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Cell-based internal standard for qPCR determinations of antibiotic resistance indicators in environmental water samples

Jaqueline Rocha, Célia M. Manaia*

Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho 1327, 4169-005 Porto, Portugal

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ABSTRACT

Quantitative PCR (qPCR) has been used to quantify antibiotic resistance genes (ARGs) in water, wastewater, soil, sediment and tissue samples. Concerns regarding the comparability of data obtained in different laboratories has been a major bottleneck to incentivize the compilation of publicly available of ARGs quantifications gathered from different reports. In this study, the influence of the DNA extraction kits (NZY Tissue gDNA Isolation kit or DNeasy PowerWater kit) and of the operator on the DNA extraction yield and on qPCR genes quantification was assessed. Since in wastewater and water samples the matrix effect can affect the DNA recovery and, therefore, gene quantification, an internal standard, consisting in a cloned gene not found in environmental samples, was tested. The aim was to assess how qPCR determinations in wastewater and water samples can be affected by the matrix effect. The results show that the DNA extraction operator did not significantly influence DNA yield. The use of distinct kits resulted in qPCR gene quantifications that did not differ in more than 1 log-unit mL^{-1}. The matrix effect, assessed based on the use of an internal standard, was associated with an underestimation that ranged 0.1–0.9 log gene copy number mL^{-1} of sample, irrespective of the water type.

The reliability on the use of a DNA extraction kit that costs about 3 times less than the most commonly used can be an incentive for the use of DNA based analyses of ARGs in environmental waters. Moreover, the fact that both the DNA extraction operator and the reduced matrix effect have little influence on the final results, are good news, encouraging the compilation of data produced in distinct laboratories. Nevertheless, harmonization efforts are still necessary to minimize bias that may be due associated with other conditions, such as equipment.

1. Introduction

The environmental contamination with antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) is widely recognized and known to assume concerning proportions in aquatic environments (Allen et al., 2010; Fatta-Kassinos et al., 2011; Kolpin et al., 2002; Kümmerer, 2009; Martinez, 2009). This situation represents a public health issue, requiring urgent measures that allow the combination of monitoring efforts and implementation of control processes (Berendonk et al., 2015; Manaia et al., 2016). The monitoring of ARB and their ARGs in the environment can be performed based on culture-dependent or culture-independent methods (Manaia et al., 2018, Manaia et al., 2016). While culture-dependent methods have the advantage of being comparable when involve the use of routine and directive-oriented procedures (ISO 7899; ISO 9308; Drinking water directive, (98/83/EC)), they have the limitations that are not designed for ARB enumeration and leave non-culturable bacteria aside the analyses. Therefore, culture-independent methods are considered an essential complement or alternative to assess the quality and safety of water environments in terms of antibiotic resistance occurrence (Manaia et al., 2018, 2016; Vartoukian et al., 2010). Quantitative PCR (qPCR) has been, in this aspect, one of the methods of choice (Kim et al., 2013; Klein, 2002; Valasek, 2005). As for other quantitative methods, an adequate implementation of qPCR involves the use of identical conditions in all assays, an objective that may be difficult to reach, given the diversity of operators, samples to analyse, reagents used, among other.

Abbreviations: ARGs, antibiotic resistance genes; ASLP, activated sludge from a municipal wastewater treatment plant; *EcmolA* + , *Escherichia coli* JM109 cloned with the plasmid containing the internal standard (*molA*); HE, hospital effluent; NZY, NZY Tissue gDNA Isolation kit; PW, DNeasy PowerWater kit; qPCR, quantitative PCR; RWWA, influent wastewater from an airport wastewater treatment plant; RWWP, influent wastewater from a municipal wastewater treatment plant; sTWWA, secondary treatment effluent from an airport wastewater treatment plant; sTWWP, secondary treatment effluent from a municipal wastewater treatment plant; UP, ultrapure water

* Corresponding author.
E-mail address: cmanaia@porto.ucp.pt (C.M. Manaia).

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In a previous study using common DNA extracts in a ring test involving five laboratories, Rocha et al. (2018) demonstrated that ARGs quantification in wastewater samples varied up to 28%, which could be attributed to a combination of the real-time thermal cycler, the respective operator or the reagents batch. In the current study, we aimed at assessing other potential influential variables. For example, the DNA extraction method, which can influence the yield or the occurrence of potential qPCR interfering agents (e.g. humic acids, heavy metals, phenolic compounds) (Foerstner et al., 2005; Venter et al., 2004), may have implications on the accurate DNA amplification and gene quantification (Bessetti, 2007). These and other potential biases may hamper the comparison of data worldwide or even in the same laboratory at different time scales (Manaia et al., 2016; Smith and Osborn, 2009).

Studies comparing the effect of filtering membrane (Djurhuus et al., 2017; Hinlo et al., 2017); sample preservation (Hinlo et al., 2017; Li et al., 2017); DNA extraction methods (Djurhuus et al., 2017; Hinlo et al., 2017; Li et al., 2017); among others, have contributed to better understand the influence of water sample processing on the final results. Nevertheless, the sample processing and qPCR protocols used vary considerably worldwide (Rocha et al., 2018). The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin, 2010; Bustin et al., 2009), aim at improving qPCR reliability, reproducibility and comparability of data. However, MIQE guidelines are focused on qPCR analysis and not on sample processing. In this context, this work was designed to assess the influence of DNA extraction kits and operators on DNA yield and on gene qPCR quantification and also to quantify the bias imposed by the matrix effect of the water sample, by using an internal standard. To our knowledge this is the first study that reports the use of a bacterial cell-based internal standard for the determination of the matrix effect of water samples on DNA extraction and qPCR determination. The evaluation of the usefulness of the internal standard to monitor ARGs in water and wastewater samples was motivated by the internationally claimed need of surveillance of antibiotic resistance to be implemented at critical control points, which include wastewater treatment plants, untreated wastewater sources (hospital effluents, areas of poor sanitation infrastructure), and water bodies impacted by these sources (Berendonk et al. 2015; Manaia et al., 2016; Huijbers et al., 2019). The results of this study aim at contributing to increase the body of knowledge that may facilitate and support the comparison of data obtained under distinct conditions in different laboratories or time scales.

2. Material and methods

2.1. Samples

Seven types of water and wastewater samples were tested in this study (Table S1). Samples were collected from the influent and secondary wastewater treatment effluent (RWWA and sTWWA) from an airport wastewater treatment plant and from the influent, secondary wastewater treatment effluent and activated sludge samples (RWWP, sTWWP and ASLP) from a municipal a wastewater treatment plant. Both wastewater treatment plants operated with primary and conventional activated sludge secondary treatments. Other samples comprised the hospital effluent and river water. All samples were collected in the Northern region of Portugal. The samples were collected in sterilized bottles, transported to the lab in refrigerated conditions and immediately processed.

2.2. DNA extraction

Total DNA was extracted from sample volumes varying from 25 mL to 100 mL, processed in triplicate. Samples were filtered through polycarbonate membranes (0.22 μm porosity, Whatman, UK) and stored at −80 °C until DNA extraction. For DNA kit extraction comparison were used the NZY Tissue gDNA Isolation kit (Nzytech, Lisbon, Portugal) and the DNeasy PowerWater kit (QIAGEN, Hilden, Germany) that at the time of the work performance was known as PowerWater DNA Isolation Kit and was commercialized by MO BIO Laboratories Inc., CA, USA. For simplicity, from this point forward, the most recent designation will be as synonymous of the previous. The approximate costs per reaction of the DNeasy PowerWater kit and the NZY Tissue gDNA Isolation kit, were 9 € and 3 €, respectively. For this comparison, four environmental and four ultrapure water-internal standard spiked samples, extracted by two different operators, were analysed. To assess the matrix effect, using the internal standard described below, a total of 14 environmental and 14 spiked ultrapure water were extracted using the DNeasy PowerWater kit) (Table S1). Both kits were used according to manufacturer instructions with the following exceptions: in the DNeasy PowerWater kit the lysis period of time was increased from 5 min (as recommended) to 15 min; an extra centrifugation of 30 s was performed before DNA elution, and DNA was eluted twice with 50 μL of elution buffer warmed at 55 °C, to increase DNA recovery; in the NZY Tissue gDNA Isolation kit, before DNA extraction procedure the membranes were inserted into a 2 mL tube to which were added 360 μL of NT1 buffer and 50 μL of proteinase K, the volume of these two reagents was increased to ensure that the polycarbonate membrane used to concentrate the biomass in the water sample was covered by the lysis solutions, and DNA was also eluted twice with 50 μL of elution buffer warmed at 55 °C. The concentration of the DNA extracts was determined using Qubit (Thermo Fisher Scientific, USA). DNA extracts were preserved at −20 °C until their use for quantitative PCR analyses.

2.3. Genes quantification using qPCR

The quantitative PCR assays targeting five chromosomal/housekeeping genes (16S rRNA, *marA*, *rpoB*, *uidA* and *ecf)*, eight ARGs ($bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$, bla_{SHV}, bla_{TEM}, bla_{IMP}, bla_{VIM}, *qnrS*, *sul1*), one gene encoding the class 1 integrons integrase related with horizontal gene transfer (*intI*1) and one internal standard (*molA*) gene used the primers and conditions listed in Table S2. The chromosomal/housekeeping genes were selected as a measurement of total bacteria (16S rRNA); *Enterobacteriaceae* (*marA*), *Escherichia coli* (*uidA*); *Pseudomonas aeruginosa* (*ecf*); and *Acinetobacter* spp. (*rpoB*) due to their association with water environments, including wastewater, and with humans (Atrouni et al., 2016; Castiglioni et al., 2008; Jang et al., 2017; Mena and Gerba, 2009). The ARGs encoding resistance to the β-lactams ($bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$, bla_{SHV}, bla_{TEM}, bla_{IMP}, bla_{VIM}), fluoroquinolones (*qnrS*) and sulphonamides (*sul1*) and the mobile genetic elements-related encoding gene (*intI*1) were selected based on their common occurrence in domestic wastewater and widespread in environmental compartments, correlation with anthropogenic pollution, and on the fact they have been reported as clinically relevant genes (Du et al., 2014; Gillings et al., 2015; Narciso-da-Rocha et al., 2018; Narciso-Da-Rocha et al., 2014; Szczepanowski et al., 2009; Varela et al., 2016; Walsh et al., 2005; Zhang et al., 2009). Quantifications were made based on the Standard Curve method as described in Brankatschk et al. (2012) using a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The qPCR results, based on three independent DNA extracts for each sample, were analysed according to the quality and acceptability criteria described in Rocha et al. (2018). Briefly, the criteria used were: standard curve efficiency between 90 and 110%; Ct values in the test samples could be interpolated in the standard curve; identical amplicon melting temperature in reference (used for the standard curve) and test samples; observation of a single and correct melting point; and absence of shoulders (increased signal in the baseline, e.g. due to primer dimers).

2.4. Internal standard

The criteria to select an internal standard to assess the matrix effect was the use of a bacterial species that might mimic the behaviour of

Table 1
DNA yield obtained with distinct DNA extraction kits or operators. DNA concentration yield obtained by two operators using the DNA extraction kits NZY Tissue gDNA Isolation kit (NZY) or DNeasy PowerWater kit (PW). UP – ultrapure water; HE – hospital effluent. All samples were spiked with *EcmolA* + .

		NZY kit	PW kit
Sample	Sample volume (mL)	DNA concentration (ng μL^{-1}) (Average ± Standard deviation)	DNA concentration (ng μL^{-1}) (Average ± Standard deviation)
UP1 – Operator 1 (n = 3)	50	3.0 ± 0.1	2.0 ± 0.8
UP1 – Operator 2 (n = 3)		3.3 ± 0.1	2.2 ± 0.4
UP2 – Operator 1 (n = 3)		4.4 ± 0.6	4.5 ± 3.3
UP2 – Operator 2 (n = 3)		4.6 ± 0.8	3.3 ± 1.6
UP3 – Operator 1 (n = 3)		3.6 ± 0.6	3.6 ± 0.9
UP3 – Operator 2 (n = 3)		4.1 ± 1.8	1.8 ± 0.1
UP4 – Operator 1 (n = 3)		4.2 ± 0.6	2.7 ± 0.4
UP4 – Operator 2 (n = 3)		4.3 ± 0.9	4.1 ± 3.9
River1 – Operator 1 (n = 3)		4.2 ± 1.0*	16.0 ± 2.6*
River1 – Operator 2 (n = 3)		4.8 ± 1.3*	11.3 ± 1.7*
River3 – Operator 1 (n = 3)		4.4 ± 0.8*	13.4 ± 0.8*
River3 – Operator 2 (n = 3)		5.9 ± 1.2	9.6 ± 1.9
HE2 – Operator 1 (n = 3)		17.4 ± 3.7	16.5 ± 4.5
HE2 – Operator 2 (n = 3)		13.2 ± 4.9	42.3 ± 10.8
HE4 – Operator 1 (n = 3)		11.6 ± 5.7	36.7 ± 16.3
HE4 – Operator 2 (n = 3)		28.1 ± 20.9	30.9 ± 20.3

*Represent statistically significant differences observed ($p < 0.01$) for the same operator between kits based on independent-samples T-test.

other water and wastewater bacteria during DNA extraction and the presence of a gene that is not expected to be found in these environments. To fulfil these criteria, was selected the *Escherichia coli* strain JM109 (NZYTech, Lisbon, Portugal), transformed with the internal standard gene *molA*. The gene *molA* (accession no. FN985594) of *Gulosibacter molinativorax* ON4^T strain encodes a molinate hydrolase, which is involved in the degradation of the herbicide molinate, used for the control of barnyard grass in paddy fields (Duarte et al., 2011; Lopes et al., 2013; Nunes et al., 2013). A conventional PCR targeting a *molA* amplicon with 1069 bp was performed using the primers F10 (5′-ACG ATCGCGATTGTCGGCGG-3′) and R1070 (5′–GGAGTTCACCCTGGGAC ATA −3′). The amplification was performed in a reaction volume of 50 μL with 2x KCl buffer, 2x $(NH_4)_2SO_4$ buffer, 0.25 mM $MgCl_2$, 2.5 mM dNTP mix (Thermo Scientific, USA), 2.5 μL dimethylsulfoxide (Applichem, Germany), 2.5 μM each primer, 7.5 U of Taq polymerase (Thermo Scientific, USA) and 4 μL of template DNA. The PCR conditions were 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1.45 min at 72 °C, and a final extension of 10 min at 72 °C. A negative control reaction, without template DNA was included. The amplicon obtained was cloned with the plasmid pTZ57R/T using the InsTAclone PCR Cloning Kit #K1214 (Thermo Scientific, CA, USA), in the commercial strain *Escherichia coli* JM109, from this point forward called *EcmolA* + . *EcmolA* + was grown in Luria Broth medium (Invitrogen, CA, USA) supplemented with ampicillin (50 mg mL^{-1}), at 37 °C overnight and plasmid DNA with the *molA* insert was extracted using the commercial kit GeneJET Plasmid Miniprep K0503 (Thermo Scientific, CA, USA) and purified for further use as qPCR standard.

To determine the optimal dose of *EcmolA* + to be used as internal standard, 6, 7, 8, 9 and 10 log-units of *EcmolA* + fresh culture suspensions were prepared. A volume of 1 mL of each suspension was spiked into 1 L of ultrapure water and environmental water samples from river and secondary treated effluent wastewater. The final doses of *EcmolA* + in UP and in the samples were of 3, 4, 5, 6 and 7 log-units of *EcmolA* + mL^{-1}. Based on this preliminary test, an optimal final dose of 6 log-units *EcmolA* + mL^{-1} was selected and the same procedure was applied to a total of 14 environmental water samples (Table S1). This internal standard dose was determined since higher doses of *EcmolA* + (7 log-units *EcmolA* + mL^{-1}) would result in an overestimation of the 16S rRNA gene in samples. Lower doses (3 to 5 log-units *EcmolA* + mL^{-1}) could lead to internal standard (*molA*) abundance below the limit of quantification or to unspecific amplification of the gene, with non-compliance with the quality criteria (e.g. amplicon melting curves with additional melting peaks or shoulders). Ultrapure water was spiked with the same dose of *EcmolA* + used in environmental samples. Viable *EcmolA* + spiked in ultrapure water were enumerated on Plate Count Agar (PCA, Liofilchem, Roseto degli Abruzzi, Italy).

The gene *molA* was analysed in spiked and non-spiked with the internal standard. The abundance of the *molA* gene recovered in ultrapure water was compared with the abundance of the *molA* gene recovered in field water samples spiked and used to determine the internal standard losses due to sample matrix effect, which were calculated as follows: A = log (*molA* gene copy number/mL of spiked ultrapure water) – log (*molA* gene copy number/mL of spiked water sample) and B = A/log (*molA* gene copy number/mL of spiked ultrapure water) × 100.

2.5. Statistical analyses

Independent-samples T-test was used to assess statistically significant differences between 1) the yield of DNA obtained using two different DNA extraction kits and between two operators ($p < 0.01$), 2) the copy number of target genes using DNA extracts obtained by two operators ($p < 0.01$), 3) the matrix effect between both kits, 4) the original and the corrected copy number of target genes ($p < 0.01$). One-way analysis of variance (ANOVA) and Tukey's and Bonferroni post-hoc tests were used to assess statistically significant differences ($p < 0.01$) between the *molA* losses due to matrix effect in different water samples. These analyses were performed with the aid of the SPSS Statistics (for Windows v.24.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. DNA extraction kits effect on DNA yield and on genes quantification

Two DNA extraction kits were compared in terms of DNA extraction yield, gene quantification and inter-operator reproducibility. One was the NZY Tissue gDNA Isolation kit, recommended for a variety of matrices, including animal cells and tissues, Gram-positive and Gram-negative bacteria, mouse tails, yeast, forensic samples and clinical samples and the other was the DNeasy PowerWater kit, recommended for filtered water samples, even water containing heavy amounts of contaminants. These comparative assays involved spiked-ultrapure water, river and hospital effluent samples. The DNA concentrations in the extracts obtained using the DNeasy PowerWater kit and the NZY Tissue gDNA Isolation kit are summarized in Table 1. In most samples, the DNA concentration yield was not statistically different for both DNA

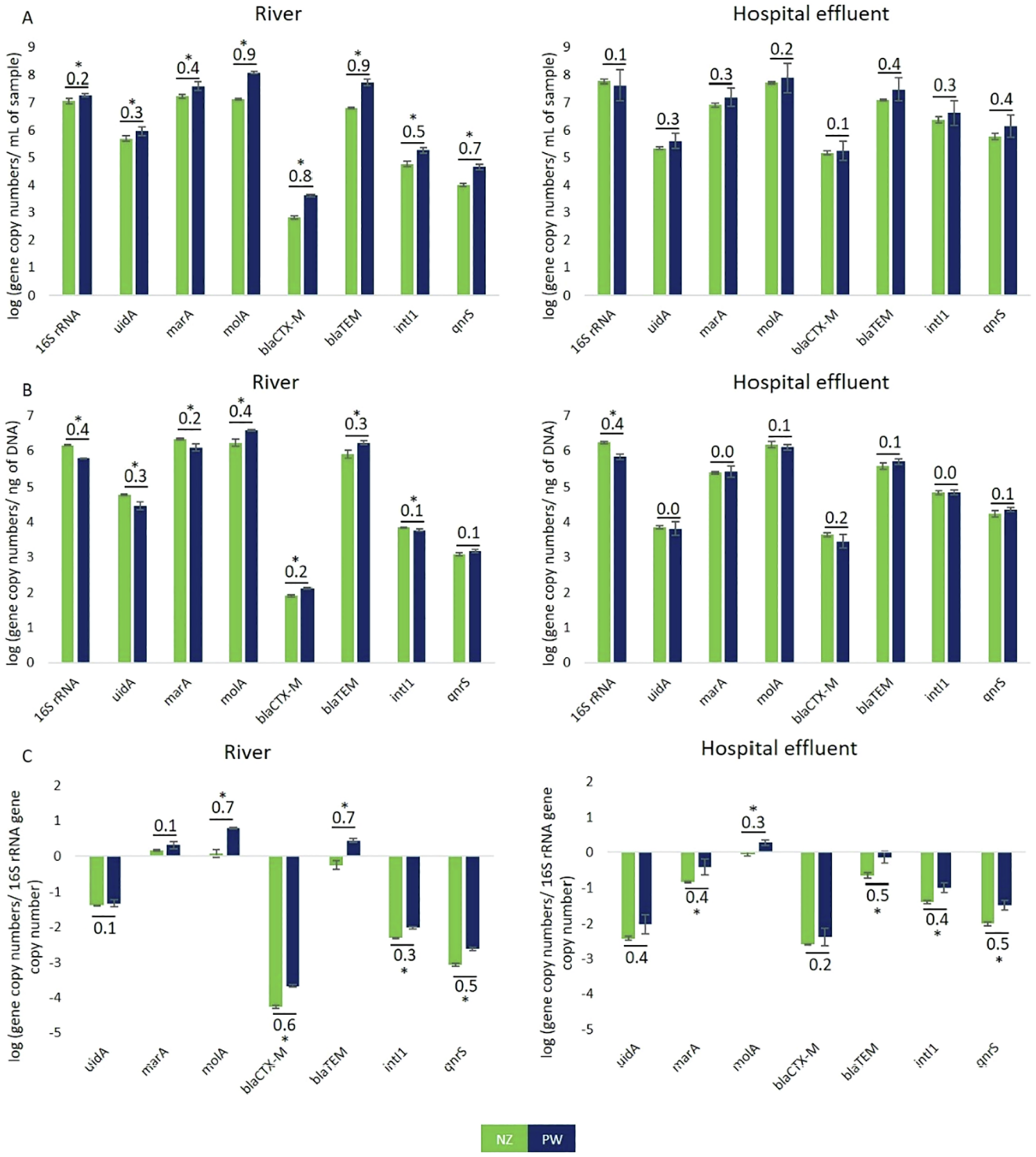

Fig. 1. Comparison of gene quantification in DNA extracts prepared with two different kits. Chromosomal (16S rRNA gene, *uidA*, *marA*), putatively plasmid associated (bla_{CTX-M}, bla_{TEM}, *intI*1, and *qnrS*), and internal standard (*molA*) genes quantification in DNA extracts prepared with the NZY Tissue gDNA Isolation kit (NZ) and DNeasy PowerWater kit (PW). These results refer to environmental water samples that were spiked with the internal standard *molA* gene. The internal standard *molA* gene was also monitored in non-spiked environmental water samples, and it was confirmed its absence in environmental samples. Results are expressed as logarithm transformed values of gene copy numbers obtained per (A) mL of sample, (B) ng of DNA or (C) 16S rRNA gene copy number. Values above the bars represent the difference between logarithmic values of genes quantification obtained after DNA extraction with both kits. * indicate statistically significant differences between both DNA extraction kits ($p < 0.01$).

extraction kits ($p < 0.01$) (Table 1). Exceptions were observed for River1 (operators 1 and 2) and River3 (operator 1) samples, with higher yields in DNeasy PowerWater kit extracts. The vulnerability to operator variations, assessed by the use of the same samples by two operators extracting DNA simultaneously, demonstrated that, also in this aspect, NZY Tissue gDNA Isolation kit and DNeasy PowerWater kit were not significantly different ($p < 0.01$).

Given the consistency of results between both operators, further assays on qPCR gene determinations used the DNA extract set obtained by operator 1. Even though the DNA yield obtained by both extraction

kits was not significantly different, it was hypothesized that differences could be observed in the gene quantification. In hospital effluent samples, no significant differences were observed for the quantification of genes abundance in extracts obtained with both kits. In contrast, in river samples, most of the genes were quantified either per water volume or per ng of DNA at significantly higher amounts in the DNeasy PowerWater kit than in the NZY Tissue gDNA Isolation kit extracts (Fig. 1). However, these differences were never higher than 0.9 log gene copies mL^{-1} of sample or 0.4 log gene copies ng^{-1} of DNA (Fig. 1). As demonstrated using the internal standard, the matrix effect for the NZY Tissue gDNA Isolation kit estimated for river and hospital effluent samples were in average 0.3 and 5.5%, respectively, not significantly different of what was observed for DNeasy PowerWater kit. Considering that both kits did not show an overwhelming performance difference, it was taken the decision to proceed with the internal standard assays using the DNeasy PowerWater kit, since it is recommended by the manufacturer for water samples.

3.2. Matrix effect in different water types

The internal standard, used to measure the matrix effect, consisted in a culture suspension of the *Escherichia coli* strain JM109 *molA* clone, *EcmolA* +, spiked simultaneously in water or wastewater and in ultrapure water, where the matrix effect was assumed to be null. In preliminary assays, different doses of *EcmolA* + (between 6 and 10 log-units *EcmolA* + mL^{-1} sample) were spiked in ultrapure water and environmental water samples to determine the adequate dose to use in further experiments. The value determined as most suitable was 9 log-units *EcmolA* + mL^{-1} to reach a final abundance of 6 log-units *EcmolA* + mL^{-1} of spiked ultrapure water or sample. The matrix effect, estimated based on the quantification of the gene *molA* in control spiked ultrapure water and in samples is shown in Table 2. Although the quantification of the *molA* gene in spiked samples was, in average, underestimated in 0.1 to 0.9 log-units, occasionally, it was overestimated in 0.05 and 0.23 log-units, in sTWWP and in river samples, respectively. In average, the matrix effect ranged of 0.1–0.5 log-units, corresponding to 1.2% in sTWWP, to 6.7% in sTWWA and RWWP. Interestingly, it was concluded that the matrix effect was not dependent on the origin of the sample ($p < 0.01$) (Table 2). The observed overestimation in two samples compared to the respective ultrapure water samples was not related with the presence of the *molA* gene in non-spiked environmental samples, since it was confirmed the absence of the gene in environmental samples. In two situations the quantification of the internal standard *molA* was higher in environmental water samples than in spiked ultrapure water, although all the quality criteria cited above were observed. This might be due to the fact that the internal standard (*molA*) could be adsorbed onto samples particles, hampering an adequate homogenization of the internal standard prior to their filtration. This is sometimes observed, with distinct replicates of the same sample presenting peaks for some genes. This effect is not uncommon and can be attributed to adsorption onto samples particles, which may influence the amount of the gene recovered after DNA extraction in these samples. This effect would never be observed in ultrapure water, explaining the apparent unexpected results.

Table 2
Matrix effect estimated based on the internal standard gene (*molA*). sTWWA and sTWWP – secondary treated wastewater effluent from sampling site A and P; ASLP – activated sludge treated effluent from sampling site P; RWWA and RWWP – influent wastewater from sampling site A and P and HE – hospital effluent. The internal standard *molA* gene was also analysed in non-spiked environmental water samples, and it was confirmed its absence in environmental samples.

DNA extraction matrices	Matrix effect	
	Average Log-units ± Standard Error (A)	Average ± Standard Error in % (B)
River (n = 6)	0.3 ± 0.2	4.5 ± 2.8
sTWWA (n = 6)	0.5 ± 0.1	6.7 ± 1.3
sTWWP (n = 6)	0.1 ± 0.1	1.2 ± 1.7
ASLP (n = 6)	0.4 ± 0.0	5.3 ± 0.5
RWWA (n = 6)	0.2 ± 0.1	2.0 ± 1.0
RWWP (n = 6)	0.5 ± 0.1	6.7 ± 1.7
HE (n = 6)	0.4 ± 0.1	4.4 ± 1.0

A = log (*molA* gene copy number/mL of spiked ultrapure water) – log (*molA* gene copy number/mL of spiked water sample).
B = A/log (*molA* gene copy number/mL of spiked ultrapure water) × 100.

3.3. Gene quantification and internal standard correction

A total of 14 samples of water (river, n = 2) and municipal and airport wastewater treatment plants (influent, secondary effluent and activated sludge, n = 10) and hospital effluent (n = 2) (Table S1) were used. These samples were characterized for the abundance (gene copy number/volume of sample) and prevalence (gene copy number/16S rRNA gene copy number) of five chromosomal genes and nine putatively plasmid-associated genes. The quantifications were corrected according to the matrix effect percentage values (Table 2 and Fig. 2). Except for samples from secondary wastewater treatment effluent (sTWWA and sTWWP), where *uidA* gene abundance per volume of water was higher than *rpoB*, the chromosomal genes abundance could be ranked as 16S rRNA > *marA* > *rpoB* > *uidA* > *ecf*. The same hierarchy was observed for genes prevalence (normalized by 16S rRNA gene abundance) (Fig. 2). Regarding the putatively-associated plasmid genes, the bla_{TEM}, *intI*1 and *sul1* were the most abundant genes in the majority of samples ranging 7–8, 6–8 and 6–7 log (gene copies mL^{-1} of sample), respectively. These genes were in higher abundance than *marA* and *uidA* genes, specific for *Enterobacteriaceae* and *Escherichia coli*, respectively (Castiglioni et al., 2008; Chern et al., 2009) and, than the genes encoding β-lactamases (bla_{CTX-M}, bla_{OXA-A}, bla_{SHV}, bla_{IMP} and bla_{VIM}), widespread in Gram-negative bacteria (Rawat and Nair, 2010). These results are in agreement with the proposal that have been made that they are widespread and that *intI*1 is considered an indicator of anthropogenic contamination (Du et al., 2014; Gillings et al., 2015; Narciso-Da-Rocha et al., 2014).

Also, the carbapenems encoding genes bla_{VIM} and bla_{IMP} presented higher abundance (6 log-units each) in hospital effluent samples compared to other samples, in which abundance ranged from 2 to 4 log-units. This might be related to the fact that these ARGs are associated with last resort antibiotics that are only administrated in hospitals (Meletis, 2016). A major question of this study was if due to matrix effect the abundance of genes could be underestimated in environmental water samples. Underestimation ranged 0.0–0.6 log-units in abundance (per volume of water) and 0.0–0.8 log-units in prevalence (per 16S rRNA gene). The significant differences on the genes abundance due to the matrix effect were more frequently observed in chromosomal than in plasmid-associated genes ($p < 0.01$) (Fig. 2). The matrix effect correction, estimated based on the internal standard *molA* associated to a plasmid which was inserted into a cell, led to significant differences with the same frequency in the prevalence of either plasmid-associated or chromosome associated genes.

4. Discussion

DNA extraction is a critical step on the genetic analyses of environmental samples. For water samples, processing usually involves a step of biomass concentration, frequently through filtration, DNA extraction, and the genetic sample characterization using targeted methods, such as qPCR or non-targeted approaches, such as metagenomics (Manaia et al., 2018). Aware of the importance of this step on the quality of genetic analyses of water samples, manufacturers have

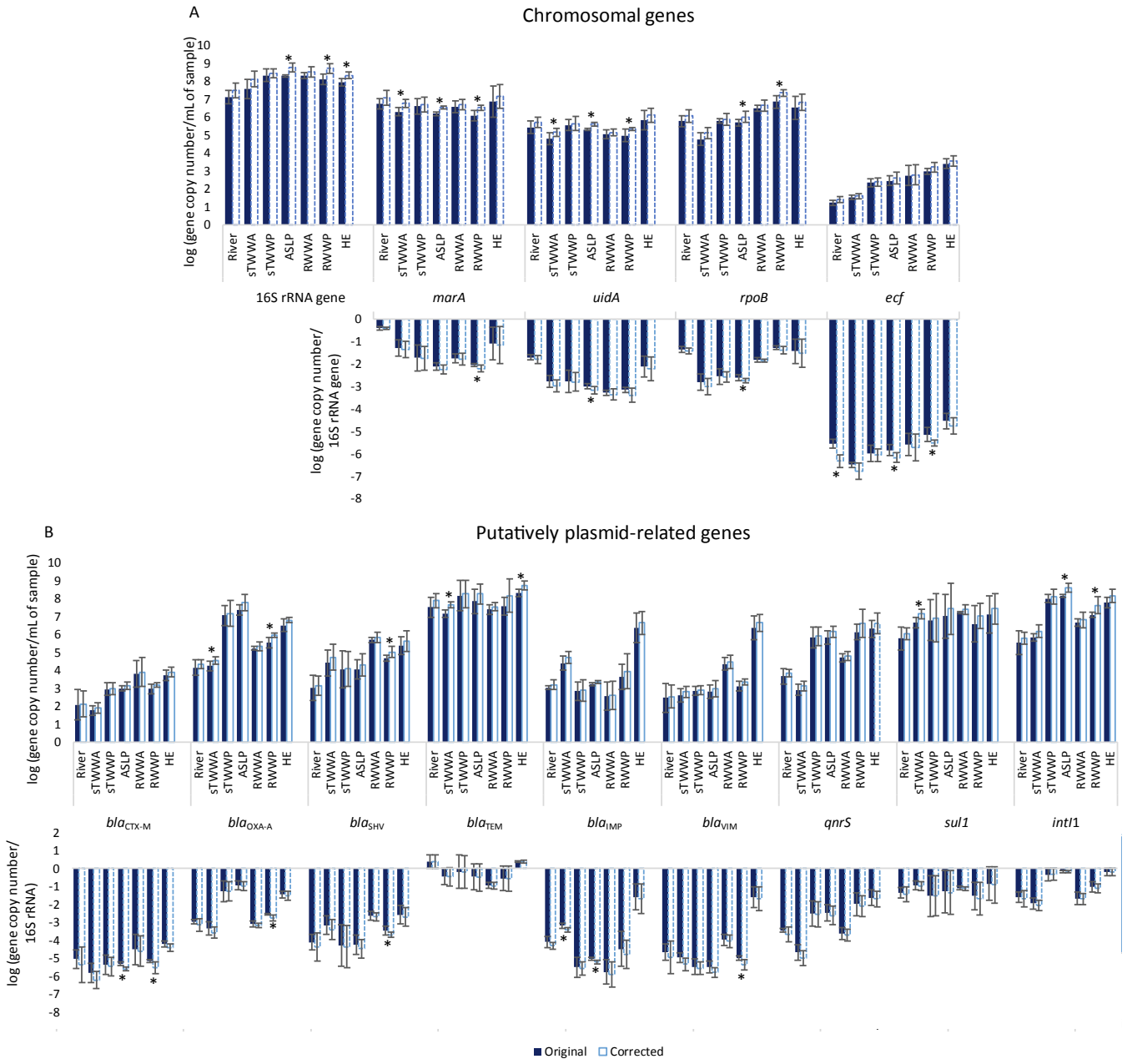

Fig. 2. Matrix effect on genes quantification assessed based on the use of the internal standard *EcmolA* +. (A) Chromosomal genes and (B) putatively plasmid-associated ARGs abundance (gene copy number/mL of sample) and prevalence (gene copy number/16S rRNA gene copy number) were determined in 7 environmental water types from different origins: river, secondary treated wastewater effluent from sampling site A and P (sTWWA and sTWWP), activated sludge treated effluent from sampling site P (ASLP), influent wastewater from sampling site A and P (RWWA and RWWP) and hospital effluent (HE). The internal standard *molA* gene was also analysed in non-spiked environmental water samples, and it was confirmed its absence in environmental samples. The results express the direct gene quantification and respective corrected value due to matrix effect percentage, estimated based on the internal standard losses. * indicate statistically significant differences, $p < 0.01$.

developed DNA extraction kits, which are designed to process filtering membranes containing the water sample biomass and to avoid the matrix effect (e.g. DNeasy PowerWater kit). Highly convenient and popular (Cacace et al., 2019; Pärnänen et al., 2019), these water-specific solutions are expensive and, naturally, cannot completely overcome all kind of matrix effect that can exist in water samples. Therefore, this work aimed to assess if a generalist DNA extraction kit could be adapted for the analysis of water samples, both at the level of sample processing and gene quantification. The matrix effect, which may influence the genetic analyses results, is in chemical analyses overcome through the use of an internal standard (Skoog et al., 2017). Therefore, a cell-based internal standard was designed for this study to measure matrix effects. To our knowledge, this is the first time a cell-based internal standard was designed and implemented for analysis of genes in water samples.

The influence of DNA extraction procedure on DNA yields has been discussed in previous studies, with distinct outcomes. For instance, Hinlo et al. (2017) did not observe differences on the DNA yield obtained using Qiagen's DNeasy Blood and Tissue kit and DNeasy PowerWater kit; Li et al. (2017) observed that the DNA yield was higher using FastDNA SPIN Kit for Soil, compared to PowerSoil DNA Isolation Kit and ZR Fecal DNA MiniPrep; and Djurhuus et al. (2017) observed

the DNA yield was extremely variable between DNeasy Blood and Tissue kit, the DNeasy PowerWater kit, and standard phenol/chloroform methods. In the current study, the fact that statistically significant differences were obtained for DNA extraction from river using DNeasy PowerWater kit compared with NZY Tissue gDNA Isolation kit may be due to the fact that DNeasy PowerWater kit is described as being optimized to remove humic acids, heavy metals, polysaccharides, among other substances in water samples. The differences observed for river sample in genes abundance (genes copy number per mL of sample or ng of DNA) might also be related with the fact that in river samples higher DNA yield was obtained with DNeasy PowerWater kit. Riediger et al. (2016) also observed a higher gene quantification using DNeasy PowerWater kit in river and ultrapure water samples, than using QIAamp DNA mini and PowerSoil DNA Isolation kits. The lack of statistically significant differences observed for DNA extraction between operators using both kits is also interesting and in agreement with the report of Li and colleagues (Li et al., 2017), in which no statistically significant differences (p greater than 0.05) (DNA yield differences up to 12 μg) were observed for DNA yield obtained by different operators. Overall, if DNA extraction kits comparison is analysed in a cost benefit perspective, the choice deserves weighting. The DNeasy PowerWater kit cost around 9 € and the NZY Tissue gDNA Isolation kit costs around 3 € per reaction. If convenience characteristics are taken into account, such as being 1) cheap, 2) user friendly or 3) fast, a simple kit as NZY Tissue gDNA Isolation kit can be adopted to encourage the survey of environmental samples.

The matrix effect, an obligatory issue when water chemical analyses are in discussion (Carbajo et al., 2015; Van De Steene and Lambert, 2008; Zhou and Kang, 2013), is an issue poorly explored in the microbiological analyses domain. The matrix effect refers to particles and organic matter that may impede the extraction of a given analyte (Schrader et al., 2012). Environmental samples matrix might be composed by substances such as detergents, phenolic compounds, humic acids, heavy metals and other contaminants that might influence DNA extraction efficiency and qPCR performance, due to, for example, bacterial interaction with samples matrix or inhibition of qPCR reactions (Kim et al., 2013; Wilson, 1997). The use of external controls spiked into DNA extracts (Cloud et al., 2003; Volkmann et al., 2007) or of internal controls added to the samples before the DNA extraction (Burggraf and Olgemöller, 2004) were reported as a way to understand the reliability of qPCR results. However, these approaches used cell-free DNA, which did not consider the losses due to filtration, an obligatory step in water sample processing, and to deficient cell lysis, a crucial step during DNA extraction which might influence the amount of DNA and genes recovered and quantified. Cell-based internal standards were used for the detection of *Helicobacter pylori* in drinking water, expectedly at low abundance (Sen et al., 2007). Therefore, to our knowledge, this is the first report of a cell-based internal standard used to assess the effect of samples matrix effect on DNA extraction and genes quantification. It was also insightful to observe that the matrix effect is not dependent on the water type, with river water samples presenting matrix effects in the same range of values as raw wastewater. Also the importance of water sample heterogeneity, with particles onto which biological analytes may adsorb, was suggested in this study.

In summary, these results contribute to debate the common assumption that quantitative PCR may have limited value for the comparison of data obtained in distinct laboratories, due to different types of technical biases. In this work, using these two kits, it was observed that the DNA extraction kit, the operator, or the samples matrix effect had a limited impact on the final genes quantification. Moreover, it is suggested that the simplification of DNA extraction procedures, through the adoption of DNA extraction kits that are cheaper and/or user friendly, may not create important bias on the comparison of the quantification of ARGs in DNA extracts. These are encouraging findings that may incentivize labs worldwide, even with low resources, to collaborate in ARGs surveillance studies in the environment.

CRediT authorship contribution statement

Jaqueline Rocha: Conceptualization, Data curation, Investigation, Methodology, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Célia M. Manaia:** Funding acquisition, Project administration, Resources, Investigation, Conceptualization, Supervision, Methodology, Validation, Data curation, Writing - review & editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106194.

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