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Research article

Assessing the abundance of fungal populations in a full-scale membrane bioreactor (MBR) treating urban wastewater by using quantitative PCR (qPCR)

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ABSTRACT

The abundance of fungi in a full-scale membrane bioreactor (MBR) treating urban wastewater and experiencing seasonal foaming was assessed by quantitative PCR (qPCR), comparing three different sets of widely used universal fungal primers targeting the gene encoding the small ribosomal subunit RNA, 18S-rDNA, (primers NS1-Fung and FungiQuant) or the internal transcribed spacer ITS2 (primers ITS3-ITS4). Fungi were a numerically important fraction of the MBR microbiota ($\geq 10^6$ 18S-rDNA copies/L activated sludge), and occurred both in the aerated and anoxic bioreactors. The numbers of copies of fungal markers/L activated sludge calculated using the NS1-Fung or ITS3-ITS4 primer sets were up to 2 orders of magnitude higher than the quantifications based on the FungiQuant primers. Fungal 18S-rDNA counts derived from the FungiQuant primers decreased significantly during cold seasons, concurring with foaming episodes in the MBR. Redundancy analysis corroborated that temperature was the main factor driving fungi abundance, which was also favored by longer solid retention time (SRT), lower chemical oxygen demand/biochemical oxygen demand at 5 days (COD/BOD_5) of influent water, and lower biomass accumulation in the MBR.

1. Introduction

Fungi are ubiquitous microorganisms and key players in a wide array of ecosystem processes, in particular the decomposition of organic matter (Dighton, 2007; Ramoni and Seiboth, 2016). They coexist and interact with bacteria in a variety of environments, where they comprise interdependent consortia exhibiting both antagonistic and cooperative behaviors, driving together several ecologically relevant functions in spite of their evolutionary, physiological and metabolic differences (Deveau et al., 2018; Frey-Klett et al., 2011).

Fungi are part of the complex microbiota of activated sludge in biological wastewater treatment plants (WWTPs); however, the analysis of microbial communities in these engineered environments is frequently focused only on bacteria, while the abundance and roles of other microbial groups remain rather unexplored. The occurrence of fungi in WWTPs has been often neglected, since they were assumed to be numerically dominant only in industrial wastewaters, where features such as acid pH, shortage of nitrogen sources and the presence of substances toxic for bacteria offer competitive advantages favoring their growth (Evans and Seviour, 2012; Zheng et al., 2011). Therefore, the roles of fungi in urban WWTPs currently remain scarcely investigated, although several recent studies have demonstrated that these organisms are fairly diverse and display a huge biotechnological potential, being able to contribute to biomass degradation (Evans and Seviour, 2012; Maza-Márquez et al., 2016b; Yang et al., 2011), removal of emerging contaminants (Nguyen et al., 2013), denitrification (Niu et al., 2017) and stabilization of activated sludge cell aggregates (Weber et al., 2009). Dimorphic and filamentous fungi have been also related to the generation of operational problems, such as clogging of pipes and filters, bulking, foaming and membrane biofouling (Liébana et al., 2015; Zheng et al., 2011).

In recent years, the application of molecular methods has allowed to significantly increase our understanding of the diversity and functions of microorganisms in a range of natural and engineered environments. Real-time PCR (qPCR) has become a valuable molecular tool allowing the direct quantification of target microorganisms using environmental DNA (Smith and Osborn, 2009). However, only a few efforts have taken advantage of this approach to explore the size of the total mycobiota in environmental samples from soil, sediments, bioaerosols, or freshwaters (ie., Luhung et al., 2015; Prévost-Bouré et al., 2011; Wurzbacher et al., 2014; Wymore et al., 2013; Zhang et al., 2015). Most of the available studies devoted to the identification or quantification of fungal

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populations in WWTPs have been performed based on cultivation-dependent approaches (i.e., Awad and Kraume, 2011; Bauer et al., 2002; Bux and Kasan, 1994; Liébana et al., 2015), while qPCR has been very seldom used (González-Martínez et al., 2018; Niu et al., 2017).

One major drawback for the implementation of qPCR to quantify fungal abundance is the difficulty in the selection of molecular markers and the design of universal primers offering complete and specific coverage of all fungal Phyla (Prévost-Bouré et al., 2011; Wurzbacher et al., 2014). The coamplification of non-target sequences when qPCR is used for quantification of fungal marker genes in environmental samples may lead to a significant overestimation of their abundance; however, to the best of the authors' knowledge, this issue has been seldom addressed in the available literature.

Previously, the diversity of the mycobiota in activated sludge samples of a full-scale membrane bioreactor (MBR) treating urban wastewater was evaluated by 454-pyrosequecing of 18S rRNA gene (18S rDNA) amplicons (Maza-Márquez et al., 2016b), which were generated using the fungal-specific primer set NS1-Fung (May et al., 2001). In the present study, a qPCR approach was used to quantify the absolute abundance of the fungal populations in the same set of MBR samples. Since a significant percentage of the amplicon reads analyzed by Maza-Márquez et al. (2016b) were affiliated to non-fungal eukaryotic organisms, the output of qPCR of the NS1-Fung primer set was compared with other two different sets of universal fungal primer pairs, in order to evaluate to which extent the amplification of non-target sequences introduces significant quantification errors. Finally, multivariate analyses (redundancy analysis, RDA) was used to evaluate the impact on fungal abundance of the fluctuations of operational temperature (OT), characteristics of the influent wastewater and operation parameters (hydraulic and solid retention times, HRT and SRT, respectively) influencing the MBR.

2. Material and methods

2.1. Description of the full-scale MBR experimental plant, operating conditions, and summary of plant performance

The MBR investigated in the study operated in pre-denitrification mode and was already described in detail by Gómez-Silván et al. (2014). It consisted of an aerated bioreactor (19.4 m^3), an anoxic bioreactor (6.8 m^3), and a filtration tank (2 m^3) comprising three polyvinylidene fluoride (PVDF) hollow-fiber ultrafiltration membrane modules (0.034 μm nominal pore size, GE Water & Process Technologies, Fairfield, USA). Part of the treated water was retained in a fourth tank (0.2 m^3) and used for the periodic backwashing of the membrane modules. The MBR was installed at the facilities of EDAR Sur-WWTP (EMASAGRA, Granada, Spain) and received urban wastewater from the pretreatment unit of the municipal plant, after being filtered through a 1-mm brush screen, as recommended by the membrane manufacturer. Air was supplied to the aerated bioreactor with a fine bubble membrane diffuser and the dissolved oxygen (DO) concentration was maintained automatically in the 0.5–1.6 mg/L range. The filtration tank was also aerated in order to control membrane fouling.

Temperature, pH, and DO concentration inside the bioreactors; level of the tanks, transmembrane pressure and flow rates were measured automatically in the MBR and registered in a database. These datasets were gathered and analyzed with the aid of the Active Factory v.9.2 software (Wonderware, Spain). The daily medium OT in the activated sludge of the MBR system was calculated by the software from data measured every second. Influent wastewater and MBR effluent samples were analyzed daily for the concentrations of suspended solids (total, iTSS; volatile, iVSS), chemical oxygen demand (COD), and biological oxygen demand at 5 days (BOD_5). Samples were automatically collected through the day using a time controller and a peristaltic pump and kept at 4 °C until taken for analysis. Concentrations of total and volatile suspended solids (TSS, VSS) were also measured daily in the activated sludge of both bioreactors. All the analyses were done in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The effluent water complied with the EU regulation legal requirements for discharge to the environment (Directive 91/271/EEC, European Council, 1991).

The abundance of fungi was monitored in 14 activated sludge samples retrieved during four different phases of operation in the MBR: Summer 2009, Autumn 2009, Summer 2010 and Winter 2012 (Sm09, Au09, Sm10, and Wn12, respectively). Different sets of HRT and SRT, different average characteristics of the influent wastewater and OT in the MBR were recorded in each phase (Maza-Márquez et al., 2016a, Table S1). Two episodes of foaming mainly affecting the anoxic bioreactor were observed in the cold seasons, from 26/11/2009 to 22/12/2009 (end of phase Au09) and from 7/02/2012 to 18/02/2012 (phase Wn12).

2.2. Activated sludge sampling for qPCR assays

Samples (200 ml) of activated sludge were taken from both bioreactors (aerobic and anoxic) using sterile plastic containers, carried from the experimental plant to the laboratory, and preserved until nucleic-acid extraction, following previously described protocols (Gómez-Silván et al., 2014). Two biological replicates were prepared for each DNA extraction. In each experimental phase, sampling did not start until steady-state conditions were reached (at least threefold the SRT since the start-up date, Maza-Márquez et al., 2015).

2.3. Nucleic acids extraction and purification

The activated sludge samples were processed for extraction and purification of total DNA using the FastDNA-2 ml SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, Santa Ana, CA, USA), following the manufacturer's instructions. The quality and concentration of the DNA extracted from the samples was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Waltham, MA, USA).

2.4. qPCR assays

qPCR was optimized using True Start Hot Start DNA polymerase (Thermo Scientific, Waltham, MA USA) and SYBR Green I (Sigma Aldrich, St. Louis, MO, USA) in a total volume of 25 μl, using a Step One Plus Real-Time PCR system (Applied Biosystems). Amplification for qPCR was done comparing the results of three primer sets previously described as universal and specific for fungi, as listed in Table 1A and shown in Fig. S1.

The NS1-Fung primer set has been used previously to quantify fungi by qPCR in sediments and WWTPs samples (Niu et al., 2017; Zhang et al., 2015), although lack of complete specificity for its target group has been often reported (Evans and Seviour, 2012; Hoshino and Morimoto, 2010; Wurzbacher et al., 2014) and corroborated in the same set of activated sludge samples used here (Maza-Márquez et al., 2016b). The primer set FungiQuant (FQ) was more recently designed by Liu et al. (2012) and specifically aimed for the quantitative amplification of fungal 18S rDNAs. The specificity of this primer pair has been supported by further studies (Biyeyeme Bi Mve et al., 2017; Wymore et al., 2013), and it has been also recommended for fungal quantification in WWTPs (Karst et al., 2016). Finally, the primer set ITS3-ITS4 (White et al., 1990), which was designed to target the fungal ITS2 region including the partial sequence of the 5.8S rRNA gene (herein ITS2 rDNA), was also included in the study, since the ITS1 and ITS2 have been proposed as the official fungal barcoding marker sequences (Bellemain et al., 2010). Nonetheless, amplification of non-fungal sequences by this primer pair in freshwater samples was also revealed previously (Wurzbacher et al., 2014).

The qPCR reaction mixtures contained 0.125 μl of DNA polymerase (50 U/μl), 0.5 μl of each primer (10 μM), 2.5 μl of 10 × Taq Buffer (with