixture all milled commercial mussels were mixed.

3. **Recovery test procedure:** In order to test the overall recovery of the sample preparation (including the matrix effect), two different sample weights where selected: 500 and 50 mg. The spike solutions addition and the overall procedure of the experiment is showed in the scheme below:

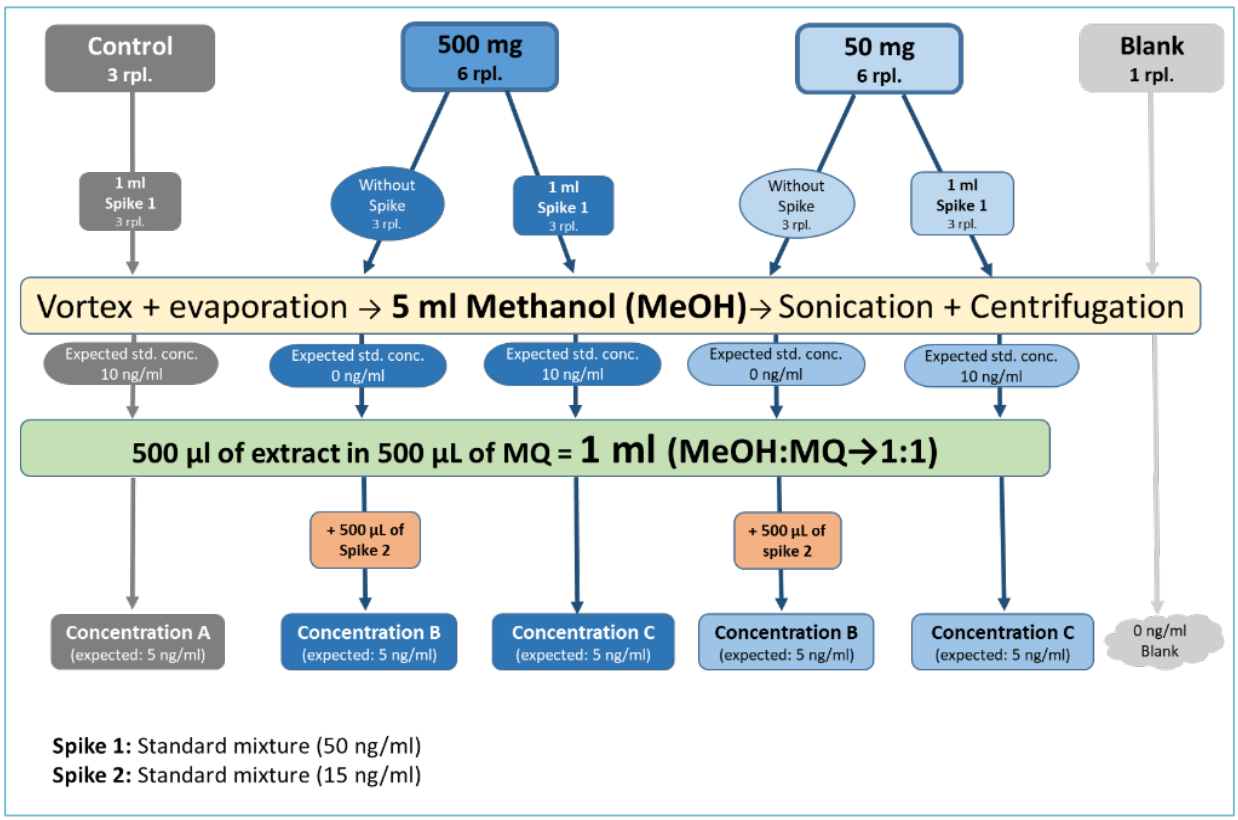


Figure 1: Recovery test experiment performed using commercial mussels, with addition of spike solution

4. **Spiking of standard mixture before extraction:** spike a standard mixture of selected tire related compounds (known concentration) inside some of the samples.
5. **Solvent evaporation:** perform a nitrogen evaporation on the samples, in order to remove the solvent of the spiking solution.
6. **Sample extraction, centrifugation and RPLC-HRMS analysis:** follow the steps from 5 to 7 already explained in Section 2.3.1.1 'Sample Preparation'.
7. **Recovery percentages calculations:** The related recovery and matrix effects percentages were calculated as reported by Matuszewski *et al*. (2003):

ME (%) = (Concentration B / Concentration A) x 100

RE (%) = (Concentration C / Concentration B) x 100

PE (%) = (ME x RE) / 100

Quality Control

To ensure the accuracy of the sample preparation procedure, two replicates of commercial mussel samples were analysed in parallel after spiking both the standard mixture of selected compounds (Spike 1) and two labelled internal standards, 6-PPDQ-d5 and DPG-d10, with initial concentrations of 2 mg/ml and 1 mg/ml, respectively.

References

Matuszewski, B. K., Constanzer, M. L. and Chavez-Eng C. M. (2003). Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. Analytical Chemistry 75, pp.3019-3030. DOI: https://pubs.acs.org/doi/10.1021/ac020361s

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