Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes

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A R T I C L E I N F O

Keywords:
Inter-laboratory calibration
Quantitative PCR
Wastewater
Antibiotic resistance gene

A B S T R A C T

Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are widely distributed in the environment where they represent potential public health threats. Quantitative PCR (qPCR) is a suitable approach to detect and quantify ARGs in environmental samples. However, the comparison of gene quantification data between different laboratories is challenging since the data are predominantly obtained under non-harmonized conditions, using different qPCR protocols. This study aimed at carrying out an inter-laboratory calibration in order to assess the variability inherent to the qPCR procedures for quantification of ARGs. With this aim, samples of treated wastewater collected in three different countries were analysed based on common DNA extract pools and identical protocols as well as distinct equipment, reagents batches, and operators. The genes analysed were the 16S rRNA, *vanA*, bla_{TEM}, *qnrS*, *sul1*, $bla_{CTXM\text{-}32}$ and *intI*1 and the artificial pNORM1 plasmid containing fragments from the seven targeted genes was used as a reference. The 16S rRNA gene was the most abundant, in all the analysed samples, followed by *intI*1, *sul1, qnrS,* and bla_{TEM}, while $bla_{CTXM\text{-}32}$ and *vanA* were below the limit of quantification in most or all the samples. For the genes 16S rRNA, *sul1*, *intI1*, bla_{TEM} and *qnrS* the inter-laboratory variation was below 28% (3–8%, 6–18%, 8–21%, 10–24%, 15–28%, respectively). While it may be difficult to fully harmonize qPCR protocols due to equipment, reagents and operator variations, the inter-laboratory calibration is an adequate and necessary step to increase the reliability of comparative data on ARGs abundance in different environmental compartments and/or geographic regions.

1. Introduction

Antibiotic resistant bacteria and antibiotic resistance genes (ARB & ARGs) are recognized environmental contaminants that can pose serious risks to human health as they reduce antibiotics therapeutic potential [1–4]. Among other sources, urban wastewater treatment plants (UWTPs) are important reservoirs of ARB and ARGs in the environment [1,3]. High quality treated wastewater is crucial for ensuring adequate protection of the environment and human health. In a world oriented to a circular economic logic and threatened by water scarcity, water reuse, either for crop irrigation or potable uses, is a central issue in modern societies. However, such a practice may increase the risks of transmission of ARB & ARGs from UWTPs to humans, via the food chain [5]. Currently, there is no legislation concerning the loads of ARB or ARGs that can be discharged into the environment from the final effluent of an UWTP [1,6]. However, it is becoming consensual that this mode of environmental contamination must be monitored in order to generate a body of information that can support the establishment of legislative policies [6]. A worldwide overview of the levels of ARGs discharged by different UWTPs is only possible if quantifications made in different

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countries, analysed in different laboratories, are comparable. For such a purpose, a method that can achieve high specificity, based on targeted detection, rendering reliable and reproducible quantifications and therefore quantifiable ranges of variation, is the best choice. Real-time quantitative PCR (qPCR) is currently the best candidate for such an application. Indeed, qPCR has gained popularity over the last decade to quantify ARGs in microbial communities, based on the interpolation of a standard curve for the same gene [7]. However, gene quantification from environmental samples may be affected by several factors, hindering the comparability of results [7,8]. One of the most important biases is due to DNA extraction processes, an issue largely discussed in the recent literature [9] and, hence, not addressed in this study where all analyses to be compared used a commercially available DNA extraction kit. Other potential factors influencing gene quantification by qPCR may be due to the presence of impurities such as humic acids, heavy metals, detergents and organic salts, which may inhibit DNA amplification [10]. In addition, qPCR analysis can also be influenced by the operator, batch of reagents used, and/or the sensitivity of the analytical equipment. In this study, the degree of variation in qPCR quantification of selected genes of interest as a function of reagents batch, operator and/or equipment was assessed. This procedure aimed essentially at assessing whether the quantification performed for a specific ARG made in different labs can be indeed comparable and if affirmative if it is possible to determine the degree of variation. To the best of our knowledge, this is the first report on an inter-laboratory calibration of qPCR-based quantification of ARGs. Methods to normalize bias due to DNA extraction is beyond the scope of this study and will be addressed elsewhere.

2. Materials and methods

2.1. DNA extractions

Samples of secondary treated wastewater effluents were collected in urban wastewater treatment plants (UWTPs) in Cyprus, Portugal, and Germany, named as CYP-Cyprus, PT-Portugal, and DD-Germany, respectively. The secondary treatment used in the UWTPs from Portugal and Germany was activated sludge, whereas in Cyprus, the treatment was performed by a membrane bioreactor. None of the UWTPs used disinfection treatments. Volumes of 150–500 mL of effluent samples were filtered through polycarbonate membranes (0.22 µm porosity, Whatman, UK) and the total community DNA was extracted using the PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA), according to the manufacturer's indication. For each sample, at least four DNA extractions were performed, each resulting in 100 µL extracts that were pooled together to reach a final concentration of at least 3.8 ng/µL of DNA. Each DNA extract and final DNA pools concentrations were measured using Qubit (Thermo Fisher Scientific, USA) and the quantification values were used by all groups in all calculations. The volume of pooled DNA extracts was divided into five aliquots and distributed by the five testing laboratories. Thus, the five laboratories tested the same extracts and, consequently, used the same DNA concentrations for the gene copies number determination. The testing laboratories designated as UCP (Universidade Católica Portuguesa), LCPME (Laboratory of Physical Chemistry and Microbiology for the Environment), TUD (Environmental Sciences Technische Universität Dresden), KIT (Karlsruhe Institute of Technology) and ARO (Agricultural Research Organization). DNA extract aliquots were shipped refrigerated at 4 °C, with ice packs in Styrofoam boxes, to each laboratory where they were immediately stored at −20 °C until further analyses.

2.2. Quantitative PCR

The genes 16S rRNA, class 1 integron integrase *intI*1 and genes encoding resistance to the antibiotic classes sulfonamides (*sul1*), quinolones (*qnrS*), β-lactams (bla_{TEM} and $bla_{CTXM-32}$) and glycopeptides (*vanA*) were quantified based on qPCR. These genes were selected based on their wide distribution in wastewater [1], in spite of the different abundance expected, varying from high to intermediate for 16S rRNA, *intI1*, *sul1*, bla_{TEM} and *qnrS* or to very low for *vanA* and $bla_{CTXM-32}$. These genes were analysed using common qPCR protocols, listed in Table S1. The quantification was performed in duplicate for each sample (DD1-3, CYP A1-A5 and PT1-3), using the Standard Curve method as described in Brankatschk et al. [11].

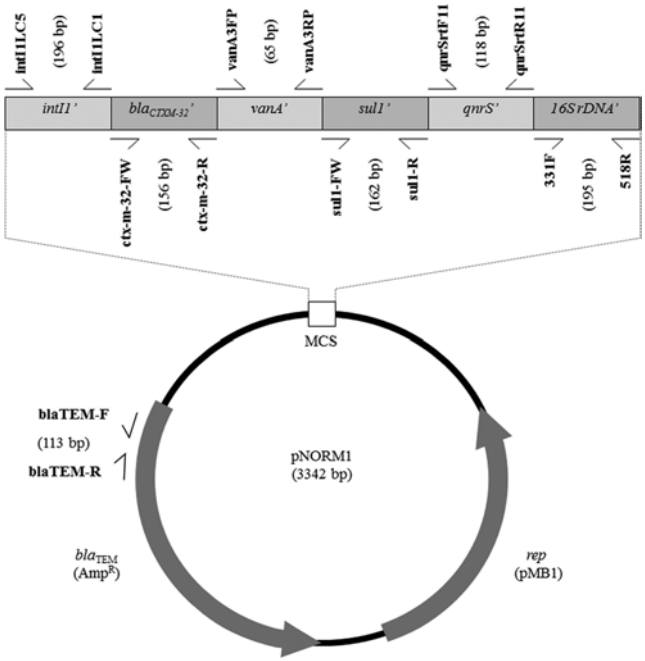

Fig. 1. Organization of plasmid pNORM1. The linearized plasmid DNA was used as a standard for all the gene quantifications. The different building blocks used for the seven target genes quantifications are represented with their cognate specific primers (small arrows).

Plasmid pNORM1 linearized by *Bam*H1 was used as a standard for all the qPCR primer sets applied in this study. This plasmid is a standard pEX-A vector containing a synthetic sequence (Eurofins, France) combining fragments that cover the seven target genes (Fig. 1). Possible qPCR inhibition was assessed by quantifying target genes using 10- and 100-fold diluted samples, as suggested by Bustin et al. [12]. For the genes *vanA* and $bla_{CTXM-32}$ inhibition was tested also by spiking a known amount of those genes in DNA extracts where they were below the quantification limit.

Different qPCR thermocyclers were used: StepOnePlus™ (Life Technologies, Carlsbad, USA), in UCP, LCPME, and ARO; Mastercycler® ep realplex (Eppendorf, Hamburg, Germany), in TUD and CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-RAD, Munich, Germany) in KIT.

In order to assess variations due to the use of different master mixes, additional 16S rRNA and sul1 genes quantifications were made, using the conditions indicated in Table S1. The 16S rRNA gene was tested with Maxima SYBR Green (Thermo Fisher Scientific, Austin, USA), besides Power SYBR Green (Thermo Fisher Scientific, Austin, USA), and the *sul1* gene was tested with SYBR Select (Thermo Fisher Scientific, Austin, USA) besides the Fast SYBR Green (Thermo Fisher Scientific, Austin, USA).

2.3. Criteria established for data analysis

The 16S rRNA gene, *sul1*, *intI*1, *qnrS*, bla_{TEM}, *vanA,* and $bla_{CTX-M-32}$ were quantified in each of the testing laboratories using distinct aliquots from the same DNA extract, using common qPCR protocols and

reference DNA (pNORM1). The qPCR results were analysed based on uniform criteria used in all laboratories: standard curve efficiency between 90 and 110%, correct T_m value, and unique melting peak. Amplifications in which the melting curves presented shoulders (increased signal in the baseline, e.g. due to primer dimers), multiple melting peaks (additional unspecific amplification) or incorrect melting temperatures (> ± 1 °C from standards) were not considered. It was considered in each case that any gene amplification product that was below the limit of quantification (LOQ) in any of the respective laboratories, would not be considered for the analysis.

2.4. Data analyses

Quantitative PCR data were expressed as the ratio of the gene copy number *per* ng of DNA. The% coefficient of variation (cv) between laboratories' analysis was calculated for each sample and for each gene as: % cv = (standard deviation of A/average of A) × 100. Where A means log (copy number of gene 1 measured in each lab per ng of DNA). The% deviation was calculated as being: % deviation = (A − average of A/average of A) × 100.

3. Results and discussion

3.1. Inter-laboratory quantification of genes

Possible qPCR inhibition was assessed by quantifying target genes using 10 – and 100-fold diluted samples. Inhibition was not observed, thus the results here presented are the results corresponding to 10-fold diluted samples, since using 100-fold diluted samples the quantification of some of the tested genes was below the LOQ.

In general, the abundance of the analysed genes per ng of DNA could be ranked as 16S rRNA gene > *intI*1 > *sul1* > *qnrS* > bla_{TEM} (Fig. 2A–E). For the 16S rRNA gene, the quantification measured at the ARO and KIT laboratories was, in general, lower than in the other laboratories (~0.4 log units) for all samples, while TUD showed higher gene copy numbers than the other laboratories (~0.4 log units), mainly for PT wastewater samples. In the case of *intI*1 gene quantifications, ARO showed lower gene copy numbers than the other partners (~0.6 log units) and TUD often quantified this target with the highest copy numbers (~0.4 log units), except for PT wastewater samples. For *sul1*, gene quantification in the KIT laboratory was lower than in the other groups (> 0.5 log units), who obtained similar results for most of the samples. For the *qnrS* gene, LCPME, UCP, and ARO obtained often similar results among them and higher than the results obtained by KIT and lower than the results from TUD group. For bla_{TEM} gene, the groups UCP and ARO presented similar gene quantifications, differing from TUD laboratory, who quantified the highest values of bla_{TEM} gene copy numbers in all samples (~0.3 log units), and from LCPME and KIT, who quantified the lowest values (~0.4 log units).

The $bla_{CTX\text{-}M\text{-}32}$ and *vanA* genes abundance were close to or below the LOQ in almost all the analysed samples, even in non-diluted DNA extracts. It was confirmed that this situation was not due to inhibition since in samples spiked with those genes the quantification led to the expected values, corresponding to the spiked amount. Gene quantification in CYP samples was often below the limit of quantification, except for the 16S rRNA gene, *sul1,* and *intI*1, possibly because these samples had lower DNA concentration (3.8-4.5 ng/μL) than DD and PT samples (5.5–75 ng/μL) and due to the fact that the other genes analysed are less abundant compared with 16S rRNA gene, *sul1,* and *intI*1. The lowest quantifications for the genes *qnrS* and bla_{TEM} were 1.74 and 0.79 log-units of gene copy number/ng DNA, respectively, suggesting that CYP samples may have a lower abundance of these genes.

Overall, gene quantifications produced higher values in TUD laboratory than in the other groups. In contrast, the results of the KIT group were most of the times lower than those obtained by the other groups. Since the observed quantification discrepancies could not be attributed to DNA extraction or qPCR protocol differences, variations were most probably due to the different qPCR equipment used. Indeed, TUD used a Mastercycler® ep realplex and KIT used a CFX96 Touch™ Deep Well Real-Time PCR Detection System, different from the real-time PCR equipment used at UCP, ARO, and LCPME (StepOnePlus™). Since they share similar equipment, the variation of genes quantification between UCP, ARO, and LCPME might be due to operator

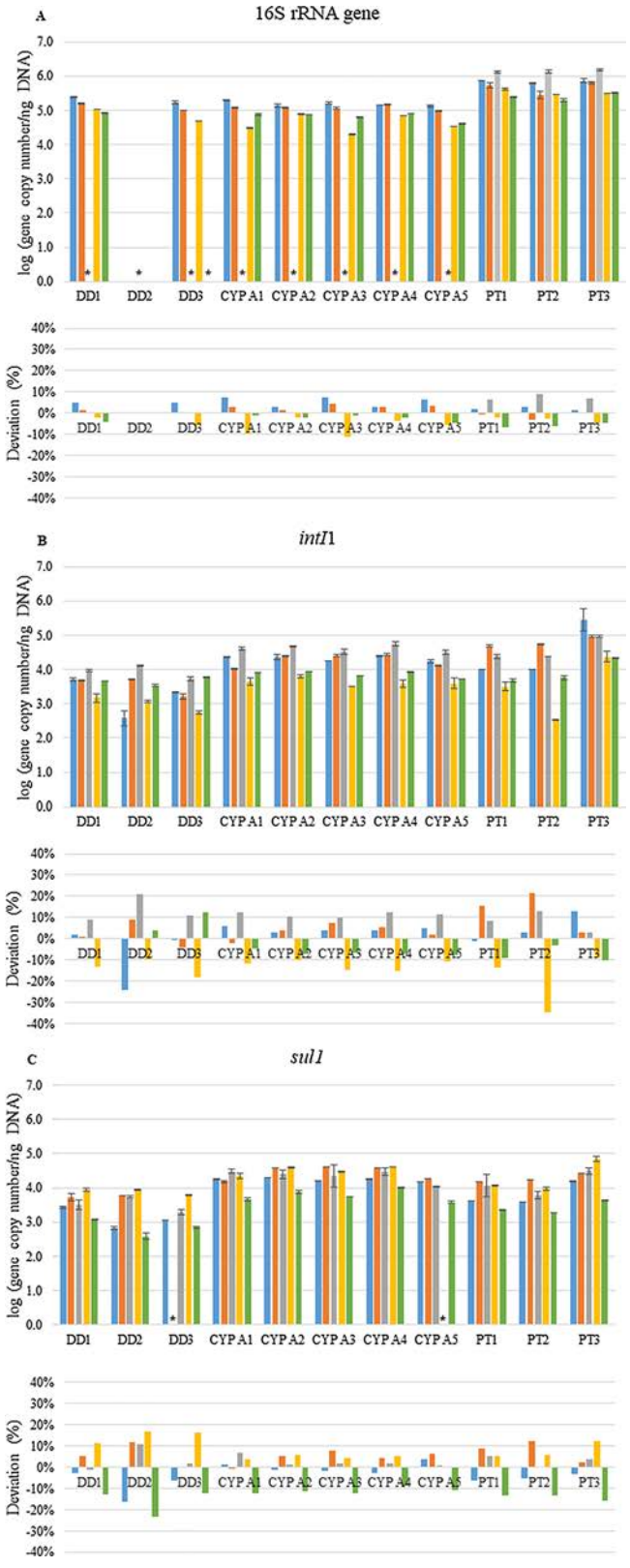

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Fig. 2. Comparison of the gene quantification (gene copies/ng DNA) in different wastewater samples from Dresden (DD1, DD2 and DD3), Cyprus (CYP A1, CYP A2, CYP A3, CYP A4 and CYP A5) and Portugal (PT1, PT2 and PT3) samples analysed in different laboratories – UCP (Universidade Católica Portuguesa), LCPME (Laboratory of Physical Chemistry and Microbiology for the Environment), TUD (Environmental Sciences Technische Universität Dresden), KIT (Karlsruhe Institute of Technology) and ARO (Agricultural Research Organization). Data correspond to average values for the genes quantification for 16S rRNA (A); integrase (B), sulfonamides resistance (C), quinolones resistance (D) and β-lactams resistance (E) and the respective deviations associated. * indicates that sample has gene amplification at a different melting temperature from standards and # indicates that samples gene quantification was below the limit of quantification. Error bars represent standard deviation from two qPCR repeats.

conditions, pNORM1 standard DNA extraction efficiency or qPCR equipment sensitivity.

3.2. Variation of inter-laboratory genes quantification

Assessing the variability of the qPCR quantification between different laboratories is important to compare quantitative data obtained for different treatment processes obtained in different studies. Globally, the deviations associated with gene quantification by different laboratories could be ranked as *qnrS* > bla_{TEM} > *intI*1 > *sul1* > 16S rRNA gene (Fig. 2). The quantification of the *qnrS* gene showed the highest percentage of deviation, in general, higher than the other genes

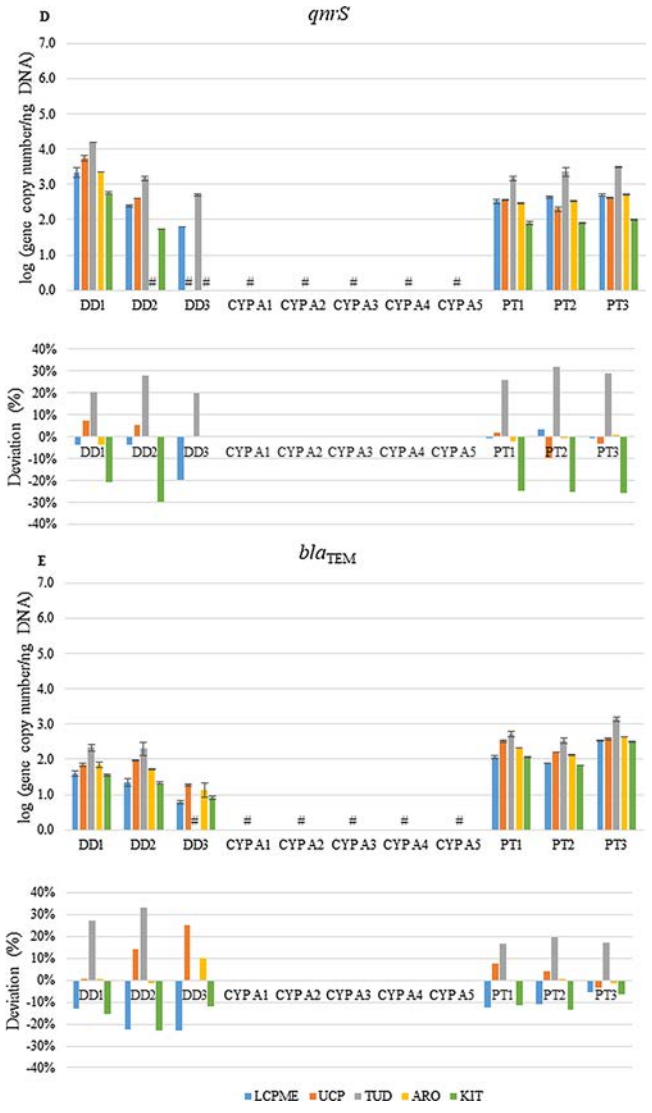

Fig. 2. (*continued*)

Table 1
Coefficient of variation in inter-laboratory comparison of gene quantification in samples from Dresden (DD1, DD2, and DD3), Cyprus (CYP A1, CYP A2, CYP A3, CYP A4 and CYP A5) and Portugal (PT1, PT2, and PT3) samples. * indicates that sample has gene amplification at a different melting temperature from standards, # indicates that samples gene quantification was below the limit of quantification.

	16S rRNA gene	bla_{TEM}	*sul1*	*intI*1	*qnrS*
DD1	4%	17%	9%	8%	15%
DD2	*	24%	18%	17%	24%
DD3	5%	21%	12%	13%	28%
CYP A1	7%	#	7%	9%	#
CYP A2	3%	#	7%	8%	#
CYP A3	8%	#	8%	10%	#
CYP A4	3%	#	6%	11%	#
CYP A5	6%	#	8%	9%	#
PT1	5%	12%	9%	12%	18%
PT2	6%	13%	10%	21%	21%
PT3	5%	10%	10%	10%	19%
Range	3–8%	10–24%	6–18%	8–21%	15–28%

(> 15%), while the 16S rRNA gene quantification presented the lowest range of deviation (< 8%). The percentage of deviation for *sul1* quantification was mostly below 10%, although it was higher for two DD samples (Table 1). For bla_{TEM} and *intI1* the deviation ranged between 10%–24% and 8%–21%, respectively (Table 1). Overall, quantifications made by different partners were reproducible and inter-laboratory variation was lower than 28% (Table 1).

Considering that TUD and KIT laboratories frequently overestimated and underestimated genes quantification, respectively, the variation of genes quantification obtained was assessed only in the other laboratories. The sharpest decrease in the variation of gene quantification was observed for *qnrS* where variation was < 6%. The variation of quantification of the genes 16S rRNA, *sul1* and bla_{TEM} were not as variable as for the genes *qnrS* and *intI*1, after removing the results from TUD and KIT. The variation of *intI1* quantification was lower than 15%, except for PT2 samples, for which ARO group obtained a substantially different result compared with the other groups, increasing the variation up to 30%. Nevertheless, the overall variation of genes quantification among the groups was below 30%. These results suggest that the variation of genes quantification between different laboratories is higher for less abundant genes than for more abundant ones, even if the same qPCR equipment is used. This may be related to the sensitivity of qPCR equipment. Thus, it is suggested that qPCR equipment may represent an important factor that influences the quantification of genes.

3.3. Comparison of qPCR master mixes

Since the master mix composition is another factor that might influence gene quantification, the 16S rRNA and *sul1* genes were analysed using two master mixes and the same equipment, thermocycler protocol, primer concentration and operator. The qPCR experiment efficiency obtained using Power SYBR Green and Maxima SYBR Green for 16S rRNA gene quantification were 95% and 101%, respectively. For *sul1* gene quantification, the efficiencies were 56% and 95%, using SYBR Select and Fast SYBR Green, respectively. The quantification of the 16S rRNA gene using two different master mixes showed that the variation between both quantifications was < 2% (Fig. 3). For the gene *sul1,* despite the low efficiency observed with the SYBR Select master mix, the variation of quantifications due to the master mix change was < 5.4% (Fig. 3). While the choice of the master mix may be critical to achieve adequate quantifications, the results obtained suggest that once specific amplification is assured (e.g., without primer dimerization or unspecific primer binding), the quantification may be reliable, irrespective of the master mix used.

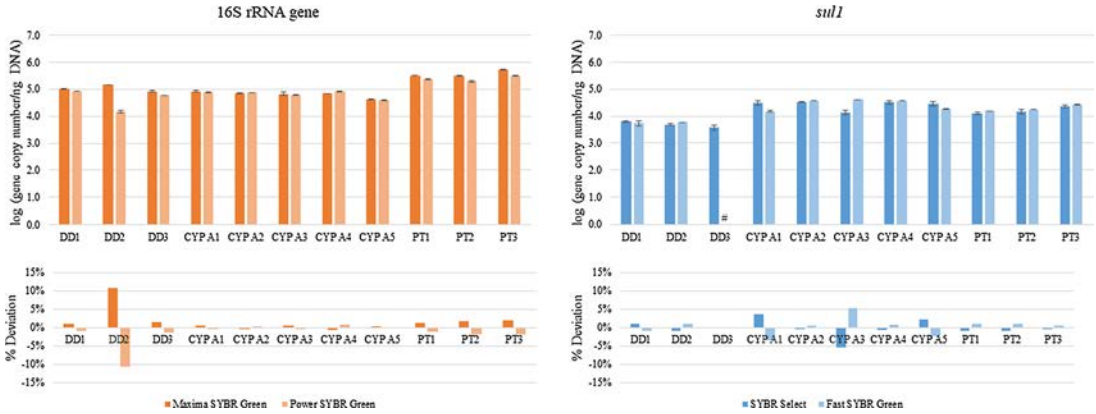

Fig. 3. Master mix comparison. A) Quantification results obtained for the gene 16S rRNA using the master mixes Power SYBR Green and Maxima SYBR Green and B) Quantification results obtained for the *sul1* gene using the SYBR Select and Fast SYBR Green master mixes. # indicates that samples gene quantification was below the limit of quantification. Deviation bars represent standard deviation from 3 qPCR repeats.

4. Conclusions

Harmonized protocols and inter-laboratory calibration are crucial for reliable quantification of ARGs in the environment. The coefficient of variation was determined to be < 28% for all laboratories involved. Although significant differences could be observed in the quantification of a single gene from one laboratory to another it should be mentioned that intra-laboratory variations (standard deviation) were kept at a minimum, therefore suggesting adequate practice in each lab. Still, such inter-laboratory variations, as presented in this study, should be noted when comparing absolute quantification values obtained in a different context by different laboratories, using different machines. The qPCR equipment played an important role in the quantification of genes, being eventually more determinant than the type of master mix used. Besides the equipment-derived bias, the nature of the environmental DNA samples may also be an influencing factor due to the composition of the natural bacterial communities and other matrix effects.

An important note refers to the establishment of the following criteria for qPCR interpretation: (A) amplicons of individual qPCR reactions using a given pair of primers and master mix should display a characteristic melting temperature (± 1 °C); (B) qPCR reactions forming shoulders and multiple melting temperatures peaks may give an overestimation of the gene amplification and must be avoided; (C) the concentration of DNA in an extract is critical; if it is too high, it may cause an underestimation and if it is too low it may hamper reliable quantifications. Thus, our data suggest the concentration should be between 0.39 and 7.2 ng/μL, in the reaction tube. Nevertheless, for less abundant genes higher DNA concentrations should be considered. In samples with low biomass loads or in which stressed cells may be resilient to lysis, such as those resulting from harsh wastewater disinfection processes, some problems may arise related with the limited efficiency of DNA extraction. In this situation, the limited DNA extraction may lower the concentration of DNA extracts obtained. Finally, the quantification of abundant genes in diluted and non-diluted samples is advisable, to assess potential inhibitor effect.

Funding

This work was supported by Working Group 5: Wastewater Reuse and Contaminants of Emerging Concern from Norman activities (http://www.norman-network.net/?q=node/106). UCP was supported by the Portuguese National Funds from FCT – Fundação para a Ciência e a Tecnologia STARE – "Stopping Antibiotic Resistance Evolution" (WaterJPI/0001/2013). Jaqueline Rocha was supported by the International PhD Programme in Biotechnology – BIOTECH. DOC (NORTE-08-5369-FSE-000007). LCPME was supported by the QualiOrne project funded by the French "Agence de l'Eau Rhin-Meuse". ARO was partially supported by the Israel Ministry of Agriculture Chief Scientist (grant # 821-0000-13). Roberto Marano is part of the ANSWER project that has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No 675530.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors wish to warmly acknowledge the support of NORMAN network, in particular, Valeria Dulio and Jaroslav Slobodnik. The authors would like to acknowledge the COST Action ES1403 NEREUS "New and emerging challenges and opportunities in wastewater reuse", supported by COST (European Cooperation in Science and Technology) for enabling the collaboration among the authors of the paper.

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