NEPTUNE

New sustainable concepts and processes for optimization and upgrading municipal wastewater and sludge treatment

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1 Introduction

WWTP are the major point source for discharge of pollutants in surface water, and consequently impact on the new focus legislated by the WFD. The emerging interest on organic (eco-)toxic compounds requires characterizing treated effluent and treatment technologies concerning ecotoxicologic aspects and micropollutants.

The potential of emerging processes in wastewater treatment to reduce the discharge of pathogens, micropollutants and their metabolites with a consequent reduction of toxicity in receiving waters can be extensively evaluated by means of combined chemical and biological analyses. By analyzing emerging chemical micropollutants, ecotoxicity assessment of whole effluents and identification of pathogens and microbial Indicators helps to identify the most promising sewage treatment technologies with greatest benefits for protection of the river and streams.

Emerging chemical micropollutants

In addition to persistent organic pollutants and WFD priority substances, the emission of so-called “emerging” chemicals is of major environmental and drinking water relevance. Due to the large number of emerging compounds, only a small subset are analyzed in the project. Therefore compounds were selected which fulfills the following selection criteria to evaluate the different treatment technologies.

- Strongly sorbing contaminants with high-to-medium persistence in sludge
- Highly dissolved compounds with high-to-medium persistence in water
- Dissolved and sorbing compounds with high-to-medium persistence in water and sludge
- Tracer compounds with already known behavior to facilitate the extrapolation of the results to other emerging groups.

Ecotoxicity assessment of whole effluents

The ecotoxicity of effluents of conventional nutrient removal plants and the different post treatment technologies were assessed using a range of screening assays (tier 1) and a set of chronic or life-cycle tests with representative taxa. Tier 1 assays were applied for Whole Effluent Testing (WET) and Toxicity Identification Evaluation (TIE) with a high number of samples. They allow the identification of (anti-)estrogenicity, (anti-)androgenicity, mutagenicity and cytotoxicity. Tier 2 was a set of biological tests, representing key species for the structure and function of aquatic ecosystems in Europe which will be applied for whole effluents. These tests consider as toxicological endpoints population-relevant parameters such as growth, development and reproduction. The taxa (algae or higher plants, annelids,

molluscs, arthropods and fish) represent more than 80 % of the biodiversity in European aquatic ecosystems so that this approach is in accordance with the WFD.

Identification of pathogens and microbial indicators

The survival rates of bacteria and pathogens in WWTP can depend on specific organism properties such as size or sensitivity to chlorination, ozonation, photochemical processes or UV inactivation. Microbial measurements at strategic intervals or after upgrading processes include the following bacterial indicators: total, fecal coliforms, *Escherichia coli* and *enterococci*, usually strong indicators of fecal pollution or possible pathogenic bacterial contamination. The effluents were tested for the presence and quantity of male-specific (F+) and somatic *coliphages*, appropriate indicators for the presence of enteric but non-pathogenic viruses as they only infect bacterial cells. Based on existing standardized methods (EN, ISO), microbial indicators were determined by fast and reliable assays (culture-depended most-probable number (MPN) or membrane filtration (MF).

Based on the outcome of whole effluent testing and toxicity identification evaluation analyses, a set of biological tests were identified to be proposed for future testing of sewage treatment effluents in Europe (D3.1). As a mid-range objective, a brief description of a mobile WET unit was developed and used for this purpose. Furthermore, instrumentation and enrichment techniques for crucial and toxic chemical pollutants as well as microorganisms and pathogens are listed, in order to allow for an on-site sample preparation followed by subsequent analysis in the lab. Although the complete development of a mobile unit is clearly beyond the scope of NEPTUNE.

A proven and tested concept for a mobile analytical unit combining on-site instrumentation for ecotoxicological evaluation, whole effluents testing, and sample preparation for chemical analysis of specific organic and inorganic pollutants are described in this report. The report is aimed to support for further studies or to serve as a draft outline for an analytical company supplying equipment for on-site sample preparation and/or on-side analysis.

Methods for WET and TIE analysis are well described in deliverable D3.1 “Ecotoxicity and microbiological testing methods (OECD and DIN/ISO guidelines) optimized for whole effluent testing”.

2 Toxicological analyses of wastewater samples with in vitro tests

A test battery based on *in vitro* bioassays is the appropriate tool for short term evaluation of toxicity in wastewater samples. Assays for the detection of estrogenicity and androgenicity (yeast estrogen/androgen screen, YES, YAS), mutagenicity (Ames and umu test) and cytotoxicity (bioluminescence inhibition test) are reasonable and feasible. The overall test duration is three days. The sample preparation includes filtration followed by solid phase extraction procedure. These tests have to be accomplished under sterile conditions (clean bench, Bunsen burner).

Therefore, the non-filtered whole-effluent samples have to be stored in sterile glass bottles. It is advisable to use gloves while sampling and not to touch the bottlenecks.
Before use, samples should be stored at 4°C in a refrigerator or cooling box with thermal packs. Samples have to be processed within 24 h after sampling.

List of technical devices:

- Microbiological cabinet (clean bench), type II
- Two incubators (temperature range 20-37°C) incl. microplate shaker
- Luminometer/Photometer for multiwell plates
- Micro centrifuge
- Magnetic stirrer
- Autoclave
- Refrigerator (4°C)
- Freezer (-21°C)
- Vacuum-manifold for SPE
- Vacuum-pump, adjustable
- Nitrogen gas cylinder
- Basic laboratory equipment (pipettes and tips, microplates, test media, (micro-) tubes, diverse glassware etc.)

3 Toxicological analysis of wastewater with standard in vivo tests

To detect ecologically relevant toxic effects of waste waters, chronic tests with organisms from different trophic levels have to be performed. The whole effluent *in vivo* tests allow the

detection of complex relationships between waste waters and the ecology in the receiving environment.

The OECD defines several standard tests for the determination of hazards to the environment, among them tests with primary and secondary consumers. Out of four selected standard tests, the fish early life stage test (OECD 210, 1992) with rainbow trout (*Oncorhynchus mykiss*) and the reproduction test with the worm *Lumbriculus variegatus* (OECD 225, 2007) proved to be sensitive test systems for detection of potential hazards in waste waters.

The duration of both tests is considerably longer than of the aforementioned *in vitro* tests, but could be easily implemented in a MAU, because of the use of common technical devices (see table below). The reproduction test with *L. variegatus* lasts 28 days, the fish early life stage test needs approximately 70 days from exposure start to end. The mentioned OECD guidelines require certain temperature ranges for each test system, thus a cooling / heating device or climate chamber has to be employed. Figure 1 and Table 1 gives an overview of a possible test set up.

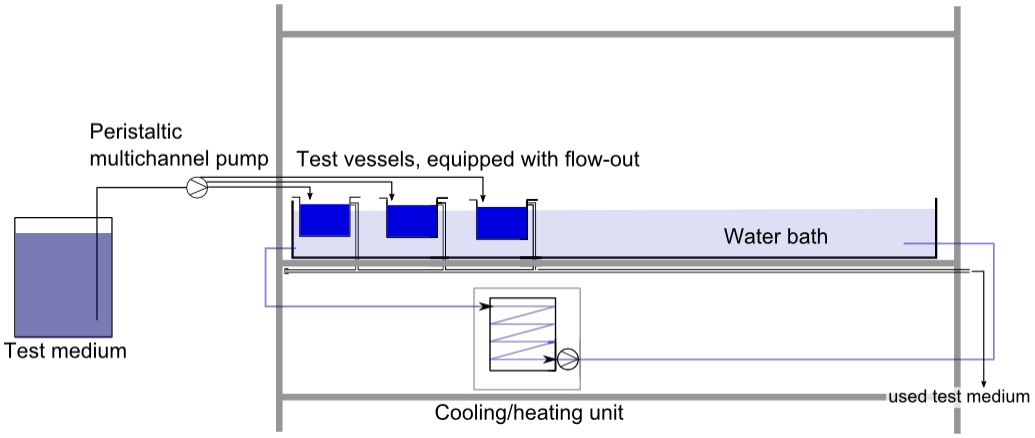

Figure 1: Exemplary set up for a flow-through test with one test medium. Temperature of the test medium is controlled indirectly via a regulated water bath.

The following technical devices are necessary for the reproduction test with *L. variegatus* and the fish early life stage test with rainbow trout:

Table 1 : Technical devices for the reproduction test with L. variegatus and the fish early life stage test with rainbow trout

Description	Specifications	
Heavy load rack to support test setup	e.g. 2 x 2.2 x 1 m (H x W x L)	
Rectangular tough for use as water bath, Alternatively climate chamber, see below	Dimensions: sufficient to hold all test vessels, e.g. 1.8 x 2 x 1.1 m Fabric: polyethylene or aluminum, water tight	
Flow through cooling / heating device, alternatively climate chamber	Fish *O. mykiss*	Temperature: min: 10±2°C, max: 12±2°C[1] Dimensions: approx. 1 m^3
	Worm *L. variegatus*	Temperature: 20±2°C Dimensions: approx. 1 m^3
Peristaltic multichannel pump	Number of channels and flow-rates depending on individual goal of investigation, e.g. 18 channels cover 3 test media plus one control, each in 4 replicates 40 mL/min allow flow-through > 5 times vessel volume (with vessel volume = 10 L)	
Test vessels with flow-out	Fish	Stainless steel vessels, Dimensions: 10 L, e.g. 20 x 27 x 25 m (H x W x L)
	L. variegatus	Glas beakers with cap, Dimensions: 0.3 L, e.g. 11 x 6 cm (H x D)

For the testing of waste water, it is implicitly necessary to perform the mentioned tests in flow-through conditions. The large volume of waste water required to accomplish this allows no other conclusion but to perform these tests on site.

Due to the bulk of equipment necessary for the tests it stands to reason whether it has to be integrated in a fully functional mobile container or if the test equipment can be set up in a room on the test site.

[1]: please note that temperature requirements differ when other fish species are used, see OECD guideline 210, 1992.

4 Microbiological analyses for the determination of bacterial and viral indicators in wastewater

Microbiological analyses are mostly based on culture-dependent methodologies. Therefore sampling itself and processing the samples requires sterile techniques to avoid secondary contaminations. To do so, the use of a laminar flow or clean bench is required.

The samples for microbiological analyses have to be non-filtered whole-effluent samples that need to be taken in sterile glass bottles. It is advisable to use gloves while sampling and not to touch the bottlenecks.
Until and during transport to the laboratory, samples have to be stored at 4°C in a refrigerator or cooling box with thermal packs. Samples need processing within 24h.

4.1 Requirements

- presterilized water bottles, 1000 mL per sample
- gloves
- refrigerator
- thermal packs for transport

5 Analysis of organic micropollutants in wastewater

For the investigation of effluents from a WWTP sampling should be performed automatically as 24-hour composite samples, which are sampled into glass vessels and cooled down to 4 °C. After sampling, the wastewater will be analysed by means of solid phase extraction (SPE). Table 2 gives an overview what kind of material is needed for an appropriate SPE and Figure 2 shows how to build-up a SPE device.
Before an extraction on-site the WWTP can be carried out, an excessive training of the involved personal is necessary in order to obtain a certain routine in SPE. After extraction the dried cartridges are send to the laboratory for the final analysis. Methods for the in-lab analysis are described in the following scientific papers.

Arne Wick, Guido Fink and Thomas A. Ternes; Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography–tandem mass spectrometry Journal of Chromatography A, 2010,1217,(14) 2088-2103

Jennifer Lynne Kormos, Manoj Schulz, Manfred Wagner and Thomas A. Ternes; Multistep Approach for the Structural Identification of Biotransformation Products of Iodinated X-ray Contrast Media by Liquid Chromatography/Hybrid Triple Quadrupole Linear Ion Trap Mass Spectrometry and 1H and 13C Nuclear Magnetic Resonance. Anal. Chem., 2009, 81 (22), pp 9216–9224

Maria Ramil, Tarek El Aref, Guido Fink, Marco Scheurer and Thomas A. Ternes; Fate of Beta Blockers in Aquatic-Sediment Systems: Sorption and Biotransformation. Environ. Sci. Technol., 2010, 44 (3), pp 962–970

Arne Wick, Guido Fink, Adriano Joss, Hansruedi Siegrist, Thomas A. Ternes; Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. Water Research, 2009, 43, (4) 1060-1074

Karoline Stein, Maria Ramil, Guido Fink, Michael Sander and Thomas A. Ternes; Analysis and Sorption of Psychoactive Drugs onto Sediment. Environ. Sci. Technol., 2008, 42 (17), pp 6415–6423

Figure 2: Build-up of a SPE equipment

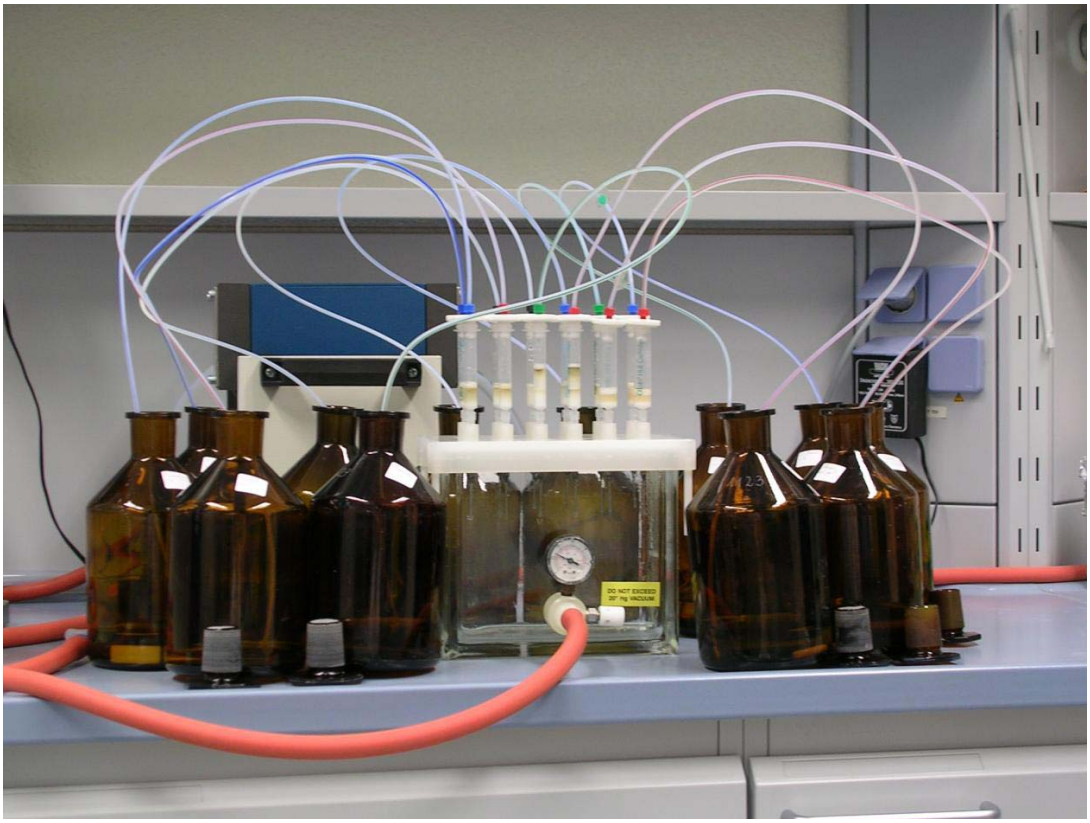

Table 2: SPE material list

Nr. 1	devices needed	number	company	Art.No.
1	SPE Box: Supelco Visiprep 12-Port	2	Supelco	5-7030-U
2	Tubes: Visiprep large Volume Sampler	6	Supelco	57275
3	Cartridge adapter for 1.3.6 mL	2	Supelco	57020-U
4	VacMaster Drying Adaptor 10-Port	2	Separtis	124-1003
5	Bottles brown 0.5L with stopper	24	VWR/Merck	215-1761
6	Bottles brown 1L with stopper	50	VWR/Merck	215-1762
7	Bottles brown 2L with stopper	5	VWR/Merck	215-1763
8	Gilson pipet 25 µL: Model Microman M25	1	Gilson	F148502
9	Gilson pipet 100 µL Model Microman M100	1	Gilson	F148504
10	Gilson pipet 250 µL Model Microman M250	1	Gilson	F148505
11	Gilson pipet 1000 µL Model Microman M1000	1	Gilson	F148506
12	Capillary for M25: CP25	2	Gilson	F148112
13	Capillary for M100: CP100	2	Gilson	F148414
14	Capillary for M250: CP250	2	Gilson	F148114
15	Capillary for M1000: CP1000	2	Gilson	F148560
16	Vacuumpump LVS 301P	1	Ilmvac	113042
17	Vacuumcontroller V-850	1	Büchi	047231
18	Vacuum filtration unit	1	VWR/Merck	516-7017
19	Vacuumbottle 15L	1	VWR/Merck	519-4224
20	Glas tube for 15L bottle	1	-	-
21	Stopper for 15L bottle	1	VWR/Merck	217-9421
22	Vacuum tube 10m, Di=6mm	1	VWR/Merck	189-9123
23	Tube adapter PP (star form) for tube with Di=5-7	4	VWR/Merck	229-0571
24	Tube adapter PP (long form) for tube with Di=5-7	4	VWR/Merck	229-0714
25	Liquid pump (Wasserstrahlpumpe)	1	VWR/Merck	181-9100
26	SPE Cartridge: OASIS HLB 200mg, Box of 30	5	Waters	WAT106202
27	SPE Cartridge: OASIS MCX 60mg 3cc, Box of 100	3	Waters	186000254
28	SPE Cartridge: Bakerbond C18 500mg/3mL, Box of 50	4	J.T.Baker	7020-03
29	ISOLUTE ENV+ Bulkmaterial, 100g	100g	Separtis	9915-0100
30	ISOLUTE ENV+ SPE-Kartuschen, 500mg/3mL, Box of 50	3-4	Separtis	915-0020-B
31	Empty cartridges for ENV+ material	50	-	-
32	Baker PTFE-Frits for 3mL Cartridges (refill), Box of 250	2	J.T.Baker	7329-03
33	PTFE magnetic stir bar l= 4cm	4	VWR/Merck	442-4527
34	PP-Stopper	3	VWR/Merck	217-9263
35	Glasfibre filter Schleicher&Schuell GF6, Box of 100	2	VWR/Merck	515-9102
36	Tweezers for GF filter	1	VWR/Merck	232-2182
37	pH-meter,WTW inoLab pH720	1	VWR/Merck	662-1127
38	pH buffer pH 2, 500mL	1	VWR/Merck	83600.260
39	pH buffer pH 7, 500mL	1	VWR/Merck	32096.267
40	pH storage solution, KCL 3mol/L, 500mL	1	VWR/Merck	83605.260
41	Magnetic stirrer Modell 205	1	VWR/Merck	444-2788
42	Balance up to 2 kg	1	-	-
43	Balance up to 200 g	1	-	-
44	Label for bottles/cartridges, 22x18mm, Box of 1200	1	Avery/Zweckform	3318
45	Nitrogen, Quality: 5.0, 50 L, 200bar	1	-	-
46	Pressure reducer for N2	1	VWR/Merck	618-3211
47	n-Heptan, picograde, 2.5L	1	Promochem	SO-1210-B025
48	Methanol, picograde, 4L	1	Promochem	SO-1142-B040
49	Aceton, picograde, 4L	1	Promochem	SO-1263-B040
50	H2SO4, 3.5mol/L , for ph adjustment	1	-	-
51	Milli Q Water	1	-	-
52	Aceton, tec., 2.5L	1	VWR/Merck	8.22251.2500
53	Pipet for conditioning the cartridges, 5 mL	4	VWR/Merck	612-1027
54	Washing bottle 500mL (Aceton)	1	VWR/Merck	215-0622
55	Washing bottle 500mL (dest. Water)	1	VWR/Merck	215-8503
56	Glas pasteur pipet (for pH adjustment), Box of 250	1	VWR/Merck	612-1701
57	Suction bulb, 2mL	2	VWR/Merck	612-1941
58	Surrogat-Solution	1	-	-
59	Spiking-Solution	1	-	-
60	Aluminium foil	1	-	-
61	Adhesive tape	1	-	-
62	Waste container for organic solvents	1	-	-
63	Fridge	1	-	-
64	Oven >>> 260°C	1	-	-

5.1 Detailed SPE procedures

Surrogate standard mixtures (Surro-Mix A-D) are provided by the responsible lab. The mixtures contains the respective internal standards for the in lab analysis.

5.1.1 Method 1: Antibiotics, Psychoactive Drugs and Biocides

- For wastewater: take **100 mL** of sample and filtrate it over a **glass fibre filter** with a pore size of **< 1µm**. The glass fibre filters must be conditioned as described in "Instructions for conditioning 1.2.1"
- Adjust the **pH** of the sample to **7 - 8** with a 3.5M **H_2SO_4**-solution
- **Add 20 µL of Surro-Mix A** (**Antibiotics**) **to each sample**. Use a Gilson Microman pipette or a Hamilton syringe to add the surrogate standard. **Avoid cross contamination!!** Stir or shake the sample after the addition of the surrogate standard.
- For the conditioning put the cartridges on a SPE box and flush them by gravity flow (without vacuum!) as described below:
- Condition the cartridges (**Waters Oasis HLB 200mg, 6mL**) by flushing them first with **1 x 2 mL n-heptane**. Then take **1 x 2 mL** of **acetone** followed by **3 x 2 mL** of **methanol**. Finally take **4 x 2 mL** of **groundwater** with the same pH as the sample (**in this case pH 7.5**). After the last 2 mL of groundwater add 1-2 mL of groundwater (pH 7.5) to the cartridge and keep it closed until the SPE starts. If groundwater is not available take non-carbonated **table water, preferably in glass bottles**.
- It is important that the **cartridges do not run dry** between the different conditioning steps!!!
- For the SPE connect the sample bottles with the cartridges on the SPE box with the SPE tubes. Open the valves of the SPE box for every cartridge and suck the samples through the cartridges. The pressure should be around **~200 mbar** which is often equal to **~20 mL/min**.
- When a sample has completely passed the cartridge close the valve of this sample.
- **Don't forget to label the samples and then the cartridges!!**
- After the SPE connect the cartridges to the drying adapter and **dry them with nitrogen for 2 h**. After drying close the cartridges with aluminium foil.
- **Store the samples in the fridge** until sending them to the project partner.

- For sending the samples put them into aluminium foil. Put always 5 cartridges together to one unit. If possible send them in a styrofoam box to be sure they are stored at constant conditions.

5.1.2 Method 2: Contrast media and acidic pharmaceuticals

- For **wastewater:** take **100 mL** of sample and filtrate it over a **glass fibre filter** with a pore size of **< 1 µm**. The glass fibre filters must be conditioned as described in "Instructions for conditioning 1.2.1"

- Adjust the **pH** of the sample to **2.8** with a **3.5 M H_2SO_4**-solution.

- First **add 40 µL of Surro-Mix B1** (**Contrast media**) to each sample. Then add **30 µL of Surro-Mix B2** (**Acidics**) **to each sample**. Use a Gilson Microman pipette or a Hamilton syringe for the addition of the surrogate standard. **Avoid cross contamination!!** Stir or shake the sample after the addition of the surrogate standard.

- For the conditioning put the cartridges on a SPE box and flush them by gravity flow (without vacuum!) as described above. Condition every single cartridge and not the combination of the cartridges (see next steps).

- Condition the cartridges (**for contrast media take Isolute ENV+ 200 mg, 3 mL; for acidics take Waters Oasis MCX 60 mg, 30 µm, 3 mL**) by flushing it first with 1 x 2 mL n-heptane. Then take 1 x 2 mL of acetone followed by 3 x 2 mL of methanol. At last take 4 x 2 mL of groundwater with the same pH as the sample (in this case pH 2.8). After the last 2 mL of groundwater add 1-2 mL groundwater (pH 2.8) to the cartridge and keep it close until the SPE starts. If groundwater is not available take non-carbonated table water preferably in glas bottles.

- It is important that the **cartridges don't run dry** between the different conditioning steps!!!

- For the SPE connect the conditioned cartridges to the SPE box. At first connect the ENV+ cartridge with the box. Then connect the cartridge adapter to the ENV+ cartridge. Connect the MCX cartridge then to the adapter on the ENV+ cartridge.

- Now connect the sample bottles with the cartridges on the SPE box by using the SPE tubes. Open the valves of the SPE box for every cartridge and suck the samples through the cartridges. The pressure should be around **~600 mbar. In any case 10 mL/min should NOT be exceeded!!!!** (Flow must be slower than for the antibiotics !!).

- When a sample has completely passed the cartridge close the valve of this sample.
- **Don't forget to label the samples and the cartridges!!**
- After the SPE connect the cartridges to the drying adapter and **dry them with nitrogen for 2 h**. After drying close the cartridges with aluminium foil.
- **Store the samples in the fridge** until sending them to the project partner.
- For sending the samples put them into aluminium foil. Put always 5 cartridges together to one unit. If possible send them in a styrofoam box to be sure they are stored at constant conditions.

5.1.3 Method 3: Betablockers

- For wastewater take **100 mL** of sample and filtrate it over a **glass fibre filter** with a pore size of **< 1 µm**. The glass fibre filters must be conditioned as described in "Instructions for conditioning, chapter 1.2.1"
- Adjust the **pH** of the sample to **7 - 8** with a **3.5 M H_2SO_4**-solution
- **Add 20 µL of Surro-Mix D (Betablockers) to each sample**. Use a Gilson Microman pipette or a Hamilton syringe for the addition of the surrogate standard. **Avoid cross contamination!!** Stir or shake the sample after the addition of the surrogate standard.
- For the conditioning put the cartridges on a SPE box and flush them by gravity flow (without vacuum!) as described below:
- Condition the cartridges (**Baker C18 500 mg, 3 mL**) by flushing them first with **1 x 2 mL n-heptane**. Then take **1 x 2 mL** of **acetone** followed by **3 x 2 mL** of **methanol**. At last take **4 x 2 mL** of **groundwater** with the same pH as the sample (**in this case pH 7.5**). After the last 2 mL of groundwater add 1-2 mL of groundwater (pH7.5) to the cartridge and keep it closed until the SPE starts. If groundwater is not available take non-carbonated **table water preferably in glass bottles**.
- It is important that the **cartridges don't run dry** between the different conditioning steps!!!
- For the SPE connect the sample bottles with the cartridges on the SPE box by using the SPE tubes. Open the valves of the SPE box for every cartridge and suck the samples through the cartridges. The pressure should be around **~200 mbar** which is often equal to **~20 mL/min**.
- When a sample has completely passed the cartridge close the valve of this sample.
- **Don't forget to label the samples and then the cartridges!!**

- After the SPE connect the cartridges to the drying adapter and **dry them with nitrogen for 2 h**. After drying close the cartridges with aluminium foil.
- **Store the samples in the fridge** until sending them to the project partner.
- For sending the samples put them into aluminium foil. Put always 5 cartridges together to one unit. If possible send them in a styrofoam box to be sure they are stored at constant conditions.

5.1.4 Instructions for conditioning the SPE cartridges:

1) Put the cartridges on the SPE Box and open the valve for each cartridge.
2) Fill in **1 X 2 mL of n-Heptane (picograde)**. Let the solution run to the cartridge until it has reached the upper PTFE-frit of the cartridge.
3) Add **1 X 2 mL of Acetone (picograde)**. Let the solution run to the cartridge until it has reached the upper PTFE-frit of the cartridge.
4) Add **3 X 2 mL of Methanol (picograde)**. Let the first 2 mL solution run to the cartridge until it has reached the upper PTFE-frit of the cartridge. Then add the next 2 mL. After the last 2 mL of Methanol add the next solution when the methanol has reached the upper PTFE-frit of the cartridge.
5) Add **4 X 2 mL of groundwater (or table water without gas - preferably in glass bottles) adjusted to the pH of the sample** that will be analyzed.

   At all steps it is important that the cartridges do not run dry!!

5.1.5 Instructions for conditioning the glass fibre filter (used for sample filtration):

1) Put all the filters into a 250 mL beaker
2) Fill in **n-Heptane (picograde)** until the filters are covered with Heptane and leave them in Heptane for 10 min.
3) Pour out the heptane and fill in **Acetone (picograde)** until the filters are covered with Acetone. Leave the filters in Acetone for 10 min.
4) Pour out the Acetone and fill in **Methanol (picograde)** until the filters are covered with Methanol. Leave the filters in Methanol for 10 min.

5) Pour out the Methanol and fill in **Milli Q Water** until the filters are covered with Milli Q Water. Leave the filters in Milli Q Water for 10 min.

6) Pour out the Milli Q Water and rinse the filters with Milli Q Water until you can't smell any organic solution.

5.1.6 Instructions for cleaning glassware

1) Rinse one time with **water**

2) Rinse one time with **distilled water**

3) Rinse one time with **acetone** (technical quality)

If a dish washer is available it can be used **BUT** only with **distilled or Milli-Q water**! And the glassware has to be cleaned **after this step** with **acetone (technical quality).**
After rinsing the glass ware it has to be heated at 260°C for 6 h (over night).

5.1.7 Instructions for rinsing the SPE tubes

1) Connect the tubes to a **water** pipe and rinse for approximately 1 min.

2) Connect the tubes to a distilled water pipe and rinse for approximately 1 min with **distilled water**, rinse also from the outside.

3) Connect the tubes to an SPE Box and suck approximately 10 mL of **acetone** (technical quality) through the SPE tube

4) **Air-dry the SPE tubes over night** or dry them with nitrogen if they are needed immediately.

5) Avoid contact of the tube with any kind of surface in the lab. Store tubes in a drawer or cup board on a tissue or aluminum foil.

6 Analysis of organic micropollutants in solids

6.1 Sample preparation for freeze-drying of suspended matter or sludge

• at least **10 g** (final weight) freeze dried material per sample is needed

• freeze sample down to -20°C

• freeze-dry sample in stainless steel or alumina cups in available freeze-dryer and follow its instructions

• seal cup and send sample to the lab

6.2 Sample preparation for tin organics in suspended matter or sludge

• take at least **20 g** homogenized wet material

• fill sample in PE flasks with screw caps

• freeze sample down to -20°C

• send sample on dry ice to ensure that material is still frozen at transport to the lab

7 Overview of the testing methods

Not all tests are suited to be done on-site a WWTP. Most of the tests have to be conducted in a laboratory that fulfils S1 requirements. An overview of what can be done on-site is given in Table 3.

Table 3 Overview of the testing methods

Test	Conclusion
Toxicological analysis of wastewater samples with *in vitro* tests	• Samples must be collected in sterile containers and transported in cooling boxes within 24 h to the respective labs (S1 laboratories).
Toxicological analysis of wastewater with standard *in vivo* tests	• Due to the bulk of equipment necessary for the tests it has to be integrated in a fully functional mobile container. Alternatively the equipment can be set up in a room on the test site.
Microbiological analysis indicators in wastewater	• Samples must be collected in sterile containers and transported in cooling boxes within 24h to the respective labs
Micropollutants in wastewater	• Samples can be extracted by means of SPE on-site after an excessive training of the personal. Dry SPE cartridges can be stored for 3 weeks at -20°C until further analysis starts. • SPE cartridges must be transported to the labs and have to be analysed in the lab.
Micropollutants in solids	• Solid samples must be frozen (-20°C) and transported in cooling boxes to the respective labs. Frozen samples can be stored for 3 weeks at -20°C until further analysis starts.

8 Conclusions

8.1 In vitro test systems

Water samples enriched via solid phase extraction can be analyzed using the following assays to test on specific modes of toxic action. The cytotoxicity assay allows the detection of non-specific toxicity.

A) (Anti-)YES. Yeast estrogen screen for the detection of estrogenic activity and anti-estrogenicity.
B) (Anti-) YAS. Yeast androgen screen for the detection of androgenic and anti-androgenic activity.
C) YDS. Yeast dioxin screen for the detection of aryl-hydrocarbon agonistic activity caused by dioxin-like compounds.
D) UmuC-test for the detection of genotoxicity of wastewater samples.
E) Ames fluctuation assay for the detection of mutagenic activity. In particular a mutagenicity increase caused by ozonation can be detected with selected tester strains.
F) Cytotoxicity assay to test on non-specific toxicity. Vertebrate cell based test systems can be applied with GH3 cells (rat pituitary cells) and RTL-W1 cells (rainbow trout liver cells).

8.2 In vivo test systems in a flow through test design

The appliance of whole organism tests on site at wastewater treatment plants enables the detection of increased toxicity, for example as a result of toxic byproduct formation during ozonation. Such byproducts are hardly extractable and readily degradable and can thus not be detected in conventional solid phase extracted samples using *in vitro* test systems. This is particularly important when evaluating the detoxication efficiency of a post treatment, as e.g. sand filtration. The following test systems are identified as suitable to test the increase of non-specific toxicity as a result of ozonation and to evaluate the possible subsequent detoxication in a bioactive sand filtration step:

A) Fish early life stage toxicity test (FELST) with the rainbow trout (*Oncorhynchus mykiss*) according to OECD-guideline 210 (OECD 1992a).
B) Sediment-water *Lumbriculus* toxicity test with the annelid *Lumbriculus variegatus* according to OECD-guideline 225 (OECD 2007).

C) Comet assay with haemolymph of the mollusc *Dreissena polymorpha* after exposure to wastewater for 70 days to detect *in vivo* genotoxicity after the different treatment steps.

8.3 Bacterial and viral indicators in wastewater

The survival rates of bacteria and pathogens in WWTP depend on specific organism properties such as size or sensitivity to chlorination, ozonation, photochemical processes or UV inactivation. The following bacterial indicators are suggested: fecal coliforms, *Escherichia coli* and *enterococci* are strong indicators of fecal pollution or possible pathogenic bacterial contamination. As viral indicators male-specific (F+) and somatic *coliphages* can be used.

8.4 Organic micropollutants in wastewater

Persistent organic pollutants, WFD priority substances and so-called “emerging” chemicals are of major environmental and drinking water relevance. Therefore the following indicator substances fulfilling the selection criteria to evaluate the treatment technologies.

- Strongly sorbing contaminants with high-to-medium persistence in sludge

Tin organics, PBDE, PAH and PCB are identified as strongly sorbing contaminants attached to suspended matter

- Highly dissolved compounds with high-to-medium persistence in water

Substances which are not eliminated in WWTPs are in general persistent in rivers and are hardly removed by biodegradation and sorption to sediments and soil. Substances of particular interest according to these criteria are the X-ray contrast media diatrizoate and iopamidol, the antiepileptic carbamazepine, the antibiotic erythromycin, and the beta-blocker metoprolol. Diatrizoate is especially known for its high persistence in both WWTP and the environment [Poseidon, 2004]. Among the compounds poorly removed in WWTP are furthermore the antibiotics clarithromycin and azithromycin, the antidepressant fluoxetine, the analgesics phenazone and propyphenazone, and the beta-blocker propranolol.

- Dissolved and sorbing compounds with high-to-medium persistence in water and sludge

Surfactants such as 4-Nonylphenols and the antibiotic ciprofloxacine can be used as an indicator for sorbing compounds with high-to-medium persistence in water and sludge.

- Tracer compounds with already known behavior to facilitate the extrapolation of the results to other emerging groups.

Organic micropollutants which are usually completely removed during basic wastewater treatment can serve as indicators of insufficient wastewater treatment, e. g. due to WWTP malfunction or overflow events.
The following compounds are fulfilling these criteria, the analgesics ibuprofen and paracetamol, the psychoactive drug codeine, the lipid regulator bezafibrate and the X-ray contrast media iopromide.

REFERENCES

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