nereus COST ACTION ES1403

Deliverable of WG1

Deliverable 4

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

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Contents

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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

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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.

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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

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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*"

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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 ARB&ARGs to be taken into account for the risk evaluation for wastewater reuse

For routine monitoring of ARB, the WG1 recommends using the cefotaxime-resistant fecal coliform (culture-based) protocol described in **Annex I** below. For more exhaustive analyses, targeting selected taxa from Table 1 is recommended.

Table 1 - 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/).

For routine monitoring of ARGs using quantitative PCR, WG1 recommends the current Norman gene list: *qnrS, vanA, bla_{CTX-M}, bla_{TEM}, sul1, intI1* (proxy), using the protocols elaborated in **Annex II**. As mentioned above, there is a lack of data regarding the scope of hazardous and persistent ARBs and ARGs in downstream environments and therefore this list may be modified over time. Currently the NEREUS Blue Circle Society is

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conducting a comprehensive literature search to pinpoint potentially relevant ARBs and ARGs and we hope this will enable amendment of this list next year.

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5. References

Kloesges, T., Popa, O., Martin, W., Dagan, T., 2010. Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths. *Molecular Biology and Evolution*, 28, 1057-1074.

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ANNEX I. Protocol for enumeration of cultivable total faecal coliforms *vs* cultivable cefotaxime resistant faecal coliforms

Culture media and reagents required:

- mFC agar (500 g; Difco ref. 267720)

- Rosolic acid (6 x 1 g; Difco; ref. 232281)

- NaOH (no specific reference needed)

- Cefotaxime (100 mg Cefotaxime sodium salt; Sigma; ref. C7039)

- Sterile saline solution (0.85% (w/v) NaCl).

- Sterile cellulose acetate syringe filters, 25 mm diameter, pore size 0.2 µm (no specific reference needed)

- Sterile nitrocellulose membrane filters with grid, 47 mm diameter, pore size 0.45 µm (no specific reference needed; suggestion Sartorius (ref. 11407--47-ACN))

Preparation of antibiotic stock solution:

Antibiotic	Antibiotic Solvent	Potency* (µg/mg)	Concentration of the stock solution (g/L)	Conc. in the culture medium (mg/L)	Volume of stock solution to add to 1 L of culture medium (mL)
Cefotaxime (CEF)	H_2O	964	5.0	4.0	0.8

* This value may be different if is used a different brand

Note: The stock antibiotic solution must be sterilized by filtration (0.2 µm porosity filters). **Prepare immediately before use.**

Culture media preparation

mFC agar will be used to enumerate the total faecal coliforms. The same medium supplemented with the antibiotic CEF (mFC+CEF) will be used to enumerate the cefotaxime resistant fecal coliforms (CEF).

The culture media (mFC and mFC+CEF) should be prepared 1 to 3 days before the first sampling day.

mFC must be prepared according to the manufacturer's instructions:

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Prepare a 1% (w/v) Rosolid Acid solution in 0.2 N NaOH. This solution is to be used fresh (**do not store**).

For 1 L of medium suspend 52 g of the powder in 1 L of distilled water in a Schott flask with a magnetic stirrer. Mix thoroughly. To dissolve the powder, introduce the closed flask inside a pan with boiling water. The level of hot water should be close to the level of the medium (be careful to avoid contact of the water with the flask cap). From time to time, remove the flask and agitate (flip flop, rotation, stirring). In this step, avoid formation of foam. When it is almost dissolved, boil for one minute to completely dissolve the powder. Add 10 mL of the 1% Rosolid Acid solution. Boil for one more minute. **DO NOT AUTOCLAVE.** Cool down the medium to < 50°C (in a water bath, or by stirring); avoid foam formation.

For mFC: Pour into 60mm Ø Petri plates when (Volume of mFC per 60 mm Ø petri plate is ~7 mL)

For MFC+CEF: When the temperature is below 50°C, add 1.6 mL of the antibiotic stock solution. Stir to homogenize. Pour into 60mm Ø Petri plates when (Volume of mFC per 60 mm Ø petri plate is ~7 mL)

Parameter	Culture media	Final Concentration of CEF (mg/L)	Volume of antibiotic stock solution
Total faecal coliforms	mFC	-	-
CEF resistant faecal coliforms	mFC+CEF	8	1.6 mL

The antibiotic stock solution must be added to mFC after cooling under agitation (temperature below 50°C).

Bacteria enumeration/isolation- preparation of dilutions and plating:

Suggested dilutions to enumerate total faecal coliforms (mFC). Note that these dilutions may need to be adjusted for each WWTP.

- for raw wastewater: prepare 1 mL of the dilutions ranging from 10^{-2} to 10^{-5};

- for treated/final wastewater: prepare 1 mL of non-diluted sample (10^{0}) and 1 mL of the dilutions ranging from 10^{-1} to 10^{-4};

Suggested dilutions to enumerate faecal coliforms resistant to CEF (mFC+CEF). Note that these dilutions may need to be adjusted to each WWTP:

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- for raw wastewater: prepare 1 mL of the dilutions ranging from 10^{-1} to 10^{-4};

- for treated/final wastewater: prepare 10 mL/ 1 mL of non-diluted sample (10^{1} and 10^{0}) and 1 mL of the dilutions ranging from 10^{-1} to 10^{-2};

Each sample should be serially diluted once. All the volumes (sample/dilutions) suggested above should be filtered in triplicate and plated as described below.

Prepare serial decimal dilutions in sterile saline solution (1 mL sample/previous dilution mixed with 9 mL of sterile 0.85% NaCl) – do not forget to vigorously vortex between each dilution and before the next step.

1. Filter triplicates for each sample/ dilution through the 0.45 µm membrane filter, using a vacuum filtration system; transfer the membrane filter onto the respective culture medium (grid upwards).
2. Incubate the plates at 37 °C for 24 h.
3. Enumerate the total number of colonies (grey, pink and the blue) and the blue colonies. Faecal coliforms form blue colonies. Grey and/or pink colonies usually show up, and may be not coliforms. The ratio between blue and total colonies will allow estimation of the selectivity of the media.

Sampling sites and procedure

Samples are to be collected on working days (avoid Mondays) once per month for three months;

Grab samples

- After the primary decantation: Raw Wastewater

- Final effluent as is it released to the environment (Secondary, tertiary or quaternary treatment)

Describe the treatment plant, according to the questionnaire bellow

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ANNEX II. qPCR protocols based on analysis of the NORMAN network WG5

Target gene	Primers	Primers sequence	qPCR product size	Conditions	Primers reference
16S rRNA	q_331F	TCCTACGGGAGGCAGCAGT	195 bp	95 °C - 10 min (1 cycle); 95 °C - 15 s, 60 °C - 1 min (45 cycles) Other: 1c	
	q_518R	ATTACCGCGGCTGCTGG			
bla$_{TEM}$	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (40 cycles) Other: 2a	Bibbal *et al*., 2007
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG			
bla$_{CTX-M-32}$	CTX-M32-Fw	CGTCACGCTGTTGTTAGGAA	156 bp	95 ºC - 7 min (1 cycle); 95 ºC - 10 seg, 63 ºC - 30 seg (40 cycles); 95 ºC - 15 seg (1 cycle) Other: 3b	Szczepanowski et al., 2009
	CTX-M32-Rv	CGCTCATCAGCACGATAAAG			
sul1	sul1-FW	CGCACCGGAAACATCGCTGCAC	162 bp	95 ºC - 5 min (1 cycle); 95 ºC - 10 seg, 60 ºC - 30 seg (35 cycles) Other: 4b	Pei *et al*., 2006
	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTG	118 bp		

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	qnrSrtR11	TGGCATTGTTGGAAACTT		95 ºC - 5 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 2d	Marti and Balcázar, 2013
intI1	intILC5_fw intILC1_rv	GATCGGTCGAATGCGTGT GCCTTGATGTTACCCGAGAG	196 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 1c	(Barraud et al., 2010)
*van*A	vanA3FP vanA3RP	CTGTGAGGTCGGTTGTGCG TTTGGTCCACCTCGCCA	65 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles) Other: 1c	Volkmann *et al*., 2004

For all protocols the constructed plasmid pNORM1 with sequences of all target genes was used. Conditions: 1) Power SYBR Green® PCR Master Mix; 2) SYBR® Select Master Mix; 3) DyNAmo ColorFlash SYBR Green Master Mix; 4) Fast SYBRTM Green Master Mix; a) 200 nM of primer; b) 300 nM of primer; c) 500 nM of primer; d) 600 nM of primer.

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