Deliverable of WG1

Deliverable 2

List of the top 10 most prevalent and persistent, and the top 5 most hazardous ARB&ARGs in treated wastewater and surrounding environment, specifically focusing on antibiotic resistance genes associated with mobile genetic elements

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Contents

ACRONYMS

AR	Antibiotic Resistance
ARB	Antibiotic-Resistant Bacteria
ARB&ARGs	Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes
ARGs	Antibiotic Resistance Genes
qPCR	Quantitative real-time Polymerase Chain Reaction
MGEs	Mobile Genetic Elements
NGS	Next Generation Sequencing
TWW	Treated Wastewater
WWTPs	Wastewater Treatment Plants

1. Introduction

Antibiotic resistance (AR) is considered to be one of the most significant human health risks of the 21st century. Although this phenomenon is strongly associated with hospitals and other clinical environments, there is a growing realization that it is also linked to anthropogenic activities such as animal husbandry and wastewater treatment that disseminate antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) to natural environments, thereby contributing to the propagation of antibiotic resistance. The capacity of ARGs to be mobilized across phylogenetic and environmental barriers, thanks to their association with mobile genetic elements (MGEs), has led to their characterization as *"contaminants of emerging concern"*. This has facilitated a myriad of studies that have developed and applied culture-independent molecular techniques and especially quantitative real-time PCR (qPCR) to assess the diversity and relative abundance of ARGs in the environment at the molecular level. Although qPCR-based assessment of ARGs enables researchers to assess the abundance of ARGs on spatial and temporal levels or as a result of different treatments, a major drawback of the method is that: (i) due to differences in standard qPCR techniques applied, it is often not possible to compare results from different studies and therefore almost impossible to make conclusions regarding ARG dynamics of a more global level, and (ii) even after standardization of the methods, it still requires some apriorism regarding the gene to monitor.

The development of next generation sequencing (NGS)-based methods has revolutionized biology and enabled the field of metagenomics where whole microbial gene profiles can be acquired from natural environments without the need for culture-based methodologies, which do not allow screening of the vast majority of the non-cultivable bacteria (up to 99.9% in certain natural environments). These high throughput-screening molecular methods are increasingly applied to assess the diversity and distribution of bacterial populations and functional genes, including ARGs, in natural and engineered environments. Nonetheless, the large data sets generated in these analyses necessitate highly exhaustive bioinformatic tools in order to identify ARGs and their associated MGEs, and to compare their abundance and diversity in different samples.

The extensive quantities of data generated by metagenomic analyses of environmental microbiomes necessitate supporting databases that can synchronize data and specifically pinpoint relevant ARGs and MGEs in these environments. In the past years, several such metagenomic datasets have emerged, including ones that specifically target ARGs and MGEs in the environment. Nonetheless, discussion among WG1 members highlighted the fact that, generally, these databases remain vastly underexploited, and often they are threatened with closure at the end of a grant period due to lack of support funding after years of implementation (for instance the insertion sequence "IS finder" database and its "IS saga" metagenome exploring module). WG1 members underscored that fact that there are almost no funding programs that support utilization and maintenance of existing data/databases and therefore the data is inevitably lost. The exploitation of existing metagenomic data for the identification of relevant/abundant ARGs and associated MGEs in many different receiving environments is possible as far as the means are provided. In-depth analyses of specialized databases to pinpoint co-linkage between ARGs and MGEs would be a means to specifically address the question of the dissemination risk of specific ARGs and identify the mobile platforms associated with the dissemination of individual ARGs. Therefore, there is an urgent need for funding agencies to open the possibility of exploitation of the data that are continuously being accumulated.

While culture-based methods are limited because they target very specific microbial communities within complex environmental microbiomes, they provide several advantages compared to molecular-based methods when evaluating antibiotic resistance dynamics in WWTPs and downstream environments, and therefore should still be applied to complement molecular and metagenomics analyses. First, characterization of MGEs and associated ARGs in targeted strains can provide vital information on ARGs transfer dynamics within these taxa. Additionally, specific fecal bacterial taxa (*i.e. Enterobacteriaceae*, *Enterococcus*) are often strongly associated with AR in clinical settings, and theretofore the culture-based approach is often more beneficial to specifically focus on AR within specific groups of higher epidemiological potential. Furthermore, certain taxa such as *Burkholderia* species and *Enterobacteriales,* have hyper-potential for acquisition and dissemination of functional genes and therefore these taxa, which represent "hubs" within gene transfer networks, may be much more indicative regarding the mobilization of ARGs than other environmental taxa (Kloesges et al., 2011). Finally,

stakeholders (*i.e* WWTP laboratories) generally do not have the resources and facilities for conducting molecular and metagenomics analyses and therefore, if optimized, the targeting of specific AR fecal indicators can be optimized to allow these stakeholders to ascertain the scope of AR in WWTPs using available methods.

2. Objectives

The overall goal of **WG1** is to ***assess the epidemiological potential of ARGs released from WWTPs and propose conventional (culture-based or qPCR-based) and state-of-the-art molecular approaches that can be applied by research scientists and stakeholders to evaluate AR potential in effluents and effluent-impacted environments, including water bodies that receive effluent discharge and soils and crops that are irrigated with wastewater effluents.*** Four specific research objectives that support this goal are being pursued within the context of this WG:

(A) Propose standardized procedures used for ARB&ARGs detection and quantification in water and soil samples
(B) Identify the most prevalent and/or hazardous ARB&ARGs with ability to persist, spread and proliferate after wastewater disposal, including under wastewater reuse scenarios
(C) Assess the fate (whenever possible quantitatively) of ARB&ARGs discharged in treated wastewater and released in surface water or soils
(D) Identify the conditions favoring ARB&ARGs persistence or proliferation

These objectives will be fulfilled based on: (i) the input and experience of the WG members who are involved in several national and international project focusing on these questions; (ii) literature searches including reviews assembled by the NEREUS Blue Circle Society; and (iii) the joint activities of the WG1 discussions.

2.1 Deliverables

The following deliverables were defined for the second year:

D.1.1. *Protocol for assessing relative abundance of mobile ARGs in effluents and downstream environments based on experience being gathered through running project"*

D.1.2. *List of the top 10 most prevalent and persistent, and the top 5 most hazardous ARB&ARGs in treated wastewater and surrounding environment, specifically focusing on antibiotic resistance genes associated with mobile genetic elements*

D.1.3. *Guidelines for the analysis of treated wastewater planned to be reused*

D.1.4. *List of ARB&ARGs to be taken into account for the risk evaluation for wastewater reuse*

3. List of the top 10 most prevalent and persistent, and the top 5 most hazardous ARB&ARGs in treated wastewater and surrounding environment, specifically focusing on antibiotic resistance genes associated with mobile genetic elements

Based on WG1 discussions in the Patras meeting (March 22-23, 2017) the following terminology was defined for different classes of ARB and ARGs:

Prevalent: ARB&ARGs that are abundant in treated wastewater (TWW).

Persistent: ARB&ARGs that are not strongly affected by the treatment processes and persist in downstream (terrestrial and aquatic) environments.

Hazardous ARB: Antibiotic-resistant clinically profuse pathogens that persist in the wastewater treatment process and therefore can reach the receiving environment and potentially be disseminated to humans.

Hazardous ARGs: Genes that can be significant epidemiological burdens if they reach pathogens. Contrary to hazardous ARB, these ARGs may present an indirect risk, which is strongly correlated to their mode of dissemination. With this respect the association of these genes to MGEs may be considered due to the increased capacity to be transferred to pathogens. But inevitably, ARGs will be defined as hazardous for their potential to be associated with therapeutic difficulties once they reach specific bacterial pathogens for which no treatment is available.

An opinion survey was conducted among NEREUS members and affiliated researchers in order to define the most prevalent, persistent and hazardous ARGs based on metagenomics and qPCR data, or any other personal experience. The compiled results are summarized in **Table 1**. When considering the ARGs lists, there are two important

points that need to be emphasized: (i) The scope of genomic data for WWTPs and especially for downstream environments is very limited and therefore, this list is not a comprehensive representation of the global picture; and (ii) ARB&ARGs are highly dynamic and newly emerging pathogens, resistance genes and multidrug-resistant MGEs are frequently reported. Therefore, although the bacteria and genes in this list are a good representation of ARB&ARGs currently considered prevalent, persistent and hazardous in European effluents and downstream environments, it should be evaluated on a periodic basis to assess the relevance of each bacteria and gene. For this reason, we propose to keep the survey open on the NEREUS website, as well as the ResearchGate site of the COST Action in order to feed our perception of the problematic ARB&ARGs with more inputs and over time.

Table 1 - List of most prevalent, persistent and hazardous WWTP-associated ARGs in downstream environments (*according to the 14 answers received from the WG1 opinion survey*).

Prevalent ARGs (rank)		Persistent ARG (rank)		Hazardous ARGs (rank)	
$intl1$	(1)	$sul1$	(1)	bla_{KPC}	(1)
$qnrS$	(1)	$intl1$	(2)	$vanA$	(2)
$sul1$	(3)	$vanA$	(2)	bla_{NDM-1}	(3)
bla_{CTX-M}	(4)	$qnrS$	(2)	bla_{OXA}	(4)
bla_{TEM}	(5)	bla_{OXA}	(2)	bla_{CTX-M}	(5)
$vanA$	(6)	bla_{TEM}	(2)	$mcr1$	(5)
$qacE\Delta1$	(6)	bla_{CTX-M}	(2)		
bla_{OXA}	(6)	$sul2$	(8)		
		$tetM$	(8)		

3.1 Format of the WG1 opinion survey

List of relevant ARGs proposed (based on WG1 member experience) : *intl1, sul1, sul2, bla$_{CTX-M}$, bla$_{TEM}$, bla$_{NDM-1}$, bla$_{VIM}$, bla$_{KPC}$, qnrS, aac-(6´)-Ib-cr, vanA, mecA, ermB, ermF, tetM, aph, tetW, qacEΔ1, msr, mcr-1, mph, mef, bla$_{FOX}$, bla$_{OXA}$, bla$_{GES}$,* other.

Prevalent ARGs : Among the relevant genes listed below, what are the top 10 most prevalent genes in WWTP effluents: please use the field to rank the ARGs by adding numbers (1=highest) and provide your rational for choosing each ARGs, i.e. what kind of data was used for ranking these ARGs.

> **Persistent ARGs** Among the relevant genes listed below, what are the top 10 most persistent genes in WWTP effluents: please use the field to rank the ARGs by adding numbers (1=highest) and provide your rational for choosing each ARGs, i.e. what kind of data was used for ranking these ARGs.
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> **Hazardous ARGs** : Among the relevant genes listed below, what are the top 5 more hazardous genes in WWTP effluents: please use the field to rank the ARGs by adding numbers **(1=highest)** and provide your rational for choosing each ARGs, i.e. what kind of data was used for ranking these ARGs.

A list of the most hazardous ARB was compiled (**Table 2**) based on the amalgamation of (i) the virulence of a specific bacterial taxa, (ii) the capacity of that taxa to acquire antibiotic resistance genes and (iii) the prevalence of these resistant taxa in nosocomial- and community-associated disease. For these reasons carbapenemase resistant (bla_{kpc}) enterobacteria (including *Escherichia coli* and *Klebsiella pneumoniae*) and vancomycin resistant (*vanA*) enterococci were at the top of this priority list.

Table 2 - List of most hazardous ARB and associated ARGs.

Hazardous ARB	Prevalent associated ARGs
Escherichia coli	bla_{KPC}, bla_{NDM-1}, bla_{OXA}, bla_{CTX-M}, bla_{OXA-48}, mcr1
Klebsiella pneumoniae	
Enterobacter sp.	
Enterococcus faecalis	*vanA*
Enterococcus faecium	*vanA*
Staphylococcus aureus	*mecA*
Pseudomonas aeruginosa	bla_{CTX-M}, bla_{GES}, bla_{OXA}, bla_{SHV}
Aeromonas spp.	bla_{CTX-M}
Acinetobacter baumanii	bla_{TEM}

* The table was compiled following rigorous discussions among WG1 members, based on current clinical data (*i.e.* http://www.who.int/antimicrobial-resistance/en/).

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