FINAL REPORT

RADAR: *Selection and dissemination of antibiotic resistance genes from wastewater treatment plants into the aquatic environment and clinical reservoirs*

PROJECT PN-III-P4-ID-PCCF 2016-0114 CONTRACT NO. 10/2018

The consortium

- Coordinator (CO): University of Bucharest (UB) – 2.000.000 RON
- Partner 1 (P1): National Research and Development Institute for Industrial Ecology (NRDI-ECOIND) – 1.625.000 RON
- Partner 2 (P2): National Institute of Infectious Diseases "Prof.Dr. Matei Balş" - 1.625.000 RON
- Partner 3 (P3): University Politehnica of Bucharest (UPB) – 1.600.000 RON
- Partner 4 (P4): National Institute of Medical-Military Research Development "Cantacuzino" – 1.625.000 RON

107 team members (36 - CO, 10 - P1, 30 - P2, 17 - P3, 14 - P4)

Project summary

Wastewater treatment plants (WWTPs) represent one of the hotspots for the enrichment, recombination and selection of antibiotic resistant "super-bugs" which could eventually be discharged into downstream aquatic systems, impacting both the environment and the human health. The RADAR project aim was to assess the prevalence and dissemination of AMR from urban, clinical and industrial wastewater into the aquatic environment *via* WWTPs and to correlate these results with the environmental and clinical resistome, thus identifying the possible mechanisms of AMR emergence and spread.

Sampling campaigns were conducted seasonally, for two consecutive years from i) seven WWTP (influent, effluent and activated sludge sampling), ii) seven hospitals (influent and effluent sampling) and iii) environmental aquatic bodies (upstream and downstream WWTP river). The sampling sites covered all Romanian geographic regions. In addition, three anthropic and one natural lake were also sampled for water and fish collection. Clinical strains belonging to ESKAPE species have been collected in the same temporo-spatial sequence, from the hospitals releasing their wastewater in the sampled WWTPs.

All collected water and sludge samples were screened for heavy metals (detected by inductively coupled plasma – mass spectrometry, pesticides (analyzed by GC-MS) and antibiotic (determined by LC-MS analysis) detection. The heavy metals contamination from hospitals to the environmental aquatic bodies, *via* WWTP was relatively constant throughout the year and not high, the aquatic bodies showing a good to excellent quality. However, the values inside the WWTP, similarly to microbial load, were significantly higher compared to those determined upstream and downstream. Both organochlorine (OC) and organophosphorus (OP) pesticides residues were below the detection limit of the method. The following antibiotics were detected in the water and sludge samples: clindamycin, tetracycline, erythromycin, and vancomycin, but within the maximum residue limits (MRLs).

The standard microbiological analysis of the environmental samples has shown that the bacterial load of hospital wastewaters was comparable with that of the WWTP influents decreasing by 2-3 logs after mechanical and biological wastewater treatment. The ratio between total and faecal coliforms has significantly decreased in WWTP downstream compared to upstream suggesting that this parameter could be used for assessing the risk of discharging human pathogens, including antibiotic resistant bacteria (ARB) in the water bodies.

The distribution of ESKAPE pathogens in different samples and their seasonal and annual variation has been similar, irrespective of the studied geographic regions. The abundance of the ESKAPE pathogens was similar in the two consecutive years, 2019 and 2020. Despite the decrease of ESKAPE counts in the effluent as compared to influent, the antibiotic resistant bacteria (ARB) had generally a significantly higher abundance in downstream compared to upstream water samples, showing that the WWTP effluents contribute to the release of ARB into the environment. Also, the ARB abundance was higher in the aeration tanks compared to the WWTPs effluent suggesting that the activated sludge treatment is efficient in removing the ARB. The abundance of ESKAPE pathogens in the hospitals effluent and influent is similar suggesting that the pre-treatment of hospital effluents before being discharged in the urban WWTP is not efficient in the removal of ARB, this probably explaining the high abundance of clinically important resistance phenotypes in the WWTPs and then downstream.

The isolation and quantification of ESKAPE pathogens was performed using the membrane filtration method and cultivation on both non-selective, for the enumeration of total *E. coli* and on antibiotic supplemented media for the selection of strains harboring ESKAPE phenotypes, with 10,535 ESKAPE strains being recovered and confirmed by MALDI-ToF-MS. The MALDI-ToF-MS species confirmation of the targeted ESKAPE strains recovered from chromogenic media varied depending on the bacterial group, being 83.97-97.45% for *Enterobacteriaceae,* 15.28% for *Pseudomonas aeruginosa,* 92.99% for *Acinetobacter baumannii*, 98.93% for *Enterococus faecium* and 89.58% for *Staphylococcus aureus*. The antibiotic susceptibility profiles (ASP) of all isolated *Escherichia coli* and *Klebsiella pneumoniae* are relatively similar for different geographic regions, but different in case of non-fermenting Gram-negative bacilli (GNB). The strains recovered from non-selective media, reflecting the natural resistome, proved to be significantly more susceptible compared to those isolated on antibiotic-supplemented media.

Irrespective of the isolation medium, all Gram-negative strains seemed to preserve a good susceptibility to aminoglycosides and the severity of ASP is increasing in the order ESBL<OXA-48<MBL. These results are clearly demonstrating the epidemiological importance of carbapenemase-producing Gram-negative bacilli, and especially of *Enterobacteriacerae* and their dissemination from hospital to environment.

The methicillin resistant *S. aureus* (MRSA) exhibited high macrolide and resistance to tetracyclines, correlated with the high consumption of these antibiotics in Romania. The vancomycin resistant enterococci (VRE) strains exhibited high resistance rates to most of the tested antibiotics. Both MRSA and VRE strains preserved their susceptibility to linezolid.

The ASP of the strains isolated from upstream and downstream the WWTP have been similar or slightly increased for quinolones and tetracyclines in downstream strains, suggesting the persistence of ARB through the treatment steps inside the WWTP.

In order to establish a link among specific pollutants and the presence of particular ARBs, an upstream-water treatment plant-downstream transect was studied by multivariate analysis tools, which suggested that occurrence of ARB is correlated with the presence of certain metal pollutants, such as aluminum, mercury and iron. However, such interdependencies are site-specific.

The genetic analysis of the ESKAPE bacteria has revealed a great number of antibiotic resistance genes (ARG) reaching over 2500 per species, the highest diversity being recorded for *K. pneumoniae* strains.

The whole genome sequences (WGS) allowed to establish the most prevalent and widely distributed ARGs (e.g., *blaSHV, blaOXA, blaTEM* and *blaCTX-M* encoding for ESBL and *blaNDM-1, blaOXA-48* and *blaKPC* encoding for carbapenemases). A higher diversity of carbapenemases genes was seen in wastewater isolates. The highest number of virulence genes (75) was recorded for the influent samples.

For the ecotoxicological studies we evaluated the presence of several metals, OC and OP pesticides and antibiotics in *Carassus gibelio* collected from anthropic and natural lakes and correlated them with the generation of oxidative stress, DNA lesions and ARG in these samples. The metal, pesticides and antibiotic bioaccumulation was different depending on the analyzed tissue and sampling site, being higher in the anthropic lakes, specifically in those located in an agricultural area. The obtained results have shown the presence of Aldrin, Dieldrin even though they were banned decades ago.

Tetracycline was the only antibiotic exceeding the MRL in some fish samples, but no ARB/ARG was found in the respective fish. The oxidative stress was correlated with a high level of several heavy metals (Cr, Cu, Fe, Ni and Fe).

The WGS revealed the most prevalent ESKAPE species subtypes (STs) and their distribution. The analyzed strains belonged to high-risk clones, such as *K. pneumoniae* ST258 and ST101, *E. coli* ST167, ST10 and ST131, *E. cloacae* ST136, *A. baumannii* ST2, ST636, T578, ST620, ST858 and ST903, *P. aeruginosa* ST357 and ST233, *E. faecium* ST117 and ST80.

The WGS analysis allowed mapping the RGS in the clinical versus the aquatic environment for different geographic regions.

Some of the identified genes could represent valuable biomarkers for studying the aquatic resistome, while others could be used as markers for the efficiency of the WWTPs treatments in removing the ARGs.

The risk of AMR spread was evaluated depending on the frequency of mobile genetic elements (such as integrons, transposons and phages). The *A. baumannii,* followed by *E. coli* and *K. pneumoniae* strains were more frequently associated with integrons and transposons.

Following the phenotypic and WGS analyses, some hundreds of samples have been selected for 16S rRNA-based metagenomic analysis of microbial communities using the Ion Torrent Personal Genomic Machine. The results obtained until now show that the number of families, genera and species are slightly higher in the effluent as compared with the influent and the analyzed samples harbored with different relative abundances bacterial pathogens, incl. *Vibrio cholerae* non-O1, non-O139.The geo-climatic conditions influence the structure of the aquatic microbiota, as well as the reservoir of virulence and pathogenicity and the microbial communities of the WWTP effluent and influent, and respectively of the natural *versus* anthropic lakes have a clearly different metagenomic signature.

A standardized questionnaire was used for evaluating IPC measures in Romanian hospitals and we had the opportunity to examine the pre-COVID-19, pandemic IPC practices (2018), by comparison with the pandemic IPC practices (2020) and the current IPC practice (2022). The main findings have indicated certain gaps in IPC measures that should be prioritized for implementation (e.g., lack or incomplete IPC programmes, lack of guidelines for transmission-based precautions, outbreak management and preparedness, prevention of transmission of multidrug-resistant pathogens, waste management, antibiotic stewardship, personnel training is offered but not mandatory etc.).

Based on the gaps in IPC practices and realities identified above, we have developed a consensus process, involving key personnel from the entire hospital network involved in the current project, which have identified the most important unmet needs described above, that should be addressed and prioritized from implementation in order to improve IPC practices.

The main results of this project are: i) the database representative data for Romania allowing us to map the distribution of ESKAPE clones circulating in different geographic regions, to correlate them with other physico-chemical and microbiological parameters and thus to predict the emergence of epidemic clones, to propose new parameters for assessing the efficiency of WWTPs treatments for removal of ARB/ARG and to set up a solid reference for the implementation of the national action plan for AMR; ii) the collection of environmental, ARB, DNA samples bridging the pre- COVID-19 and pandemic period that could be further studied in future projects; iii) the optimized protocols for the detection of pesticides and antibiotics in different matrices, for the WGS investigation of the transmission of ESKAPE clones from hospitals to the environment (using this set-up, we have demonstrated the transmission of multidrug resistant *Klebsiella pneumoniae* ST101 clones from hospital to wastewater and their persistence after chlorine treatment), for the multivariate analysis of the interdependency among specific pollutants and the presence of particular ARB/ARG and for the metagenomic analysis of microbial communities changes induced by different anthropic activities that could be further correlated with the abundance of resistance and virulence; iv) last but not least, crystallization of a multidisciplinary team able to provide state-of-the art training, research, interventions, and data-gathering activities for different stakeholders involved in the mitigation of the AMR problem. The team published together 21 papers indexed in WOS (19 in Q1/Q2 journals, with a cumulative impact factor of >75), 5 papers indexed in IDB, 4 chapters in international databases and presented the results in 27 international (27) and national (12) conferences, 5 PhD theses, 6 MSc theses, 17 Bachelor theses.They organized one national workshop and one session during one international conference dedicated to RADAR, gave press interviews about the project and developed a certified laboratory for the detection of pesticides, heavy metals and antibiotics in different matrices, including food and waste, drinking and surface water samples.

We therefore consider that despite the huge burden posed by the COVID-19 pandemic, all the objectives were accomplished and all the expected results and deliverables have been achieved, although some of the activities are still ongoing.