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# Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: Applications and considerations

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ABSTRACT

Quantitative real-time PCR (qPCR) has been widely used in recent environmental microbial ecology studies as a tool for detecting and quantifying microorganisms of interest, which aids in better understandings of the complexity of wastewater microbial communities. Although qPCR can be used to provide more specific and accurate quantification than other molecular techniques, it does have limitations that must be considered when applying it in practice. This article reviews the principle of qPCR quantification and its applications to microbial ecology studies in various wastewater treatment environments. Here we also address several limitations of qPCR-based approaches that can affect the validity of quantification data: template nucleic acid quality, nucleic acid extraction efficiency, specificity of group-specific primers and probes, amplification of nonviable DNA, gene copy number variation, and limited number of sequences in the database. Even with such limitations, qPCR is reportedly among the best methods for quantitatively investigating environmental microbial communities. The application of qPCR is and will continue to be increasingly common in studies of wastewater treatment systems. To obtain reliable analyses, however, the limitations that have often been overlooked must be carefully considered when interpreting the results.



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## 1. Introduction

Biological processes have long been used in treating municipal and industrial wastewaters with different physicochemical characteristics. Performance of a biological wastewater treatment process depends primarily on the concerted activity of microorganisms involved, which underlines the importance of understanding microbial community structure and dynamics. A wastewater treatment system has been regarded as a black box for decades due to the complexity of microbial community compositions and a lack of methods for exploring individual microbial populations. Culture-dependent approaches, based on isolation and cultivation in a laboratory, were applied in the early days to the study of microbial communities. Such methods, however, inevitably lead to a significantly distorted view of community structure because the majority of environmental microorganisms cannot be cultured ex situ (Amann et al., 1995; Sievert et al., 1999), which indicates a high likelihood of missing important populations. In recent years, the application of culture-independent molecular techniques in microbial ecology studies has been extensively reported, providing deeper insights into microbial ecosystems. By eliminating the error-inducing cultivation step, culture-independent methods can provide a closer look at the true diversity of a microbial community. Such molecular approaches are frequently used today to study wastewater treatment systems, providing fundamental information for transforming the black-box description into a transparent description.

The molecular techniques used to explore wastewater microbial communities can be roughly grouped into four categories: clone library, molecular fingerprinting, hybridization, and quantitative real-time PCR (qPCR). After a pioneering work by Giovannoni et al. (1990), clone library assays, especially targeting the 16S rRNA gene, have been widely used to examine various wastewater treatment systems and are still often employed when comprehensive taxonomic information is needed (Sanz and Kochling, 2007). However, this laborious and time-consuming method is unsuitable for high-throughput analyses dealing with large numbers of samples. Furthermore, to ensure the representativeness of a clone library, a large number of clones must be constructed, which increases the cost and time required for the analysis. Among today's most popular methods for investigating environmental microbial communities are molecular fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism analysis (T-RFLP). In these techniques, PCR fragments of different sequences and/or sizes are separated by their differing mobility on a gel or in a capillary (Gilbride et al., 2006). Based on the assumption that each band or peak represents one microbial species, the patterns generated (i.e., molecular fingerprints) can directly reflect the community diversity. At the same time, phylogenetic information can also be obtained by sequencing the DNA fragments recovered from the bands or peaks of interest, although the resolution is limited by the sequence length. Nucleic acid hybridization employs short oligonucleotide probes (approximately 15–25 bases) that are specific to target microorganisms. This probing can be conducted with or without extracting nucleic acids from cells. Dot blotting, an ex situ method, has often been employed to explore the metabolic activity of a community by assessing relative changes in gene expression levels. Given that gene expression patterns vary significantly with growth and environmental conditions, this technique seems to be of limited applicability to microbial quantification. Fluorescence in situ hybridization (FISH), a probing method that does not require nucleic acid extraction, has recently been applied to various wastewater treatment systems. Due to its in situ nature, FISH-microscopy can visualize the abundance and distribution of microorganisms in true environmental community structures (Amann and Fuchs, 2008; Wagner et al., 2003). These hybridization techniques, however, have a common limitation of being low-throughput methods, largely due to difficulties in optimizing the reaction conditions, particularly for the hybridization and washing steps (Talbot et al., 2008). A newer technique, qPCR, has been available since the late 1990s (Heid et al., 1996) and is currently regarded as the most precise method for detecting and quantifying nucleic acids. Contrary to conventional PCR-based methods, such as DGGE and T-RFLP, this method not only detects the presence of target sequences but also quantifies their absolute numbers. In environmental studies, qPCR was first applied to the detection of harmful microorganisms (Bowers et al., 2000; Kuhnert et al., 2000; Lin et al., 2000). More recently, its application to wastewater has been extensively studied as the analysis has become more affordable and widely available (Zhang and Fang, 2006).

Applications of qPCR quantification have helped us gain more in-depth insight into fixed and suspended microbial communities in various wastewater treatment processes under both aerobic and anaerobic conditions (Lee et al., 2011; Parameswaran et al., 2009; Saikaly et al., 2010; Tang et al, 2013; Yang et al., 2012a). Such quantitative information facilitates linking changes in microbial community structure to changes in process performance because the functional attributes of a biological process are heavily affected by the composition of microbial community (Akarsubasi et al., 2005; de los Reyes, 2010). Although a substantial amount of quantitative data on wastewater microbial ecology has accumulated in the last decade due to the use of qPCR, there are limitations to be considered for more reliable analysis. This article reviews the applications of qPCR quantification in wastewater microbial ecology studies and discusses the limitations of such approaches.

## 2. qPCR quantification

### 2.1. Detection chemistry

Although PCR has revolutionized the detection of nucleic acids, its application to quantitative analysis is generally not recommended because conventional PCR can only measure the final concentration of amplicon. A target sequence is ideally amplified exponentially during PCR; however, in reality, the end-point concentration is not proportional to the initial concentration of template DNA due to some inherent limitations and biases of PCR (Zhang and Fang, 2006). Contrary to conventional PCR, qPCR can monitor the progress of DNA amplification in real time and visualize the phase of exponential amplification (Heid et al., 1996). This real-time monitoring, which is the key to the absolute quantification of target sequences, is achieved by continuously measuring the fluorescence emitted as the amplicons accumulate. There are several detection chemistries available that involve various fluorescent molecules, including non-specific DNA-binding dyes, hydrolysis probes, hybridization probes, light-up probes, molecular beacons, sunrise primers, and scorpion primers (Lim et al., 2011). Although each detection method has unique features, in all the methods, the level of fluorescence signal reflects the cumulative amount of target amplicons. Among the various detection chemistries, SYBR Green I and TaqMan assays (Fig. 1) are most widely used methods in current environmental microbial ecology studies.

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