nereus COST ACTION ES1403

Deliverable of WG1

Deliverable 3

Guidelines for the analysis of treated wastewater planned to be reused

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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.

The extensive quantities of data generated by metagenomic analyses of environmental microbiomes necessitate supporting databases that can synchronize data and specifically

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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

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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*

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

Within the framework of the meeting held in Patras (March 22-23, 2017) members discussed dividing guidelines and recommendations for ARBs and ARGs in WWTP effluents into two categories: **(i)** ***recommendations for risk assessment*** and **(ii)** ***recommendations for monitoring ARB&ARGs***.

Recommendations for risk assessment relates to the analyses to carry out before reusing wastewater on a routine basis. Considering that (i) the persistence of ARB/ARGs during the wastewater treatment may vary from one situation to another (process, local parameters, origin of wastewater, etc.), (ii) the persistence of ARB/ARGs in the receiving environment may also vary according to usage (*i.e.* aqueous *vs.* terrestrial, climate, flow rates, local conditions, etc.), there is a need to conduct in-depth analyses to assess the fate of ARBs/ARGs, throughout the reuse process (treatment and reuse) on a case by case basis. This could involve applying exhaustive high-throughput metagenomics approaches, in addition to simpler qPCR and culture-based approaches, in order to identify and monitor the fate of potentially hazardous ARB/ARGs along effluent discharge pathways. To address these issues, protocols of interest and lists of ARB/ARGs to consider are being compiled. It should be noted that such in-depth analyses may self-amend the lists of ARBs/ARGS to monitor for routine analyses of the treated wastewater quality.

Recommendations for monitoring water quality and the receiving environment during water reuse consists of series of protocols relatively easy to implement, to monitor ARBs/ARGs in wastewater treatment effluents and the receiving environments once the wastewater reuses is applied. The idea will be to provide the means to guarantee an acceptable level of hazardous ARB/ARGs, or any accumulation of ARB/ARGs accumulation in the final receiving product/environment. This is not a substitute for carrying out analyses with classical indicators for water quality (e.g. fecal coliforms and other water quality microbial indicators). A list of specific ARB/ARGs to be taken into consideration for such analyses will be provided (D1.4). These specific ARB/ARGs (i)

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should be regarded as indicators (not necessarily hazardous for instance), (ii) they may evolve over time as the antibiotic resistance risk does, and (iii) may be amended by specific markers or bacterial species identified as particularly relevant in the context of the specific reuses considered during the preliminary risk assessment of the setting.

3.1. Approaches for risk assessment

Shotgun metagenomics provides a broad overview of ARGs and allows monitoring them at different stages of the reuse process and thereby pinpoint persistent ARGs. For example a recent study that applied shotgun metagenomics to assess ARGs at various phases of a Swedish WWTP (Bengtsson-Palme et al., 2016), found that the relative abundance of the bla_{OXA-48} gene was consistently enriched in surplus and digested sludge. The authors found this worrying because the carbapenem resistance gene bla_{OXA-48}, is still rare in Swedish clinical isolates, which suggests that it could potentially emerge with the risk of reaching a pathogen in the future.

Targeted metagenomics approaches. These methodologies specifically target known "generic" ARGs (amplified by PCR) that are subsequently sequenced to get additional information (sub-type/alleles). This approach was recently applied to assess the composition of integron-associated gene cassettes in WWTP effluent (Gatica et al., 2016). Applied to a genetic platform frequently involved in ARGs capture (integrons), it could be used to draw up an inventory of ARGs present in a given community.

Functional metagenomics. This approach consists of cloning genomic DNA extracted from complex environments (*i.e.* wastewater biosolids and soils), cloning them into large vectors (BACs, YACs) and subsequently transforming them into cultivable bacteria where the acquisition of resistances to antibiotics is then tested. This method is highly exhaustive, it provides phenotypic evidence that a gene is actually functional and therefore can be used to identify new ARGs (Udikovic-Kolic et al., 2014).

EpicPCR and inverse PCR. These are two PCR-based techniques that when coupled to each other, can aid in establishing correlation between ARGs, MGEs and specific bacterial phyla thereby providing important insight into the mobilization of specific ARGs in WWTPs and downstream environments. In inverse PCR (Pärnänen et al., 2016), DNA is reduced to ~5 kb fragments and ligated to create circular structures. Primers amplify selected target ARGs or MGEs from within the gene outwards thereby creating linear

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amplicons from the circular nucleotide sequences. Subsequently, high throughput sequencing platforms that generate long reads (i.e. PacBio) amplify the targeted ARGs as well as MGEs that flank them that are potentially involved in their transfer. In Epic-PCR (Spencer et al., 2015), single cell-containing droplets are created by emulsification, bacteria are lysed within the droplets and cell DNA is amplified with a primer targeting the 16S rRNA gene (determines phylogeny) and a functional gene (targeted ARGs) and subsequently sequenced to acquire a hybrid sequence comprised of the functional gene and the 16S rRNA gene fragments. In this way we can specifically determine which bacteria phyla harbor specific targeted ARGs

Culture-based bacterial isolation approach. Conventional bacterial isolation protocols can be coupled with resistance typing and whole genome sequencing. While this approach only targets a specific bacterial taxa, it provides an indicative overview of antibiotic resistance dynamics within or along a specific environment (Leonard et al., 2015). This is especially informative when targeting clinically associated taxa such as *Enterobacteriacae* and *Pseudomonas*.

Permissiveness to HGT. Recently a novel fluorescent-based approach was reported for estimating the permissiveness of bacterial communities to plasmid transfer. Here, a plasmid of interest is tagged by a *gfp* gene, itself under the control of a P*lac* promoter (that is not expressed in a *lacI*^{q} bearing bacteria). The plasmid is introduced in a donor bacterium that has been modified to contained both a *mcherry* gene (red fluorescence) and a *lacI*^{q} gene. Bacterial community are allowed to mat with the donor bacteria and transconjugant cells are isolated by FACS coupled with cell sorting based on their green fluorescence. 16s rDNA is further sequenced to determine the transconjugant species (Klümper et al., 2015).

3.2. Protocols for routine monitoring ARB&ARGs

Section D.1.3.1 provides a comprehensive array of tools that can significantly enhance the understanding of antibiotic gene dynamics in WWTPs and downstream environments. Nonetheless, these methods are generally too complex and exhaustive to apply on a routine basis, and therefore simpler easily-applicable protocols such as the ones outlined below are required.

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Culture-based approach for enumerating specific resistant bacterial species can specifically evaluate the antibiotic resistance phenotypes of select hazardous bacteria such as those listed in Table 1, or specifically focus on resistance to a specific resistance phenotype in an indicator bacterial strain. While the first approach is more informative, this approach can be highly exhaustive in routine monitoring schemes. An alternative to specifically targeting pathogens is to test the scope of resistance to a specific antibiotic, in indicator bacterial strains such as fecal coliforms or enterococci. Within the framework of WG1 we devised a simple protocol for assessing the relative abundance of cefotaxime-resistant fecal coliforms (Annex I). This protocol was disseminated and applied by 30 research laboratories in 24 countries to establish the "***global cefotaxime-resistant faecal coliform project***" (Figure 1; also see summary of Patras meeting below). Faecal coliforms resistant to cefotaxime (a clinically-relevant third-generation cephalosporin) are compared to total fecal coliform abundance to determine the absolute and relative abundance of cefotaxime-resistant faecal coliforms. To date samples from different WWTP zones and effluents were sampled in December 2016, January and February 2017 and another sampling period is planned for the summer of 2017. Collected data will be analyzed and correlated to various factors including WWTP characteristics, climatic conditions, geography and antibiotic use practices. We believe that this method can be very useful for assessing the resistome of WWTP effluents and downstream environments because it is simple and implements techniques that are already readily applied by routine monitoring laboratories.

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/).

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Figure 1 - Overview of countries participating in the global cefotaxime-resistant faecal coliform project.

qPCR-based protocols for quantifying molecular indicators/genes. This approach may be more difficult to implement in some routine monitoring laboratories and may be difficult to combine with threshold values for bacterial indicators proposed by legislation. Although this approach does not exactly refer to the same risk as culture-based approaches (persistence of resistance genes in the environment without considering the host, pathogen or not), it can provide a wider and eventually more sensitive measurement of AR occurrence. It can be proposed as collaborative work with academic/research laboratories, or can be offered as a service. Currently, we recommend application of the **NORMAN Protocols** for quantifying the following ARGs: *qnrS, vanA,* $bla_{CTX\text{-}M}$, bla_{TEM}, *sul1, intI1* in wastewater effluents. These are comprehensively elaborated in Annex II below. Due to a lack of knowledge regarding the persistence of specific WWTP effluent-associated ARGs in downstream (terrestrial and aquatic) environments, we currently recommend using the same set of protocols for these environments. The protocols have already been successfully implemented for downstream sediments in river environments. In water samples this may require optimization through filtering larger quantities of water

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prior to DNA extraction, and for soil/rhizosphere samples, the application of soil DNA extraction kits.

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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:

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

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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:

- 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};

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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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