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Deliverable Report

D1.2 Analytical procedures

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1 Introduction to the project SERPIC

The project *Sustainable Electrochemical Reduction of contaminants of emerging concern and Pathogens in WWTP effluent for Irrigation of Crops – SERPIC* will develop an integral technology, based on a multi-barrier approach, to treat the effluents of wastewater treatment plants (WWTPs) to maximise the reduction of contaminants of emerging concern (CECs). The eight partners of the SERPIC consortium are funded by the European Commission and by six national funding agencies from Norway, Germany, Italy, Spain, Portugal and South Africa. The official starting date of the SERPIC project is 1. September 2021. The project has a duration of 36 months and will end 31. August 2024.

The overall aim of the SERPIC project is to investigate and minimise the spread of CECs and antimicrobial resistant bacteria/antibiotic resistance genes (ARB/ARG) within the water cycle from households and industries to WWTPs effluents, and afterwards via irrigation into the food chain, into soil and groundwater and into river basins, estuaries, coastal areas, and oceans with a focus on additional water sources for food production.

A membrane nanofiltration (NF) technology will be applied to reduce CECs in its permeate stream by at least 90 % while retaining the nutrients. A residual disinfection using chlorine dioxide produced electrochemically will be added to the stream used for crops irrigation (Route A). The CECs in the polluted concentrate (retentate) stream will be reduced by at least 80 % by light driven electro-chemical oxidation. When discharged into the aquatic system (route B), it will contribute to the quality improvement of the surface water body.

A prototype treatment plant will be set-up and evaluated for irrigation in long-term tests with the help of agricultural test pots. A review investigation of CECs spread will be performed at four regional showcases in Europe and Africa. It will include a detailed assessment of the individual situation and surrounding condition. Transfer concepts will be developed to transfer the results of the treatment technology to other regions, especially in low- and middle-income countries.

2 Report summary

Analytical methods have been developed and optimized for the identification and quantification of the selected organic CECs according to D1.1: the four chemical compounds diclofenac, iopromide, sulfamethoxazole and venlafaxine, *E. coli* as antibiotic-resistant bacteria and *sul1* as antibiotic resistance gene. They were developed and validated on the equipment available at the UCLM laboratory, where the analyses of real samples of the different matrices (raw wastewater, treated effluent, soil and crops) were carried out at a bench scale. In addition, techniques to analyse the content of the selected microbial CECs were also developed and validated.

3 Deliverable description as stated in the Project Description

The protocols for CECs sample preparation and analysis that were developed by SU and collaborators in *T1.2 Analysis of bench-scale processes* will be described in detail.

4 Introduction

SERPIC project aims to utilize a multi-barrier strategy that combines membrane filtration processes with electrochemical production of potent oxidants to significantly reduce the concentration of organic and microbial CECs in the secondary effluent of a municipal wastewater treatment plant, commonly based on activated sludge process. In order to test the effectiveness of the developed polishing treatment at a prototype scale, the occurrence of the selected CECs must be monitored before and after the polishing treatment.

Due to the complexity of the (waste)water matrices and the presence of interfering components that may mask or disrupt the compounds of interest, direct analysis may not be feasible (Pavlović et al., 2007). Consequently, the utilization of clean-up and extraction techniques is crucial to obtain purified extracts suitable for sensitive analysis, being an important procedure prior to analysis (Amato et al., 2021; Kostopoulou and Nikolaou, 2008). The analysis of organic compounds in aqueous environmental matrices is usually carried out by Liquid Chromatography-Mass Spectrometry (LC-MS) after sample clean-up and pre-concentration of the analytes by solid-phase extraction (Buchberger, 2007)(Buchberger, 2007). In the case of antibiotic resistance gene (ARG), prior DNA extraction of the gene is required for subsequent quantification by quantitative real-time Polymerase Chain Reaction (q-PCR). Antibiotic resistant bacteria (ARB) do not require prior extraction, but a careful selection of a suitable selective medium is necessary to identify and quantify them.

In this sense, the aim of this deliverable is to report the main issues related to the developed and optimized analytical methods for the identification and quantification of the four organic CECs, one ARB (*E. coli*) and one ARG (*sul*1) in water matrices. In addition, the analytical methods are validated using different real aqueous (or water) matrices from the existing bench-scale facility at UCLM. The bench-scale setup consists of a reverse osmosis unit, a photoreactor unit, and an ozone disinfection system. Thus, this deliverable describes in detail the validated analytical methods for the quantification of the selected CECs with regard to sampling, pretreatment, extraction steps as well as analysis.

5 Results

5.1 Sampling

Water samples are taken from different sampling points in bench scale plant corresponding to different types of matrices (Figure 1): Secondary effluent and treated effluent (permeate, concentrate, ozonated effluent, photoreactor effluent). Each sample is collected in sterile 1L plastic bottles and transported for subsequent storage at -20°C until handling and analysis.

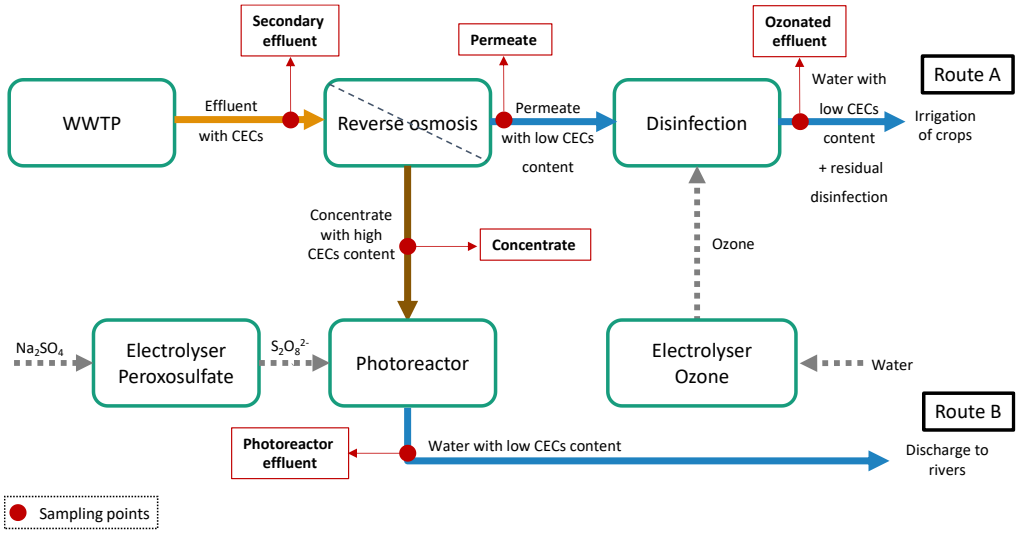

Figure 1: Sampling points in the bench scale process.

5.2 Procedure A. Organic CECs

5.2.1 Sample pretreatment

Samples (250 mL) are previously filtered through a 0.45 µm pore size membrane to remove the suspended matter present in the water and increase the extraction efficiency.

5.2.2 Extraction and concentration of organic CECs by solid phase extraction

Organic compounds (diclofenac, iopromide, sulfamethoxazole, and venlafaxine) are extracted from water samples using solid phase extraction (SPE) with polymer sorbent cartridges. This technique has been widely used to extract analytes from complex samples. Its main application is the preconcentration of the analytes and the removal of matrix effects through clean-up (Badawy et al., 2022). The SPE procedure is performed using Oasis HLB cartridges (200 mg, 6cc Waters). The water samples are extracted by loading HLB cartridges with 100-200 mL of filtered water. Before the samples are loaded, HLB cartridges are preconditioned with a combination of methanol (MeOH) and water. Then, the water samples are passed through the cartridge, which acts to retain the analytes. Subsequently, the cartridge is subjected to a drying step and the elution of analytes is conducted with 5 mL of MeOH. The fraction obtained in the elution is evaporated to dryness under a gentle stream of nitrogen. Finally, the final extracts are reconstituted in 100 µL of MeOH:water (10:90 v/v) for subsequent measurement and quantification by Liquid chromatography time-of-flight mass spectrometry (LC-MS/TOF).

5.2.3 LC-MS/TOF analysis

The samples extracted from the SPE procedure are injected and analysed on an Agilent 1260 Infinity coupled to an Agilent time of flight mass spectrometer (LC-MS/TOF 6230) with an electrospray ionization source (ESI). Ionization is carried out in positive or negative mode depending on the organic compound, as indicated in Table 1. The Liquid chromatographic separation of organic compounds is performed with a Luna C18 100A column (50 mm x 2 mm, 3µm) maintained at a constant temperature of 35ºC and 0.3 mL min^{-1}. The mobile phases consist of 1 mM ammonium fluoride in MeOH (A) and 1 mM ammonium fluoride in ultrapure water (B). The gradient is as follows: 0-0.5 min, 2% (A); 0.5-9.5 min, linear gradient to 100% (A); 11.5-11.6 min, 100% (A) and finally 11.6-25 min, 2% (A). Nitrogen is used as drying gas with the flow rate of 10 L min^{-1}, nebulizer of 50 psi, skimmer voltage of 65 V, source temperature of 350 ºC and capillary of 4000 V. Quantification of organic compounds is performed from linear regression calibration curves using internal standards to reduce or correct for matrix effects. The LC-MS TOF method is validated in terms of linearity, sensitivity, extraction recovery, matrix effect, instrumental detection limit, method detection limit, method quantification limit, and repeatability.

Table 1: ESI mode of organic compounds in LC-MS/TOF.

Compound	**ESI mode**
Diclofenac	Negative
Iopromide	Positive
Sulfamethoxazole	Positive
Venlafaxine	Positive

5.3 Procedure B. Antibiotic resistant bacteria (ARB)

5.3.1 Microtrac® analysis

Escherichia coli (*E. coli*) bacterial counts are determined with an indirect impedance method using a Microtrac® 4200 system (SY-LAB). This system records changes in the standard impedance signal resulting from microbial metabolism. Thus, the standard impedance value registered by the equipment is correlated with the bacterial concentration in colony forming units (CFU) per mL obtained through plate counting (Dupont et al., 2004; Zhu et al., 2012). Therefore, an initial calibration using the membrane filtration technique (ISO 8199) is needed to correlate the impedance values of samples with the bacteria concentration. The conditions used in the Microtrac® are shown in Table 2. The measurement procedure consists of transferring aliquots water samples (1 mL) into a measuring cell containing selective medium, which is introduced into the Microtrac® system to obtain the *E. coli* CFU count.

Table 2: Conditions for quantifying *E. coli* in the Microtrac® 4200 system.

Target bacteria	Nutrient media	Temperature	Threshold / h
E. coli	BiMedia 155A (Selective medium for the determination of *E. coli*)	37ºC	5

5.4 Procedure C. Antibiotic resistance gene (ARG)

5.4.1 Sample pretreatment and extraction of gene

Samples (100 mL) are previously centrifuged obtaining the supernatant liquid and the pellet. Cells (and the contained DNA) are retained in the pellet. Total DNA is extracted from pellet by using the Urine DNA Isolation Kit (Norgen Biotek) according to manufacturer's instructions.

5.4.2 qPCR analysis

Gene copy numbers of the *sul1* (ARG) DNA extracted from the water samples is determined by amplification by quantitative real-time Polymerase Chain Reaction (qPCR). Table 3 displays the primer sets used, enabling the amplification of short amplicons (210 base pairs). The qPCRs are performed using a QuantStudio 5 Real-Time PCR System (Thermo Fischer Scientific, Spain). Each reaction sample contains 2 µl of each 2.5 µM primer, 5 µl of template DNA, and 1 µl PCR-grade water and 10 µl of 2×SYBR Green supermix (Thermo Fischer Scientific, Spain) to a total volume of 20 µl. The PCR conditions are 95 ºC for 10 min (initial phase), followed by 39 cycles of 95 ºC (denaturation) for 15 s and 60 ºC for 60 s (annealing). Additionally, a melting curve analysis is performed to verify the purity of the amplicons generated. Each sample and standard is analyzed in triplicate. The calibration process involves serial dilutions of a known quantity of linearized plasmid containing gene fragments.

Table 3: qPCR parameters for the analysed gen *sul1*.

Target gene	Primers sequence (5´-3´)	Amplicon size (bp)	Annealing temperature (ºC)	Reference
sul1	Forward: TGTCGAACCTTCAAAAGCTG Reverse: TGGACCCAGATCCTTTACAG	210	60	(Wang et al., 2014)

6 Publications and other dissemination activities

None

7 Literature

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