

Annex 3

Template for Mid-Term Progress Report

AquaticPollutants Joint Transnational Call 2020

Risks posed to human health and the environment by pollutants and pathogens present in water resources

This Template should be used by the Project Coordinator for the reporting of the progress/results of the entire project consortium.

AquaticPollutants Joint Transnational Call Mid-Term Progress Report

Risks posed to human health and the environment by pollutants and pathogens present in water resources

Potential of decentralized wastewater treatment for preventing the spread of antibiotic resistance, organic micropollutants, pathogens and viruses (PRESAGE)

This document must be filled in by the project coordinator with the help of all project partners and must be sent to the AquaticPollutants Follow-up Secretariat by **1/03/2023** (for Consortium **PRESAGE**).

The AquaticPollutants Follow-Up Secretariat will ensure distribution to the concerned national funding agencies. The project coordinator is responsible for sending a copy of the report to all project partners.

PROJECT TITLE AND ACRONYM

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Project code: AquaticPollutants-PRESAGE

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I. Publishable Summary

Maximum 1 page

New approaches are needed to reduce the emission of contaminants of emerging concern (CECs). Some sources contribute strongly to such emissions, which has driven the focus of PRESAGE on innovative decentralized wastewater treatment (WWT), based on anaerobic and aerobic compact systems. An integrated analysis of the behaviour of organic micropollutants (OMPs), antibiotic resistant microorganisms and genes (ARMs/ARGs) and pathogens (viruses and bacteria) will be carried out. This will allow better understanding the relation between the operational parameters of reactors, the microbiological evolution in the system, the removal of OMPs and pathogens, and the development of ARMs and ARGs. The contribution of such a complex mixture on the final effluent ecotoxicity will be assessed.

The technologies will be validated at 4 demopites treating black and grey water, and effluents from hospitals and an antibiotic industry, in close collaboration with the industrial sector. This high readiness level anticipates a good impact of project results on wastewater innovation.

So far, prototypes have been developed based on anaerobic, anoxic and aerobic hybrid biological treatments, with different reactor configurations (membrane bioreactors, fixed and moving bed biofilms, plug-flow and stirred reactors, etc.). In addition, a prototype has been set-up for sustainable water disinfection based on the antimicrobial activity of functionalized particles that carry a biocide.

Standardized operation procedures for the monitoring of OMPs, ARMs/ARGs and pathogens have been adapted to the water and sludge matrixes generated in the project. First standardized ecotoxicity tests have been applied to wastewater containing CECs.

PRESAGE impacts society and economy, boosting the water industry and protecting the environment from effluent discharges containing CECs. In the proposed treatment strategy a minimum global impact is targeted, preferentially promoting the onsite water reuse.

2. Work Performed and the Results achieved during the reporting period

Maximum 10 pages (including figures and tables).

a. Scientific and technological progress

The work performed is described below following the same numeric order of tasks as in the proposal, focusing only on those planned during the first half of the project.

WPI. Domestic wastewater: Separated Black water & Grey water treatment.

Objectives: 1) Evaluate the removal of CECs; 2) Assess the influence of operational conditions; 3) Propose the optimum treatment strategy based on the integrated analysis of OMPs, pathogens, ARM and ecotoxicity.

Task. TI.1. Operation of the anaerobic membrane reactor treating the BW at different HRT (1-5 d) and temperatures (15-25 °C).

The decentralised treatment system of separated BW and GW was operated for 226 days. In BW line, the AnMBR was operated at an HRT of 2.4 ± 0.2 d, treating 42.7 ± 3.9 L h⁻¹. A removal of 92.4 ± 2.5 % of the influent COD, 1720 ± 933 mg COD L⁻¹, was achieved in the system.

OMP's characterization was carried out in two sampling campaigns, without spiking the selected compounds in the system. The results of the first sampling campaign showed a low influent concentration of the compounds: below 1 ng L⁻¹ except for ibuprofen (23.8 ± 6.3 ng L⁻¹). Removal efficiencies for the detected compounds in this line are presented in Figure 1. Moderate removal efficiencies were found for trimethoprim and naproxen, both easily biodegradable in anaerobic environments. Low removal efficiencies were achieved for the rest of compounds, probably due to their low influent concentration (hormones E1 and E2) or by the non-optimal redox conditions (ibuprofen).

Regarding ARGs, two sampling campaigns were also performed at the beginning and at the end of the operation. The results of the first sampling campaign were used to carry out a selection of the genes (**WP5**) that are being monitored in the next sampling campaigns in the decentralised system. The first screening showed the presence of different ARGs, and other genetic elements involved in antibiotic resistance. Among them, 34 ARGs were selected as the most abundant in the influent, effluent, and sludge samples. From this 34 ARGs, a final selection was achieved taking into account the methodology proposed by Zhang et al., 2021¹. It is based on the potential health risk that comes with each ARG. The seven ARGs were selected according to this methodology are gathered in Table 1. Their relative abundance found on the SmartChip qPCR is shown in Figure 2. The highest relative abundance of almost all detected ARGs was found in the anaerobic sludge, and it is reduced along the system. The opposite behavior was also found for some genes, such as *mcr* or *intl1*. The quinolone resistance gene *qnrS* was not detected neither in the sludge nor in the permeate.

Finally, it was also performed a sampling campaign to characterise the presence of pathogens in the system (**WP6**). Deliverable 1.1 includes the information related to the optimization of BW treatment in the AnMBR stage.

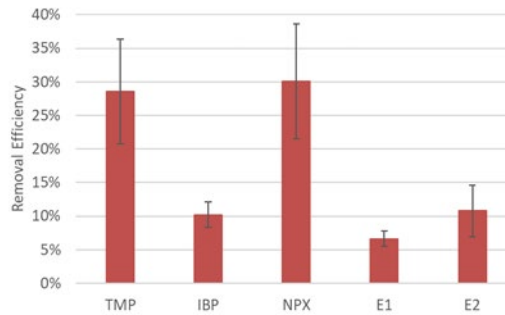


Figure 1. Removal efficiencies of OMPs in the AnMBR achieved in the first sampling campaign.

Table 1. Selected genes to be followed by qPCR after the first screening.

Gene class	ARG
Class I integron integrase	<i>intI</i>
Sulfonamides	<i>sulI</i>
Beta-lactams	<i>bla_{CTX-M}</i>
Colistin	<i>mcr</i>
Macrolides	<i>ermB</i>
Fluorquinolones	<i>qnrS</i>

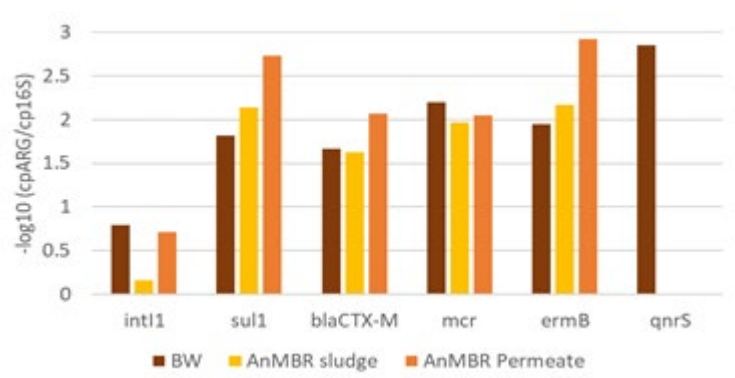


Figure 2. Normalised relative abundance of the selected genes in the initial characterization of the BW treated in the AnMBR.

Task. T1.2. Operation of the hybrid anoxic/aerobic membrane biological reactor with the mixture of GW and anaerobic effluent.

Regarding GW line, the H-MBR was operated at an HRT of 5.7 ± 0.1 h, treating 10.1 ± 0.1 L h^{-1} . COD removal efficiency achieved was 89.7 ± 3.5 % from an influent concentration of 146.9 ± 43.1 mg COD L^{-1} . As a low strength wastewater, GW contained 4.1 ± 1.1 mg N L^{-1} , from which up to 54 % was able to be removed in the system. Two OMPs sampling campaigns were also carried out to characterise their occurrence and fate in the GW treatment line. Due to the characteristics of this matrix (composed by sink water), only two compounds were detected at low concentrations in the first sampling campaign: 1.03 ng L^{-1} of trimethoprim and 18.6 ± 6.1 ng L^{-1} of ibuprofen. The removal efficiencies achieved were 9.8 ± 16.7 % for trimethoprim and 22.5 ± 5.31 % for ibuprofen. Regarding ARGs (**WP5**), there were also performed two sampling campaigns to assess the presence of selected compounds along this treatment line. Pathogens (**WP6**) were also characterised in a sampling campaign.

WP2. Industrial wastewaters: hospital and pharmaceutical effluents.

Objectives: 1) Evaluate different flow patterns on the removal of CECs in anaerobic biofilm reactors; 2) Comparison of aerobic suspended biomass and biofilms in terms of CECs removal.

Task: T2.1. Lab-scale operation of the Plug-flow Anaerobic Reactor (PF-AnFBR) and Stirred Tank Anaerobic Reactor (CS-AnFBR).

Polyurethane foam was used as support media to be inoculated with sludge from a UASB reactor treating poultry slaughterhouse. A synthetic WW containing easily degradable organic matter (500 mg COD/L) and OMPs was used as influent. OLR and HRT were the main operating parameters, with HRT ranging from 16 to 8 h.

The experiments were finished, and the obtained results are under evaluation for a deep discussion, considering the main phenomena and the comparison with the literature. Figure 3 shows the average removal efficiencies of the 12 antibiotics (ciprofloxacin-CIP, norfloxacin-NOR, pefloxacin-PEF, ofloxacin-OFL, enrofloxacin-ENR, sulfamethoxazole-SMZ, sulfamethazine-SMX, sulfamerazine-SMR, sulfadiazine-SDZ, sulfacetamide-SCT and sulfadimethoxine-SDX, and trimethoprim-TMP) incorporated into the synthetic wastewater in the PF-AnFBR and CS-AnFBR reactors, applying HRT of 16, 12 and 8 h. Ciprofloxacin (CIP), Sulfamethoxazole (SMX), and Trimethoprim (TMP), which are widely prescribed in hospitals around the world, were chosen by the PRESAGE research consortium to compare wastewater treatment technologies. It was observed that, when applying the HRT equal 8 h to the PF-AnFBR reactor, the highest removals were observed for these 3 antibiotics, while for the CS-AnFBR reactor there was an improvement in removal only for CIP and SMX. It was concluded that the Plug-flow reactor presented better performance for micropollutants removal. Hydraulic retention time of 8 h provided best results of micropollutants removal. These results are explained in more detail in Deliverable 2.3 and will be used for validation of the treatment strategy with hospital wastewater in task 2.2.

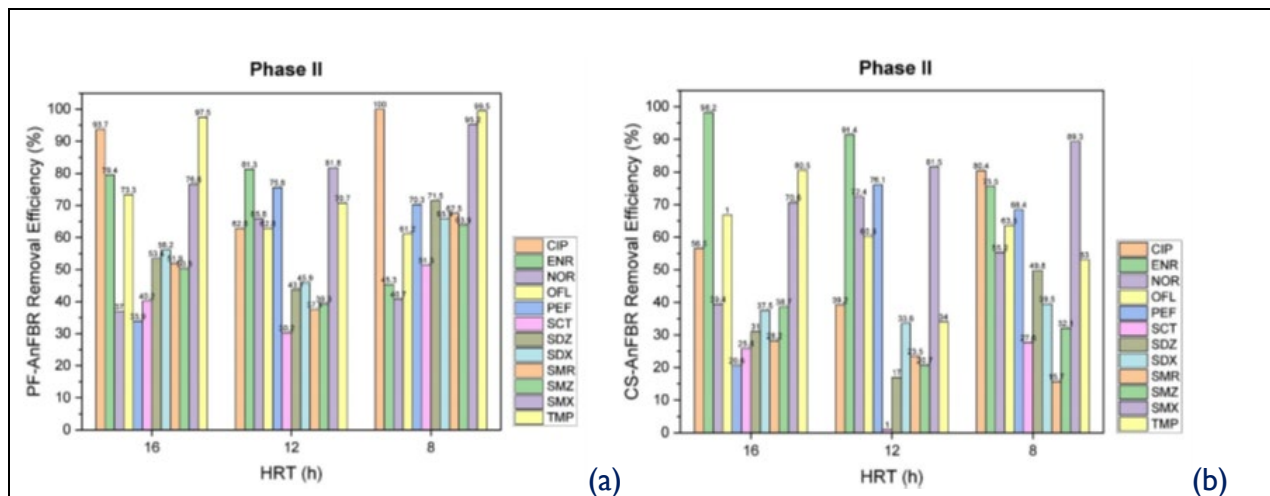


Figure 3. Average removal efficiency of the 12 antibiotics (ciprofloxacin-CIP, norfloxacin-NOR, pefloxacin-PEF, ofloxacin-OFL, enrofloxacin-ENR, sulfamethoxazole-SMZ, sulfamethazine-SMX, sulfamerazine-SMR, sulfadiazine-SDZ, sulfacetamide-SCT and sulfadimethoxine-SDX, and trimethoprim-TMP) for the 16, 12 and 8h HRTs applied to the PF-AnFBR (a) and CS-AnFBR (b) reactors during Phase 2, respectively.

Task. T2.3. Operate the IFAS-MBR and the MBR-MBBR at the hospital site in North Zealand.

Two treatment trains were established to treat the wastewater from hospital. In the first treatment trains integrated fixed-film activated sludge (IFAS) system combined with membrane bioreactor (MBR) were installed in the 500L tank followed by 200L polishing MBBR tank. In the second treatment train, MBR reactor was established in the 500 L tank followed by 200L polishing MBBR tank. The objective of this was to see the effect on the removal of antibiotics-resistant bacteria (ARBs) in IFAS-MBR reactor in series I and MBR reactor in series II. Furthermore, removal of OMPs from both pilot units in the two treatment trains will be compared. This study also investigates the antibiotic resistant genes (ARGs) that passes through the permeate water from IFAS-MBR unit and MBR unit will be taken by the biofilms in the polishing MBBR tank or biofilms in the later stage to develop the resistance towards the antibiotics. Pilot reactors were installed in the WWTP of Herlev hospital (Denmark). Wastewater after grit/grease chamber are used to feed the pilot units. Both series were running with 2 days of hydraulic retention time (HRT). Different chemical parameters such as: COD, TSS, NH₄-N, NO₃-N, PO₄-P, pH were measured regularly from pilot units to access the performance of pilot units. ARBs and ARGs described in the task 5.1 were enumerated from untreated hospital wastewater (Figure 4). DNA extraction was completed from samples from IFAS-MBR, MBR tank, permeate water from IFAS-MBR & MBR tank and from both polishing MBBR for enumeration of ARBs and ARGs.

COD removal from the IFAS-MBR & MBR units was 97% and 94%, respectively, with 2 days HRT. Similarly, COD removal from polishing MBBR tank receiving permeate water from IFAS-MBR unit was 94% and polishing MBBR tank receiving permeate water from MBR tank was 93% (Figure 5). Continuous optimization of loading of wastewater to the pilot unit is done until a stable process is achieved and results will be updated regularly in the meeting with partners.

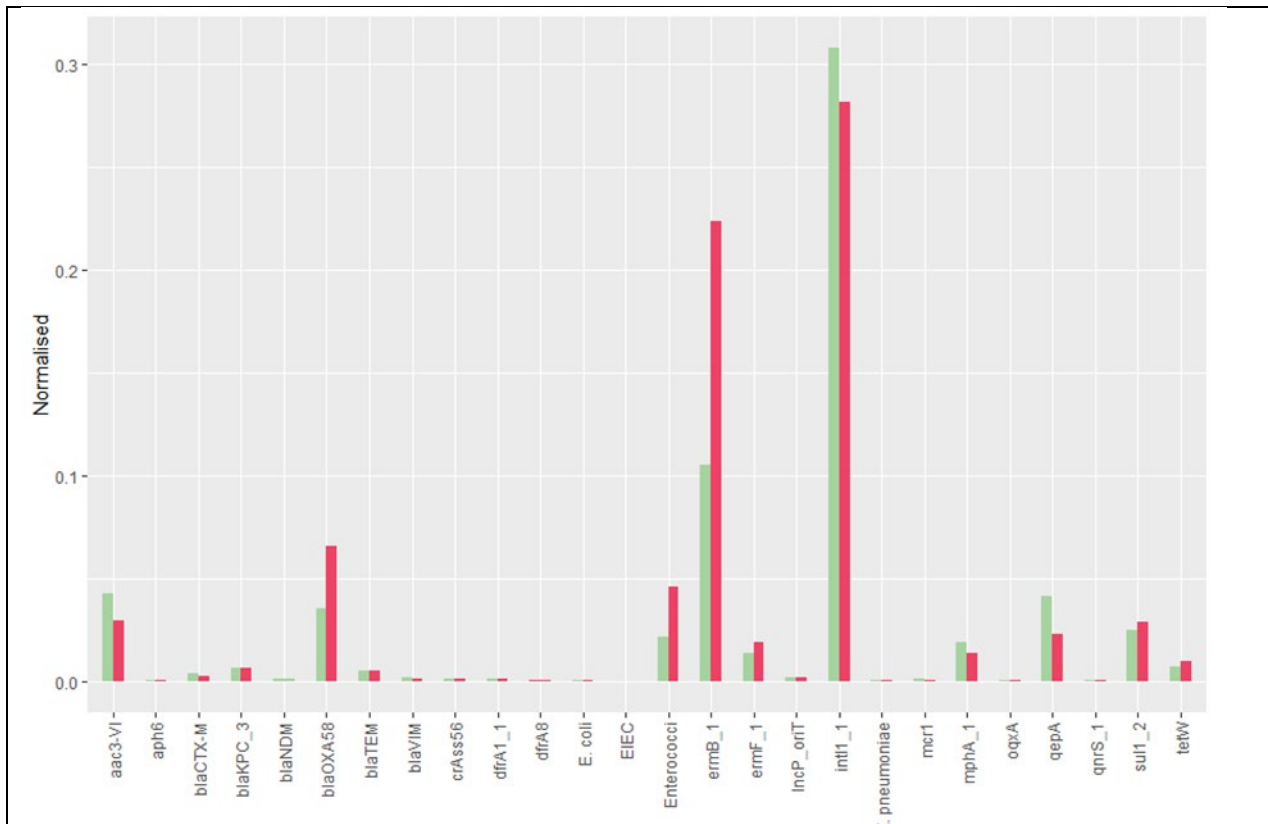


Figure 4: Quantification of ARBs and bacteria from the influent hospital wastewater from Herlev hospital. Green and red bar in the graph represent the duplicate quantification of ARBs and bacteria from influent wastewater.

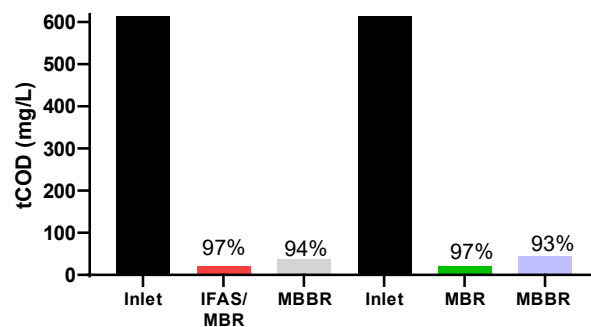


Figure 5: Total COD removal from IFAS-MBR tank, polishing MBBR tank, MBR tank and polishing MBBR tank from wastewater from Herlev hospital.

These pilot units will be moved to DTU to treat wastewater from fermentation based antibiotics production industry for Task 2.4.

WP3. Post-treatment

Objectives 1) Validate the PBBR for the minimization of the risk of Pathogens and ARM;

Tasks **T3.1.** Reactor tested and optimized at lab-scale with synthetic WW.

The more common approach in industry is to apply dissolved biocides (e.g., chlorinated products), a significant part of which is carried away in the outlet water. The PRESAGE alternative offers a new approach based on immobilizing biocides on small particles contained in a continuous flow bed reactor. For that purpose, it is needed to produce durable (micro)particles with stable biocidal activity along time.

The first step was to develop functionalized particles to select the ones with the best performance. Three different microparticle cores were used: hydroxyapatite (5 micron), calcium carbonate (5 micron) and glass beads (100 micron). The particles were functionalized with a chlorinated quaternary ammonium compound (BDMDAC- benzyldimethyldodecyl ammonium chloride), by using the Layer-by-Layer (LbL) method. The glass beads were first activated with a chlorine solution, and then went through a silanization process using APTMS ((3-Aminopropyl)trimethoxysilane). To increase the specific surface area of the glass beads, silica nanoparticles (functionalized with a biocide) were directly synthesized on the top of the glass beads. All the “biocidal”/functionalized particles were tested to determine: (a) the release of biocide along time; (b) their bacteria killing efficiency. No release of biocide was detected after two weeks in an agitated suspension. As regards the bactericidal activity, this was only detected when using the hydroxyapatite (Ha) and calcium carbonate (CaCO₃)-based particles (Figure 6): The concentration of biocide (200 mg/L in this example) is determined as the mass of biocide immobilized in the particles divided by the volume of the liquid. The value is not high considering that there is no release of the biocide from the particles to the water. In these lab tests synthetic water was used.

The hydroxyapatite functionalized particles were selected for the assays in the continuous bed reactor (PBBR) due to their stability and to the fact of being practically insoluble in water. Initially, the reactor was operated with the particles without biocide, with a flowing bacterial suspension (inlet: 10⁸ cells/mL) for 30 hours. The non-functionalized particles did not have any effect on bacterial survival. When using functionalized particles, two different conditions were tested: a fixed bed that managed to reduce bacterial concentration by 3 Log, and a fluidized bed where total elimination of culturable bacteria was achieved (Figure 6).

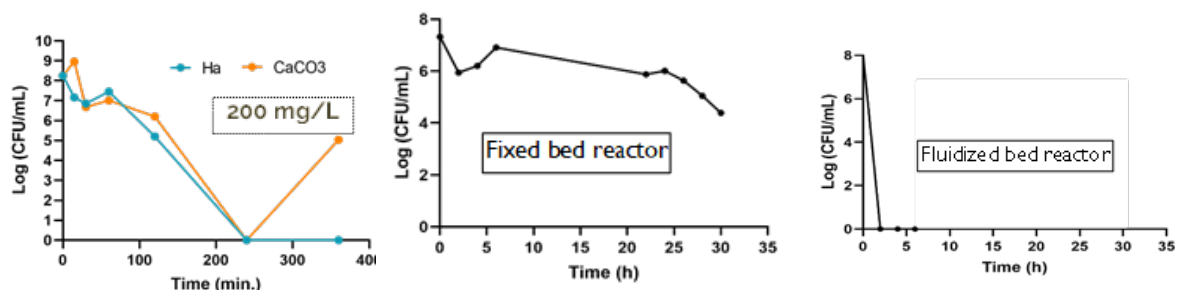


Figure 6: Elimination of bacteria attached in the different experiments.

The following steps of the work will be: (1) optimize the reactor in the lab, mainly for long duration operation; (2) design and build a pilot-plant version of the reactor to be tested in the Demosite 1. Finally, the effect of the functionalized particles on the AMR pattern will be assessed and compared with the free biocide effects.

WP4. OMPs fate & removal.

Objectives 1) Validate the analytical methodologies for measuring OMPs in the solid and liquid phases.

Task. T4.1 A list of representative OMPs was agreed by the partners.

A selection of relevant OMPs to be monitored in the different demosites was carried out in agreement with all the project partners. Three antibiotics present in the EU Watch list of 2020 were selected for monitoring: ciprofloxacin (CIP), sulfamethoxazole (SMX) and trimethoprim (TMP). Besides, other compounds are monitored in each demosite according to the characteristics of the matrixes.

Task. T4.2 The analysis of OMPs will be carried out onsite by each partner after an intercalibration of methods.

In order to compare the results among the different demosites, an intercalibration of methodology was defined (Deliverable 4.1). For the selected compounds, it consisted on the characterization of three different matrixes: WWTP influent, WWTP effluent and MilliQ water. The additions considered includes: blank, 2.5, 5 and 10 ng L⁻¹ of each selected antibiotic. After a prefiltration step (0.45 µm) and the addition of the standards, each demosite proceeded with its analytical methodology. The results are currently being compared in terms of % of recovery of the different compounds in each matrix.

WP5. ARMs/ARGs fate & transmission.

Objectives: 1) Validate the analytical methods for ARM identification and ARG quantification. 2) Analyze the prevalence of ARMs and ARGs and compare the results for each treatment strategy.

Task. T5.1 A list of representative ARMs (*E. coli*, *Enterococcus* sp., *Pseudomonas* sp. and *Klebsiella* sp.) and ARGs conferring resistance to β-lactams, macrolides, quinolones and sulphonamides will be agreed between partners. Additional microcosms will be performed and analyzed for the specific ARM/ARG.

A list of 36 genes that could give resistance to different families of antibiotics was selected based on i) their previously reported abundance in water bodies in Europe, ii) clinical relevance, iii) antibiotics chosen to be monitored by the participating demosites. Some genes that allowed the screening of selected ARM were also included (Deliverable 5.1b),

Based on the HT-PCR results and the risk categories proposed by Zhang *et al.* a subset of these genes was selected for further qPCR (Table 2).

Microcosm experiments have been initiated.

Task. T5.2 ARMs/ARGs analyses for the different demosites are centralized in WP5. The feed, sludge and effluents will be analyzed for ARMs and ARGs. The resistance profile will be ranked based on the results from task T5.1.

DNA extracted from initial samples from different matrixes (sludge, black water, grey water, reactor inlet, reactor outlet) from every demosite was screened using HT-PCR. Every gene was detected in the expected relative abundances according to previous reports. Once

selected the genes for qPCR, every sample was analysed using this technology. The higher sensitivity of this technique allowed us to detect higher relative abundances than the ones shown by HT-PCR, in spite of this, the observed proportions in the HT-PCR experiments were maintained. These results support the use of qPCR in the quantification of ARGs of interest in the following stages of the project.

Table 2: Left: Selected genes for HT-PCR screening. Right: Subset of genes selected for qPCR.

Gene	Family
16S rRNA	
<i>E. coli</i>	Taxonomic
EIEC	Taxonomic
<i>K. pneumoniae</i>	Taxonomic
Enterococci	Taxonomic
crAss56	Phage
IS26	MGE
IncP	MGE
<i>int11</i>	Integrans
<i>sul1</i>	Sulfonamide
<i>sul2</i>	Sulfonamide
<i>dfrA1</i>	Trimethoprim
<i>dfrA8</i>	Trimethoprim
<i>oqxA</i>	Quinolone
<i>qepA</i>	Quinolone
<i>qnrS</i>	Quinolone
<i>aph-(3'')-Ib</i>	Aminoglycoside
<i>aph-(6')</i>	Aminoglycoside
<i>aac-(6')-Ib</i>	Aminoglycoside
<i>aac-(3)-VI</i>	Aminoglycoside
<i>tetA</i>	Tetracycline
<i>tetW</i>	Tetracycline
<i>vanA</i>	Glycopeptides
<i>ermF</i>	MLS
<i>ermB</i>	MLS
<i>mphA</i>	MLS
<i>blaTEM</i>	Beta Lactam
<i>blaKPC-2</i>	Beta Lactam
<i>blaKPC-3</i>	Beta Lactam
<i>blaCTX-M</i>	Beta Lactam
<i>blaOXA-48</i>	Beta Lactam
<i>blaVIM</i>	Beta Lactam
<i>blaNDM</i>	Beta Lactam
<i>blaCMY-2</i>	Beta Lactam
<i>blaOXA-58</i>	Beta Lactam
<i>mcr1</i>	Colistin

Gene	Relevance
<i>int11</i>	Class 1 integron integrase
<i>sul1</i>	Sulphonamide resistance
<i>bla_{CTX-M}</i>	beta-lactam resistance
<i>ermB</i>	MLS resistance
<i>qnrS</i>	Quinolone diminished susceptibility
<i>mcr-1</i>	Colistin resistance
<i>ddl</i>	Enterococci marker

WP6. Emission of pathogens (bacteria and viruses).

Objectives: 1) Assessment of the load of pathogens in the WW. 2) Evaluate the efficacy of different treatment combinations for the elimination of Norovirus and Sapovirus.

Task. T6.1 The fate of representative opportunistic pathogens will be determined for each influent.

During the initial meetings of the project it was decided that the pathogens to be analyzed are *Klebsiella* and *Enterococcus*. We have prepared and circulated the SOP: Standardized protocol for detection of resistant *Klebsiella* and *Enterococcus* by culture-dependent methods (Deliverable 5.1a), and started to carry out experiments for the counting, detection, isolation and characterization of these two pathogens in the Spanish location. A total of 16 strains were isolated and subjected to sequencing of the whole genome. In parallel, the phenotypic characterization is being performed. Similar studies for bacterial pathogens will be applied to the other demosites.

Task. T6.2. Recovery and quantification of NoV GI, GII and SaV before and after different decentralized treatment combinations.

We have prepared a protocol for concentration and detection of enteric viruses from the samples. Samples from Spanish location were processed for virus concentration and RNA extraction. Recovery and quantification of NoV (GI, GII), SaV and HEV is in progress. Again, the standardized procedure for virus will be applied to the samples of other locations.

WP7. Ecotoxicity.

Objectives: 1) Evaluate the efficiency of the proposed treatments in comparison with conventional treatment plants; 2) Assess their long-term safety; 3) Understand the relationship between the chemical pressure and ARG dissemination.

Task. T7.1 Testing on standardised monospecific tests of treated effluent as described in WP1, WP2 and WP3.

Testing mixtures of antibiotics determined by the consortium at different concentrations and based on the EU watchlist has been realized. In accordance with international standard ISO 21427-1 guidelines, *Xenopus laevis* larvae have been exposed 12 days to 10 $\mu\text{g}\cdot\text{L}^{-1}$ of cocktail 1 (six antibiotics CPX, AZM, AMX, ERY, TMP, SMX) or cocktail 2 (three antibiotics CPX, TMP, SMX), and with or without biofilm (from WWTP outlet, or from FW). Morphometric parameters (length, growth rate), genotoxicity (micronucleus test), and oxidative stress biomarkers data have been collected in larvae exposed in different conditions compared to control. No morphometric parameters have been affected after 12 days exposure except for larvae exposed to cocktail 1 with both biofilms (WWTP, FW). No genotoxicity has been reported in larvae exposed to cocktails conditions except with cocktail 2 and WWTP biofilm (Figure 7). However, genotoxicity have been reported in larvae exposed to biofilms from WWTP and FW. Based on current data from this study, hypothesis can be drawn on ecotoxicity of biofilms but need further research. Oxidative stress biomarkers analysis is currently ongoing. Investigations of potential link between gut microbial communities and genotoxicity will be explored by further analysis of microbial diversity in gut larvae and biofilms. ARGs quantification are under analysis.

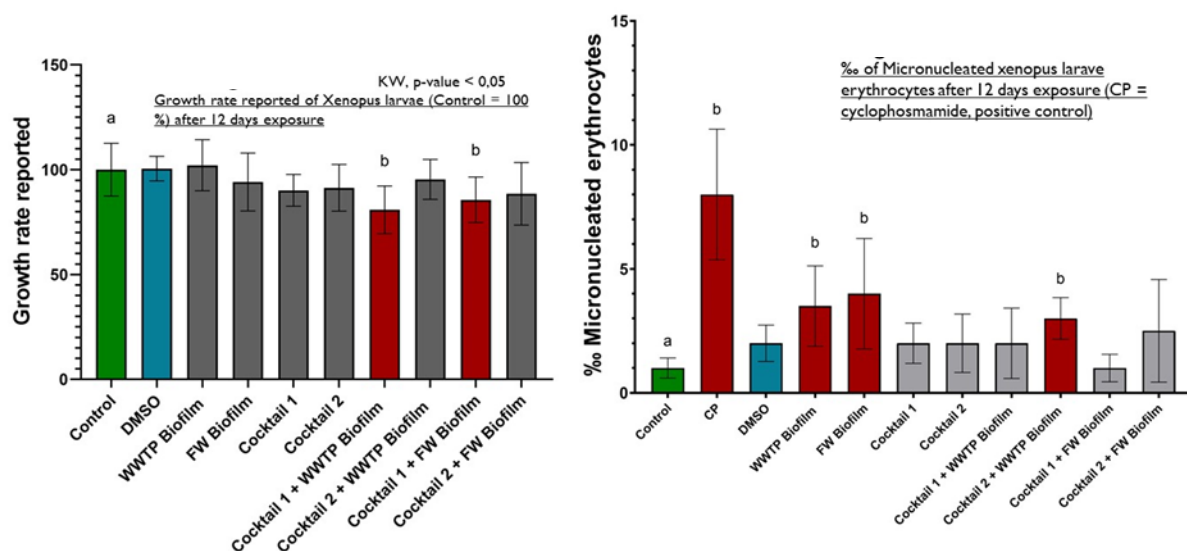


Figure 7. Growth rate and micronucleated erythrocytes.

b. Collaboration, coordination and mobility

PRESAGE is a project in which different disciplines are necessary: i) Environmental engineering for the development of the innovative technologies for wastewater treatment and disinfection in WP1, WP2 and WP3 (DTU, USC, USP and UP); ii) Microbiology to monitor ARMs/ARGs as well as pathogens during the treatment and its further emission to water bodies (TU Dresden, USC); iii) Ecotoxicity for the development of tests to evaluate the acute, chronic and genotoxicity of wastewater containing mixtures of CECs (INP Toulouse). As stated in section 2a, these tasks have been developed according to the project work plan.

The WPs comprise transversal monitoring tasks for ARM/ARG fate (WP5), emission of pathogens (WP6) and evaluation of ecotoxicity (WP7) that will be applied to wastewaters and sludges produced at the different demsites in WP1, 2 and 3. This implies the need of shipping wastewaters or sample extracts. To do so, the partners agreed during dedicated meetings the analytical protocols and needs for sample preservation and shipping. In fact, as stated in task 5.2 samples from USC and DTU have been shipped to TUD for the initial ARMs/ARGs screening. In the coming months, USC and DTU will send 35L of effluents from their demsites to INP Toulouse to evaluated ecotoxicity with real samples.

The consortium shares the target of reaching high quality effluents in terms of OMPs, ARM, pathogens and ecotoxicity during decentralized wastewater treatment. This implies the need to deeply understand the technological and microbiological factors involved in such treatments, as well as the cross-influence of one over the other. This would allow to answer questions as: Which design and/or operation parameters of wastewater treatment plants could be optimized to minimize the release of biological pollutants to the environment? How behaves ARM generation and transmission in bioreactors? What factors determine the global ecotoxicity of the effluents? The comprehensive understanding of this issues is only possible through a joint discussion and interpretation of project results, which is the main target of the regular project meetings held every 6 months.

In the proposal also inter-disciplinary exchanges of researchers have been foreseen in a mobility plan. The first one will take place in the coming months, with the PhD researcher from USP moving to DTU for 6 months in order to evaluate a different reactor configuration for hospital wastewater treatment (aerobic vs. anaerobic).

Members of the PRESAGE project have participated in all meeting organized within the Transfer Project. In addition, the recruited PhD and postdocs have joined the PhD forum to network also at an early-stage career level. Until know, first contacts and possible common methods have been identified to further explore during the project.

c. Impact and knowledge output

Several European initiatives aim at reaching good environmental status of aquatic ecosystems. In December 2019, the European Green Deal (COM(2019) 640) reinforced the need to protect the health and well-being of citizens from environment-related risks and impacts.

PRESAGE aims at understanding the integrated behaviour of selected contaminants of emerging concern (CECs) comprising organic micropollutants (OMPs), antibiotic resistant microorganisms/genes (ARMs/ARGs) and pathogens (including bacteria and viruses), during decentralized wastewater treatment (WWT) in different innovative treatment schemes. Separated domestic black and grey water (BW and GW, respectively) and hospital wastewater is currently being treated in WP1 and WP2, to assess the influence of operating parameters on the fate and behaviour of the target CECs. The ultimate aim is to find an optimal treatment strategy that provides a final effluent suitable for reuse, complying with safe chemical and biological water quality. With this aim, in WP3 water disinfection by means of a Particle Bed Biocidal Reactor (PBBR) is proposed.

Standardized protocols for selection, detection and monitoring of OMPs, ARGs and pathogens have been developed in WP4, WP5 and WP6, that once validated through samples analysed at the different demosites could be of high value to set new monitoring strategies in future policies.

The good status of aquatic environments must be approached synergistically with other important environmental strategies. In this sense, innovative solutions for WWT cannot be based on higher impacts on other environmental compartments. PRESAGE looks for sustainable technologies for CEC removal, including hybrid biological treatment processes in WP1 and WP2, and new reactor configurations that minimize biocide use/release for disinfection in WP3. The developed technologies could give raise to exploitable results for the wastewater treatment industry.

The project proposes optimal and competitive management strategies for hospital, pharmaceutical industry and domestic wastewater that reduce the risk of CECs spread in the urban aquatic environment. For this purpose, ecotoxicity tests are being developed in WP7. Currently, mixtures of antibiotics at different concentrations have been tested by standardized tests, being other mores specific assays planned for the second part of the project.

3. Table of Deliverables

Please indicate whether the planned deliverables are completed, delayed or readjusted. Explain any changes/difficulties encountered and solutions adopted. Please add/delete rows, as necessary in the table below.

Deliverable name	Lead partner (country)	Date of delivery (dd/mm/yyyy)	Status (completed/delayed/rescheduled)	Changes, difficulties encountered and new solutions adopted
WP1				
D1.1 Optimised AnMBR performance treating BW	USC	15/01/2023	Completed	
WP2				
D2.3 Report on the ASBR operation	USP	5/12/2022	Completed	
WP4				
D4.1 Analytical methods (OMPs)	USC	15/01/2023*	*Partially completed	Samples were sent for OMPs analysis, but results are pending
WP5				
D5.1a Standardized operation procedures – Culture-based methods (pathogens) D5.1b Standardized operation procedures – Culture independent methods (ARM/ARG)	TU Dresden	15/01/2023	Completed	Deliverable D5.1a was created by USC Microbiology group (WP6) Deliverable D5.1b was created by TUD group (WP5)

4. Budget and schedule review

The following table shows a budget breakdown on the concepts included in the proposal and an indication below on how the funding has been used so far.

Organisation	Personnel	Travel	Equipment	Subcontractts	Consumables/Other	Funding
USC	125,000	19,900	7,500	0	47,600	200,000
Used so far	15,267	769	0	0	2,399	18,435
USP	0	2,500	0	0	43,000	45,500
Used so far		0			12,200	12,200
DTU	171,494	4,000	20,127	0	10,857	297,328
Used so far	3,097	0	0	0	16	3,113
UP	43,704	12,000	0	0	24,200	99,880
Used so far	17,742	188	0	0	744	18,674
INP Toulouse	118,600	10,000	40,000	20,500	42,250	249,858
Used so far	53,658	2,726	0		6,015	62,398
TUD	161,012	4,733	4,844	7,140	30,896 (+41,725 Overhead)	250,350
Used so far	44,631.06	0	0	1,725	1,156.87	47,513

As can be observed, the budget used so far is quite low (average of 14%). This is specially relevant for USC and DTU, because around 60% of their budget was planned for contracting researchers that operate the pilot plants at the different demsites. However, during the first year of the project design and setting-up of such reactors was carried out by staff members at USC and DTU. In both cases, during the second half of the project recruited staff will be mainly in charge of the project execution and full budget will be used.

5. Consortium Meetings

Please list below the Consortium meetings which took place during the reporting period, by filling in the table below. Add/delete rows as necessary in the table below.

N°	Date	Location	Attending partners	Purpose/ main issues/main decisions?
1	30 November 2021	MS Teams	All	Kick-off meeting. - Brief presentation of participants. - Discuss Administrative issues (Consortium Agreement, Logo, Contact database, Deadlines, etc.) - Overview of project planning - Discuss on networking issues
2	26 January 2022	MS Teams	All	Decide how and when samples will be transported from the demosites to the laboratories carrying out analyses of emerging pollutants: TUD (ARG), USC (pathogens) and INP Toulouse (ecotoxicity). Decide on common protocols for DNA /RNA extraction and sample conditioning for pathogen analyses. Establish criteria for selecting representative antibiotics and ARMs/ARGs.
3	10 June 2022	School of Engineering, Santiago de Compostela (Spain) + MS Teams	All	Overview of the advances made in the different WPs.
4	12 December 2022	MS Teams	All	- Administrative check: state of deliverables and milestones; participation in TransNet project; reminder on correct acknowledgement procedures, etc. - Dissemination planning - Discuss on networking issues (protocols, sample shipping, mobility plan, etc.)

6. Stakeholder/Industry Engagement

Maximum 1 page

PRESAGE includes 4 associated partners, aware of the importance of actively involving industry for implementing the new solutions in the actual market. The industrial stakeholder engagement so far reached these partners, namely:

Kruger (Veolia), Denmark, will collaborate with DTU in the operation of demosites based on IFAS-MBR and the MBR-MBBR for hospital and antibiotic production industry effluents. They have supported the design of the pilot MBBR reactors. Moreover, Kruger support this project by finding the industry that will supply the antibiotics rich wastewater.

In Spain, Zona Franca de Vigo (ZFV) are the owners of the building in which demosite considered in WPI has been installed, providing their support for the auxiliary services needed to make the pilots operative. In addition, FCC Aqualia provided their anaerobic membrane pilot plant for the BW treatment. USC regularly discusses the results with them, to jointly agree on optimization strategies.

Adict Solutions, France, is a consulting company specialized in the development of collaborative decision support tools for the transfer of knowledge between researchers, industrial sectors, and managers in the field of water and the environment. Adict Solutions keeps updated on the project results in order to promote wastewater management methods through source separation.

7. List of Publications produced by the Project - Open Access

International	Peer-reviewed journals	<p>1. Barros A.C., Melo L.F. and Pereira A., A Multi-Purpose Approach to the Mechanisms of Action of Two Biocides (Benzalkonium Chloride and Dibromonitropropionamide): Discussion of <i>Pseudomonas fluorescens</i>' Viability and Death, <i>Frontiers in Microbiology</i>, Feb. 2022, Vol. 13, Article no. 842414. doi:10.3389/fmicb.2022.842414</p> <p>2. Barros, A.C., Melo, L.F., Pereira, A. <i>Pseudomonas fluorescens</i> Cells' Recovery after Exposure to BAC and DBNPA Biocides. <i>Antibiotics</i>, 2022, 11, 1042. https://doi.org/10.3390/antibiotics11081042</p>
	Books or chapters in books	
	Communications (presentations, posters)	<p>1. A. Thibodeau; F. Mouchet; M. Barret; X. Van Nguyen; L. Gauthier E. Pinelli. Ecotoxicity of combined exposure of antibiotics and biofilms to <i>Xenopus laevis</i> larvae SETAC, Dublin 2023.</p> <p>2. M. Rivadulla, A.X. Elena, S. Suarez, T.U. Berendonk, F. Omil and J. M. Garrido. Occurrence and fate of Organic Micropollutants and Antibiotic Resistance Genes during Separated Decentralised Treatment of Black Water and Grey Water. Submitted for oral presentation to EcoSTP Conference (Girona, Spain, June 2023)</p>
Dissemination initiatives	Popular articles	
	Popular conferences	
	Others	Introduction of PRESAGE project and the whole Aquatic Pollutants Call for publication in HEALTH EUROPE quarterly issue. (in progress)

8. Knowledge output transfer

For each of the Knowledge Output arising from the project so far, please complete the following table. (outputs with relevance for practitioners from the sectors mentioned in the table)

Short Title	Membrane-based treatment strategy for separated black and grey water
Knowledge Output Description	Black water will be first treated in a membrane anaerobic reactor. The effluent will be mixed with grey water for further treatment in a hybrid anoxic/aerobic MBR. The use of a mobile plastic support is considered in the case influent COD is too low for fast biomass growth. The presence of the ultrafiltration membrane will ensure the retention of biomass. The efficiency for removing OMPs, ARMs and pathogens will be optimized. The substitution of the mobile plastic support with Powdered Activated Carbon (PAC) in order to promote OMP removal through sorption and enhanced biotransformation will be assessed.
Knowledge Type	Prototype
Link to Knowledge Output	Once the treatment strategy is optimized and validated at pilot scale, the knowledge output will be made available to interested industry partners.
Sectors & Subsectors	<ul style="list-style-type: none"> • Emissions and Water Reuse • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...) • Others <ul style="list-style-type: none"> ○ Governance ○ Stakeholder Involvement
End User	<ul style="list-style-type: none"> • Environmental Managers & Monitoring • Industry • Policy Makers / Decision Makers • o Scientific Community
IPR	"n/a"
Policy-Relevance	Relevant to any initiative dealing minimization of water pollution (WFD, Zero-pollution, etc.)
Status	Experimental work is still in progress and performance of pilot units are being optimized.

Short Title	Anaerobic biofilm-based prototype for treatment of hospital wastewaters
Knowledge Output Description	<p>Anaerobic reactors have been extensively studied for domestic and industrial wastewater treatment world wide, mainly in countries with tropical and subtropical climates. In such reactors, microorganisms convert organic matter mainly into methane and carbon dioxide (biogas), thus mitigating the impact of the wastewater release in water bodies. Moreover, the generated biogas can be applied to produce heat and electricity. Compared to the traditional aerobic process, anaerobic biotechnology is less intensive in energy requirements, since aeration is not necessary, and does not generate a large amount of solid waste (excess sludge). So, this technology emerges as a sustainable alternative for wastewater treatment, mainly to remove organic matter, which can be converted into added-value products such as biogas, organic acids, and solvents. However, the potential of this technology to remove organic micropollutants (OMP) is still under scientific study. Considering the several microbial species involved in anaerobic digestion and their metabolic versatility, it is expected that anaerobic systems are able to remove a broad spectrum of OMPs. Nevertheless, there are no extensive results presented in the literature about the application of anaerobic reactors in the removal of micropollutants, mainly pharmaceuticals. So, this research focused on the constructive aspect of an anaerobic reactor and its influence on the removal of OMPs from hospital wastewater. The results demonstrated that reactors without mixing the liquid phase are the best choice in this case. This finding will help engineers to properly design full-scale reactors aiming at OMPs removal.</p>
Knowledge Type	Scientific publication
Link to Knowledge Output	The achieved data are being analysed and at least a scientific publication will be generated in a Journal in the area of Environmental Engineering.
Sectors & Subsectors	<ul style="list-style-type: none"> • Emissions and Water Reuse • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...)
End User	<ul style="list-style-type: none"> • Environmental Managers & Monitoring • Industry • Scientific Community
IPR	n/a
Policy-Relevance	Relevant to any initiative dealing minimization of water pollution (WFD, Zero-pollution, etc.)
Status	This study was performed in a lab-scale unit with lab-made wastewater. Although the results are promising, experiments with real (hospital) wastewater are required to validate the achieved results. Moreover, the scale-up of the anaerobic reactor must be carried out, at least on a pilot scale, to consolidate the knowledge.

Short Title	Membrane-based prototype for treatment of antibiotic rich wastewaters (hospitals, production industry)
Knowledge Output Description	The fate of ARMs and ARGs on two series of pilot units consisting IFAS-MBR and MBR will be reported. Moreover, removal of OMPs from two series of pilot units will be compared and reported.
Knowledge Type	Scientific publication
Link to Knowledge Output	Sufficient results and data are not available yet to compile in the <i>scientific publication</i> . Once the research articles are published it will be shared among partners.
Sectors & Subsectors	<ul style="list-style-type: none"> • Emissions and Water Reuse • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...) • Others <ul style="list-style-type: none"> ○ Governance ○ Consumer Health & Welfare ○ Socio-Economics ○ Stakeholder Involvement
End User	<ul style="list-style-type: none"> • Education & Training • Environmental Managers & Monitoring • Industry • Policy Makers / Decision Makers • Scientific Community • Civil Society
IPR	"n/a"
Policy-Relevance	Relevant to any initiative dealing minimization of water pollution (WFD, Zero-pollution, etc.)
Status	Experimental work is still in progress and performance of pilot units are being optimized. Once sufficient results and data are available those will be compiled in the <i>scientific publication (research articles) and reports</i> .

Short Title	Prototype for sustainable water disinfection
Knowledge Output Description	A continuous flow reactor for water disinfection is being developed, based on the antimicrobial activity of functionalized particles that carry a biocide. The main goals of this study are: (1) to inactivate microbes in the reactor without releasing the biocide from the particles to the flowing water; (2) to assess the effect of this disinfection system on the antimicrobial resistance of the microorganisms present in the water. The first goal has been already achieved at lab scale.
Knowledge Type	Scientific publication
Link to Knowledge Output	The outputs will be available in scientific publications.
Sectors & Subsectors	<ul style="list-style-type: none"> • Drinking Water • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...) • Consumer Health & Welfare
End User	<ul style="list-style-type: none"> • Environmental Managers & Monitoring • Industry • Scientific Community
IPR	n/a
Policy-Relevance	It is relevant to the circular use of safe water.
Status	Results are still being obtained to assess the long-term efficacy of the reactor system.

Short Title	Standardized operation procedure (SOP) and workflow for the detection and quantification of relevant ARGs
Knowledge Output Description	An SOP for the optimal extraction of DNA from influent, effluent and sludge samples was written. A list of relevant ARGs was developed and used for screening in every sample via HT-PCR. An in house script was produced to quality filter the results but also to calculate relative abundances. qPCR was used to make an absolute quantification of selected genes in every sample, allowing for the comparison of ARG removal efficiencies in each system.
Knowledge Type	RTD protocol/technical manual
Link to Knowledge Output	Deliverable 5.1 (will be made publicly available once validated experimentally)
Sectors & Subsectors	<ul style="list-style-type: none"> • Basin Management • Emissions and Water Reuse • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...) • Governance
End User	<ul style="list-style-type: none"> • Environmental Managers & Monitoring • Industry • Scientific Community
IPR	n/a
Policy-Relevance	WFD (monitoring methods for new priority pollutants)
Status	The protocol is finalised, but has still to be applied to samples generated in the project.

Short Title	Ecotoxicological assessment
Knowledge Output Description	<p>Our results show that the presence of biofilm modulates the toxicity of the tested antibiotics. The two antibiotic cocktails have no direct effect on genotoxicity, but it induces a growth inhibition only in the presence of biofilm from WWTP. These results must be completed by analyses of microbial diversity in the continuum biofilm-gut microbiota. The induction of antibiotic resistance genes will be carried out to better define the risks associated with the presence of these antibiotics in natural environments.</p> <p>Evaluation of the USC MBR effluent is currently ongoing.</p>
Knowledge Type	Scientific publication
Link to Knowledge Output	Will be made available once published
Sectors & Subsectors	<ul style="list-style-type: none"> • Emissions and Water Reuse • Adaptation to Global Change • One health approach (antimicrobials, multiresistance, biofilms, enzibiotics...) • Stakeholder Involvement
End User	<ul style="list-style-type: none"> • Education & Training • Environmental Managers & Monitoring • Industry • Policy Makers / Decision Makers • Scientific Community • Civil Society
IPR	n/a
Policy-Relevance	WFD (aquatic ecotoxicity tests to identify lists of priority substances)
Status	Currently on going

9. Open Data

In relation to Open Data, the funded projects will be requested to submit metadata on all the resources directly generated by the project, as well as additional information on how these data will be exploited, if and how data will be made accessible for verification and re-use, and how it will be curated and preserved. Metadata on all project publications and resources are required to be submitted as part of the final reporting. This will be done via the **Open Data & Open Access platform**, available at: <http://opendata.waterjpi.eu/> (also accessible from the bar menu of the Water JPI website).

10. Problems Encountered during project implementation

- Please indicate if any problems were encountered during the Project Implementation.
- Did any of the partners find difficulties related to the grant agreement, the availability of funds at national level or other similar issues not specifically related to the technical part of the project?

11. Suggestions for improvement regarding project implementation

ⁱ Zhang, AN., Gaston, J.M., Dai, C.L. *et al.* An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nat Commun* **12**, 4765 (2021). <https://doi.org/10.1038/s41467-021-25096-3>