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Time to act – assessing variations in qPCR analyses in biological nitrogen removal with examples from partial nitritation/anammox systems

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Abstract

Quantitative PCR (qPCR) is broadly used as the gold standard to quantify microbial community fractions in environmental microbiology and biotechnology. Benchmarking efforts to ensure the comparability of qPCR data for environmental bioprocesses are still scarce. Also, for partial nitritation/anammox (PN/A) systems systematic investigations are still missing, rendering meta-analysis of reported trends and generic insights potentially precarious. We report a baseline investigation of the variability of qPCR-based analyses for microbial communities applied to PN/A systems. Round-robin testing was performed for three PN/A biomass samples in six laboratories, using the respective in-house DNA extraction and qPCR protocols. The concentration of extracted DNA was significantly different between labs, ranged between 2.7 and 328 ng mg^{-1} wet biomass. The variability among the qPCR abundance data of different labs was very high (1−7 log fold) but differed for different target microbial guilds. DNA extraction caused maximum variation (3–7 log fold), followed by the primers (1–3 log fold). These insights will guide environmental scientists and engineers as well as treatment plant operators in the interpretation of qPCR data.

1. Introduction

Fluorescence-based quantitative real-time PCR (qPCR) is a popular tool in environmental microbiology and biotechnology (Sanz and Köchling, 2007; Yoo *et al.*, 2017). It allows investigations into the microbial community and functional ecology of complex and multi-species biosystems. In qPCR, the template DNA is initially denatured, followed by annealing of specific primers and subsequently extending the complementary strand by a DNA polymerase, which, in a series of cycles results in an exponential increase in amplicon numbers. The use of a fluorescent dye binding to the double stranded DNA enables quantification of the amplicon in every PCR cycle. Therefore, the success of qPCR relies mainly on (1) the DNA extraction efficiency which influences the quality of DNA templates, because coextraction of humic substances has been reported to severely inhibit qPCR (Roh *et al.*, 2006; Han, Li, *et al.*, 2013), and (2) the availability and selection of appropriate primers. Many studies emphasize the importance of careful primer selection, with a special focus on primer specificity and a targeted amplification product (Frank *et al.*, 2008; Smith and Osborn, 2009; Guo and Zhang, 2013a; Orschler *et al.*, 2019).

Its simplicity, combined with its potential for high analytical sensitivity for absolute quantification of target genes and specificity, makes it the method of choice for a suite of applications (Orschler *et al.*, 2019). Many fields, like biological, microbiological, immunological, medical, agricultural, environmental, and engineering sciences employ PCR methods to detect specific genes (usually as a proxy for a specific microbe or a group of microbes) in a background of genes (extracted from a microbiome sample). More than 166,000 published studies collected using “Real time PCR” as keyword in the SCOPUS database (accessed 08/04/2020) have used this well-recognized

analytical procedure for the quantification of phylotypes and genes in biological samples.

The lack of *a priori* knowledge of a certain microbiome limits the application of qPCR on poorly or newly-examined microbiomes as a primary probing step (Orschler *et al.*, 2019). Rather, open-format metagenetic (e.g., based on 16S rRNA gene amplicon sequencing) or metagenomic (based on shotgun sequencing) approaches must first be employed to provide necessary information for primer choice or design (Pester *et al.*, 2014; Fumasoli *et al.*, 2017). However, such approaches are vastly more costly and analysis-intensive, and furthermore, they do not provide absolute abundance information (Widder *et al.*, 2016). qPCR has therefore been heralded as the 'gold standard' for quantification of specific microbial populations or functional genes.

Wastewater microbiomes, such as activated sludge, granular sludge or biofilms, have been extensively examined using qPCR methods. The additional information that becomes available by qPCR analyses is particularly important for systems with delicate metabolic interactions due to the required syntrophy between different microbial groups. Partial nitritation/anammox (PN/A), a process whose success largely depends on such a delicate balance between multiple microbial functions (Vlaeminck *et al.*, 2012; Agrawal *et al.*, 2018), has thus been widely investigated by qPCR (De Clippeleir *et al.*, 2013; Hu *et al.*, 2013; Gilbert *et al.*, 2014; Pellicer-Nàcher *et al.*, 2014; Persson *et al.*, 2014; Ma *et al.*, 2015; Zhang *et al.*, 2017). qPCR provides the opportunity to quantitatively follow and understand the microbial community, its functionalities and the competition for resources during the conversion of nitrogen from ammonium to dinitrogen gas. Notwithstanding the apparent simplicity and quantitative nature of qPCR, its application to identical microbial communities using different primer pairs can result in different relative abundances of target microbial groups. For example,

Orschler *et al.*, (2019) reported up to 40% and Han, Huang, *et al.*, (2013) up to 22% variation in the relative abundance of anoxic ammonium-oxidizing bacteria (AnAOB) in a wastewater microbiome depending on the primers used. Due to the discrepancies in qPCR results in PN/A studies, Zhang and Okabe (2020) also emphasized that the comparison of AnAOB compositions between studies may not be entirely accurate. One of the reasons for these discrepancies is a general lack of standardization of qPCR analyses in the field of wastewater treatment. To allow for better comparability between reported results, similar to other research fields like plant pathology (Braun-Kiewnick *et al.*, 2016), surface water for pathogens (Shanks *et al.*, 2012), or soil microbiomes (Pan *et al.*, 2010), benchmarking efforts have to be made. Until now only one study (Rocha *et al.*, 2018) has performed inter-laboratory assessments of qPCR analyses in the field of wastewater treatment, however, focused only on antibiotic resistance genes.

The standardization in reporting of individual qPCR assays and their analysis has been accelerated by the introduction of the framework “minimum information for publication of quantitative real-time PCR experiments” (MIQE guidelines) which suggests to provide information about the qPCR assay reagents, primer sequences, qPCR conditions, and data analysis software in the publications (Bustin *et al.*, 2009). However, the accuracy and precision of qPCR based analyses of microbial community compositions have rarely been questioned or examined, which is in clear contrast to analytical methods for environmental or water chemistry (Eaton *et al.*, 2005; European Commission, 2009). We posit that the qPCR assay, *per se*, is not the primary cause of inaccuracy and imprecision. Most laboratories to date, if not explicitly stated, adhere to the MIQE guidelines. However, we postulate that deviating results, to a larger extent, derive from differences in preparatory steps (such as DNA extraction (Guo and Zhang,

2013b)) and the use of differing qPCR primer sets for the same target groups that vary widely in selectivity (Dechesne *et al.*, 2016), and the resulting conversion between gene copy numbers and inferred community fractions (Kembel *et al.*, 2012).

Here we aimed to assess biases and imprecisions associated with qPCR quantification of the core guilds in a community of multiple interacting functional groups, *in casu* PN/A communities: where aerobic ammonia oxidizing bacteria (AOB), AnAOB, and aerobic nitrite oxidizing bacteria (NOB) all interact. To this end, round robin testing was set up by six laboratories for three different biomass samples. Each laboratory employed literature-documented procedures (i.e., qPCR primers and conditions) and adhered to the *in-house* standard operating protocols (SOPs). The results are intended to raise awareness of the need for global harmonization of the SOPs in qPCR and data analyses – and wet-lab / dry-lab molecular biology methods in general – in order to substantially decrease the levels of analytical variations, and thereby increase comparability.

2. Materials and Methods

2.1. Sample collection and exchange

We collected 50 ml fresh sample from three different lab-scale PN/A reactors in three different countries. These three reactors employed different technologies, i.e., one was a sequencing batch reactor, one a fixed bed biofilm reactor, and one a rotating biological contractor. All systems had been operated for at least several months or even years under PN/A conditions. The samples were stored in a freezer (-80°C) until shipping to the six participating laboratories, here listed in alphabetical order: Columbia University, USA; Ghent University, Belgium; Northwestern University, USA; Technical

University of Darmstadt, Germany; Technical University of Denmark, Denmark; Tokyo University of Agriculture and Technology, Japan. Participating laboratories were blinded to sample collection methods or sample types until consolidated data release at the end of the baseline experiment.

2.2. DNA extraction

All laboratories upon receipt of the samples froze the sample sets at −80°C before DNA extraction. The majority of laboratories (4/6) used the FastSpin soil kit for DNA extraction, and one used the Qiagen QIAamp kit. One laboratory used an in-house protocol. See Supplementary Data (DNA extraction protocols.xlsx) for the detailed DNA extraction protocol of each laboratory.

2.3. qPCR

Quantification was based on (i) 16S rRNA genes targeting AnAOB, *Nitrobacter*, *Nitrospira*, and total bacteria, and on (ii) functional genes for hydrazine synthase (*hzsA*) and hydrazine oxidoreductase (*hzo*) for AnAOB, ammonia monooxygenase (*amoA*) for AOB, nitrite oxidoreductase (*nxrA*) for *Nitrobacter*, and nitrite oxidoreductase (*nxrA* and *nxrB*) targeting *Nitrospira.* These genes were selected based on their wide usage in WWTP microbiome studies (Agrawal et al., 2018). All participating laboratories performed qPCR using their established protocols. Primers used by each lab are provided in the supplementary information (List of primers used.xlsx).

2.4. Data Analysis

Comparative analysis of extracted DNA, as well as qPCR data, was performed in order to determine the extent of variation. The Box-plot analysis was performed in ggplot2 (3.1.1) and vegan (2.5.4) R packages for variance assessment (Wickham, 2016). Bray–Curtis dissimilarity index was used to identify which laboratories showed more consensus in qPCR data, across and within the samples, for each target gene, respectively. Two-way ANOVA analysis was performed to determine the significant factors (i.e., DNA extraction, primers, labs) responsible for the observed variance, and to compare qPCR data interpretation approaches (i.e., absolute quantification vs. proportionality) to determine the approach with less variance.

2.5. Data Availability

The authors declare that all the data supporting the findings of this study are available within the article and its supplementary appendix.

3. Results and Discussion

To evaluate the reproducibility of qPCR results, six labs carried out qPCR analyses on the same three biomass samples (S1 – S3) employing their routine protocols. The biomass samples originated from three laboratory PN/A-reactors at three different labs and were distributed amongst all six labs. We first compared the protocols used in each lab to determine the similarities and differences (Figure 1) and their impact on qPCR measurement outputs.

3.1. Yield of DNA

190 All labs extracted DNA according to their established methods. Lab 1 to Lab 4 used
191 the Fast DNA Spin kit (physical extraction method); (2) Lab 5 used an *in-house*
192 prepared Fast prep method (physical extraction method); and (3) Lab 6 used the
193 QIAmp DNA kit (enzymatic lysis) (Figure 1). The yield of extracted DNA varied
194 significantly (p-value is < .00001) between the labs, and was affected by the selected
195 extraction kit and by protocol particularities between labs that used the same extraction
196 kit, i.e. labs 1, 2, 3 and 4 (Figure 2 A). Although labs 1, 2, 3 and 4 used the same
197 extraction method, the mass fractions of extracted DNA ranged from 3 – 173 ng mg^{-1}
198 wet biomass for S1, 34 – 226 ng mg^{-1} wet biomass for S2, and 3 – 123 ng mg^{-1} wet
199 biomass for S3. These four labs used the same protocol, the only difference being the
200 duration of the homogenization step. Labs 1, 2, and 3 performed homogenization only
201 for 40 seconds, whereas lab 4 for 1 minute. The longer homogenization yielded more
202 DNA, as shown in Figure 2 A. This increase in recovery of DNA was likely due to better
203 lysis of microcolonies and separation of cells from the extracellular polymeric
204 substances (EPS). Both have been reported to impact the recovery of DNA (Guo and
205 Zhang, 2013b; Albertsen *et al.*, 2015).

206 Lab 5 used an *in-house* Fastprep method which resulted in an overall lower recovery
207 of DNA ranging from 4 – 13 ng mg^{-1} wet biomass for S1, 42 – 63 ng mg^{-1} wet biomass
208 for S2, and 7 – 10 ng mg^{-1} wet biomass for S3, even though it also used a physical
209 extraction method similar to those labs using the commercial kits. The commercial kits
210 include a spin column for purification and recovery of DNA. The *in-house* Fastprep
211 method included chloroform/isoamyl alcohol purification and isopropanol precipitation.
212 This procedure requires several transfer steps for purification of the DNA, and thus,
213 results in loss of DNA during the multiple transfer stages, which is unavoidable (Merk
214 *et al.*, 2001). Moreover, lab 5 used agarose gel electrophoresis as a final purification

step, which could also result in a lower DNA yield (Miller *et al.*, 1999). Previous studies comparing DNA extraction methods with activated sludge samples report higher DNA yields using physical over enzymatic extraction (Vanysacker *et al.*, 2010; Guo and Zhang, 2013b). Our data does not distinctly support this. Figure 2 A reveals that lab 6, using the enzyme-based extraction, recovered the most DNA for S1 (i.e. 205 – 328 ng mg^{-1} wet biomass) and was second to lab 4 for S2 (i.e. 141 – 224 ng mg^{-1} wet biomass) and S3 (i.e. 54 – 76 ng mg^{-1} wet biomass). The different architecture of the PN/A biomass compared to that of activated sludge flocs might be one reason for the different performance of the enzyme-based method. Due to the lack of consensus in the performance of the different extraction methods, it is time to reassess DNA extraction methods according to the needs and analytical goals of various environmental biotechnology applications, such as the PN/A system.

3.2. Quantification of AnAOB, AOB, and NOB

All six labs performed qPCR analysis for AOB, AnAOB, *Nitrobacter, Nitrospira* and total bacteria (EUB), but the primers used for each target group varied between the labs (Figure 1). The slopes of the standard curves, Y-intercept values and amplification efficiencies are provided in the supplementary information. Figure 2 B clearly shows the impact of this matter. The different microbial guilds were affected differently by the DNA extraction method and primer choices of the laboratories, respectively. In the case of AnAOB, the measured abundances varied even between the labs that used the same primers (i.e., 3 log fold difference between lab 3 and lab 6). At the same time, the results were relatively similar between labs using different primers (i.e., labs 1 and 2). Although labs 1, 2, 3 and 4 used different primers, the measured abundances for sample S2 and S3 were relatively similar. Similar observations were made when comparing abundances measured using functional genes (S.Figure 1). The AOB

abundance results, however, varied significantly, exhibiting up to 6 log fold variations (Figure 2 B), even though all labs used the same primer pair. Also, the total bacteria (EUB) concentrations measured by labs 1, 4, and 6 varied significantly between 2.27E+02 gene copies/ng DNA (lab 1) to 2.87E+09, gene copies/ng DNA (lab 6), although the same primer pair was used with different DNA extraction methods. For *Nitrobacter* and *Nitrospira* there was an up to 7 log fold variation between abundances measured in the different laboratories (Figure 2 B, S.Figure 1), suggesting that both, DNA extraction method and primer choices, affected their quantification. Overall, lab 6 reported a higher abundance of all the microbial targets in comparison to the other labs (S.Figure 2). This suggests that the use of enzyme-based DNA extraction led to either over-estimation or the physical extraction methods led to an under-estimation of the target microbial groups.

3.3. Variation due to the DNA extraction method

DNA extraction methods are a known source of variation between assays aiming at quantifying the composition of microbial communities (Smith and Osborn, 2009; Bonot *et al.*, 2010; Albertsen *et al.*, 2015). We separated the variations caused by the different DNA extraction methods from the variation due to the different samples by comparing the global abundance (across all three samples) measured by the labs using the same extraction method, *i.e.*, FastDNA Spin Kit (S.Figure 3 A). The largest variation was observed for AOB concentrations (6 log fold variation) even though all labs used the same primer set. For AnAOB, EUB, and *Nitrobacter* the observed variations were 5 log fold, for *Nitrospira* 3 log fold.

The abundance variation due to different extraction methods was also compared between labs (S.Figure 3 B). The two-way ANOVA revealed a significant effect of the

DNA extraction method ($p<0.0001$) on the observed abundances compared to the primer pairs. The most significant effect of the extraction method appeared for AnAOB quantification. Previous studies have only focused on the evaluation and design of primer pairs for AnAOB identification and quantification (Harhangi *et al.*, 2012; Han, Huang, *et al.*, 2013; Sonthiphand and Neufeld, 2013) but neglected the impact of DNA extraction on the quantification of AnAOB. There is no disagreement about the need for good primer pairs. However, there is a lack of studies to anticipate whether the most commonly used DNA extraction methods for activated sludge are also suitable for PN/A microbial communities containing AnAOB. The specific nature of PN/A communities, be it the dense granules or biofilm that is formed in these systems or the specific cell morphology of AnAOB, could be an important factor. The same goes for other specific factors of other microbial communities which differ from activated sludge in terms of composition and structure (for example, the differentiation between more planktonic or more biofilm favoring environments).

3.4. Sample-specific effect on variation

To quantify the sample effect on the measured abundances, we evaluated within- and between-sample variations (Figure 3). Each of the box plots in Figure 3 summarizes gene abundances for each functional guild, regardless of the extraction method and the applied primer sets. Overall, variations differed between samples, with the maximum variation in sample S1 followed by S2 and S3 (Bray-Curtis dissimilatory index: S1= 0.93; S2= 0.76; and S3= 0.69). However, the variations varied for each microbial target in the samples, respectively. The largest variation for AnAOB and

287 *Nitrobacter* occurred in S1, while the abundances of EUB, AOB, and *Nitrospira* varied
288 mainly in S2.

289

290 **3.5. Quantification using ratios**

291 Studies on developing and troubleshooting of PN/A systems require quantifying the
292 amounts of AOB, AnAOB, *Nitrobacter,* and *Nitrospira*, respectively. Although the
293 absolute quantity of the target microorganisms in a PN/A system is desired, the results
294 are often also interpreted as the proportion of AnAOB relative to other microbial groups
295 (i.e. AOB, *Nitrobacter, Nitrospira).* For example, Winkler *et al.*, 2012; Shi *et al.*, 2016
296 and Wang *et al.*, 2019, discussed the performance of their reactors in terms of the ratio
297 of AnAOB to other microbial groups. Therefore, we also compared the ratios of
298 AnAOB:AOB; AnAOB:*Nitrobacter* and AnAOB:*Nitrospira* resulting from the
299 quantitative analyses of the different labs. Figure 4 shows that the variation between
300 the ratios of AnAOB:AOB was smaller than the variation between the absolute
301 abundances of AnAOB and AOB by the different labs. However, this was not true for
302 the AnAOB:*Nitrobacter* and AnAOB:*Nitrospira* ratios. Proportionality could help to
303 reduce the impact of the systematic variations, because ratios are unaffected from the
304 scale of the data (van den Boogaart and Tolosana-Delgado, 2008), but one needs to
305 be careful and determine whether usage of ratios is valid only for an individual qPCR
306 analysis or for all the qPCR analyses.

307

308 **3.6. Guidance for dealing with the current situation**

309 This collaborative effort was stimulated by the need for reproducibility and
310 transferability of qPCR analyses of PN/A systems, in order to establish common and

311 generic process insights as well as a consensus on the approaches to perform
312 analyses and data interpretation. Meta-understanding of the process should be as
313 generic as possible, moving beyond multiple individual case studies (Agrawal *et al.*,
314 2018; Li *et al.*, 2018; Orschler *et al.*, 2019). We found that each step of qPCR
315 quantification, including sample handling, DNA extraction, primers, qPCR kits, and
316 data analysis, has the potential to introduce variations of comparable effect size to that
317 of sample differences. Along with previous studies related to molecular
318 characterizations of activated sludge systems (Bru *et al.*, 2008; Smith and Osborn,
319 2009; Albertsen *et al.*, 2015; Keene-Beach and Noguera, 2018), our results indicate
320 that carrying out qPCR analyses is challenging at present because almost any protocol
321 choice has the potential to yield unique results.

322 Therefore, the goal of this research was to assess the extent of variation in qPCR
323 analyses of BNR systems using PN/A as an example and provide information that
324 allows environmental scientists, engineers, and WWTP operators to make informed
325 choices. Therefore, answers are needed for the following questions: (1) How should
326 data analysis and interpretation occur?; (2) What is the tolerance range for the
327 variations and how can data be interpreted within the given variation ranges?; (3) In
328 which case can such measurements be used, and in which case not? Or simply: can
329 we even rely on this data?; (4) Can we compare studies with each other?; (5) How do
330 we consider the uncertainties in these results, e.g., when such data is used in
331 mathematical models.

332 In the end, one has to be aware of variation ranges, and integrate them in data
333 interpretation, as practically feasible. Summarizing our qPCR analyses of PN/A
334 systems, we herewith advocate a decision tree (Figure 5) that can help to objectively
335 interpret and compare qPCR results. The decision tree intends to visualize and

integrate the scale of the uncertainty associated with the qPCR data analysis. The fix-point for the interpretation is the availability of a reference gene - here we define reference gene as a gene that represents the total bacterial population of a sample.

Previously published PN/A studies that also focus on the microbial community can be divided into two groups: (1) studies that use the 16S rRNA gene or other housekeeping genes like rpoB gene as reference gene (Dahllöf *et al.*, 2000), to determine the total bacterial population and use those as a basis to quantify the total microbial community in a reactor (for example Park *et al.*, 2010; Pellicer-Nàcher *et al.*, 2014; Blum, 2018); (2) studies that do not quantify the total bacterial population and only focus on certain target microbial groups (for example De Clippeleir *et al.*, 2013; Persson *et al.*, 2017; Zhao *et al.*, 2018). Such uses of qPCR are common and permeate the literature for quantification of target microbial groups. Therefore, as a first step, it is essential to determine whether a reference gene, which represent the total bacterial abundance, is quantified or not; and whether the presence of the reference gene is as expected or not. This can serve as a first base to determine the extent of uncertainty in different quantification approaches or aims: (a) absolute quantification of target genes; (b) relative quantification of target genes to a reference gene; and (c) proportional quantification of different target genes associated with different microbes (Figure 5). For instance, we found that the abundances of our reference gene, (EUB - based on the quantification of the 16S rRNA gene) reported by the labs were less than the sum of the abundances of the target genes for the microbial groups of interest (i.e., AnAOB, AOB, *Nitrobacter* and *Nitrospira*; Figure 2 B). However, the abundance of the reference gene (which represent the total microbial population) must always be higher, after accounting for the target gene copies per genome, than that of any target gene (associated with a specific microbial group) or their total sum. Thus, in such a scenario,

361 it is clear that either unintentional selective DNA extraction has occurred or the primer
362 choice has introduced bias in quantification. Absolute as well as relative quantification
363 depends upon the measured abundance of the reference gene because it is
364 associated with total microbial abundance in PN/A and also other engineered
365 ecosystems. Therefore, we suggest to only use absolute or relative quantifications
366 when the abundance of the reference gene is higher than that of the microbial group
367 specific target genes (Figure 5). Alternatives to the 16S rRNA gene as reference gene
368 for quantification of total bacterial populations, other reference genes such as the *rpoB*
369 gene could be considered, to overcome the limitations of the 16S rRNA gene (Orschler
370 *et al.*, 2019). For example, Case *et al.*, (2007) showed that the *rpoB* gene can
371 complement results obtained by the 16S rRNA gene.

372 When using the ratios of microbial group specific target genes, we suggest, as shown
373 in Figure 5, that with the present state of standardization, the ratios of the target genes
374 might still be used even if the abundance of the reference gene was not as expected.
375 However, such proportionalities may not necessarily help to overcome uncertainties
376 due to presence of large variations between the data, like we show in Figure 4.

377

378 **4. Conclusions**

379 Using the PN/A microbial community as an example, we emphasize the need for
380 standardization of qPCR analysis, so that qPCR-based assessment of microbial
381 community compositions can become comparable and a reliable decision-making tool
382 for monitoring and operation of wastewater treatment plants. In our view, DNA
383 extraction methods, and primer selection have large effects on the variations in the
384 qPCR analyses, resulting in lack of reproducibility. Furthermore, we found that a DNA

extraction method may perform better for one type of sample and microorganism but not for another sample or microorganism. Therefore, as the next phase, we expect to carry out systematic surveys (1) of the DNA extraction protocols for different types of PN/A biomasses such as suspended biomasses, (small) granules and substratum-based biofilms, to understand how different extraction methods perform in different biomass or community types; (2) of available primers for their performance for different set of samples. These surveys will be used to determine the extent of uncertainties of qPCR quantification approaches, which are carried out in different laboratories, including our own, to develop and further improve a community-based standardization.

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