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Quantitative response of nitrifying and denitrifying communities to environmental variables in a full-scale membrane bioreactor

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HIGHLIGHTS

- A full-scale MBR treating urban wastewater was operated for nine months.
- Gene markers specific of AOB, NOB and denitrifiers were quantified by qPCR.
- Both abundance (DNA) and transcription level (cDNA) of gene markers were analyzed.
- MDS was used to link population abundances to changes in operation parameters.
- N-cycle bacterial groups showed varying trends of response to operation parameters.

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ABSTRACT

The abundance and transcription levels of specific gene markers of total bacteria, ammonia-oxidizing Betaproteobacteria, nitrite-oxidizing bacteria (*Nitrospira*-like) and denitrifiers (N_2O-reducers) were analyzed using quantitative PCR (qPCR) and reverse-transcription qPCR during 9 months in a full-scale membrane bioreactor treating urban wastewater. A stable community of N-removal key players was developed; however, the abundance of active populations experienced sharper shifts, demonstrating their fast adaptation to changing conditions. Despite constituting a small percentage of the total bacterial community, the larger abundances of active populations of nitrifiers explained the high N-removal accomplished by the MBR. Multivariate analyses revealed that temperature, accumulation of volatile suspended solids in the sludge, BOD_5, NH_4^+ concentration and C/N ratio of the wastewater contributed significantly (23–38%) to explain changes in the abundance of nitrifiers and denitrifiers. However, each targeted group showed different responses to shifts in these parameters, evidencing the complexity of the balance among them for successful biological N-removal.

1. Introduction

One of the main objectives of biological wastewater treatment (WWT) is the removal of N compounds from water bodies, due to their otherwise harmful effects in water ecosystems, such as eutrophication or ground-water contamination. Pre-denitrification is among the most efficient strategies reported (Le-Clech, 2010). Under this mode of operation, the wastewater first enters an anoxic phase, where denitrifiers use NO_3^- to oxidize the organic matter in a four-step reductive pathway generating N_2 as end product. The water then enters an aerated phase where the nitrifiers, ammonia oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB), sequentially transform NH_4^+ into NO_3^-. Finally, NO_3^- is supplied into the anoxic phase by partial recirculation of the nitrified effluent.

Among the abovementioned bacterial populations, nitrifiers have slow growth rates, complicating the correct development of a community capable of highly efficient N-removal (Le-Clech, 2010). Membrane bioreactors (MBRs) have been established as an alternative technology to the conventional activated sludge (CAS) processes, showing several advantages that make them

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attractive for wastewater reclamation and reuse. MBRs apply membrane filtration for the separation of particulate material, avoiding the need for a secondary clarifier (Judd, 2011). Compared to CAS, MBRs are characterized by a high solids retention time (SRT), and an independence between SRT and hydraulic retention time (HRT). These features strongly influence the biology of the system and improve the development of slow-growing microorganisms (Le-Clech, 2010).

The oxidation of NH_4^+ to NO_2^- is regarded as the rate-limiting step for the removal of N compounds (Limpiyakorn et al., 2005). In WWT plants, the prevalent ammonia oxidizers are evolutionally related to a monophyletic group consisting of two genera of the Betaproteobacteria, *Nitrosomonas* and *Nitrosospira* (Purkhold et al., 2003). The actual contribution to nitrification of ammonia-oxidizing archaea (AOA) in engineered systems is still unclear (Calderón et al., 2013). Among the microorganisms responsible for the second step of nitrification, *Nitrobacter*-like bacteria were classically acknowledged as the most relevant NOB group in WWT plants, but cultivation-independent molecular techniques revealed *Nitrospira* spp. as the most diverse, abundant, and ubiquitous NOB group (Lücker et al., 2010; Winkler et al., 2012). *Nitrospira* have slower growth rates but are more competitive than *Nitrobacter* when long SRT are set (Yu et al., 2010b), and under lower NO_2^- and O_2 concentrations (Winkler et al., 2012). Despite their decisive roles, AOB and NOB are commonly detected in only relatively low abundance in WWT systems (Geets et al., 2007; Kim et al., 2011; Xia et al., 2012; Zhang et al., 2010).

It is well known that the variations in the operational parameters and environmental variables in WWT systems affect the ability of bacteria to biologically transform N compounds (Kim et al., 2011). Adequately linking the changes in quantitative composition of bacteria involved in the N-cycle to such variables may contribute to further improvements in WWT design and operation. Nevertheless, few studies are currently available taking advantage of multivariate analysis methods for this purpose (Huang et al., 2010; Kim et al., 2006). Furthermore, the number of studies quantifying simultaneously AOB, NOB and denitrifying bacteria in full-scale WWT plants is limited (Geets et al., 2007; Kim et al., 2011), and do not include the evaluation of the levels of gene expression.

Because of these concerns, in the present work, quantitative PCR (qPCR) and reverse-transcription qPCR (RT-qPCR) were applied to evaluate the abundance (qPCR) and levels of transcription (RT-qPCR) of gene markers specific for Bacteria, AOB, NOB, and denitrifiers (N_2O-reducers). The targeted bacterial groups were quantified during a time frame of nine months, spanning three seasons of the year (spring, summer, and autumn), in both the aerated and anoxic bioreactors (BRs) of a full-scale MBR operated in pre-denitrification mode, and fed urban wastewater. Throughout the experimental period, multivariate analyses of the data (non-metric multidimensional scaling, MDS, and BIO-ENV) were used to evaluate the effect of fluctuations in the characteristics of the influent wastewater, temperature, and biomass concentration in the BRs on the abundance of the targeted bacterial populations.

2. Methods

2.1. Description of the full-scale MBR experimental plant, operational parameters and performance

The experimental MBR plant, operational parameters, and performance were previously described (Gómez-Silván et al., 2013). Briefly, the system consisted of an aerated BR (19.4 m^3), an anoxic BR (6.8 m^3), and a filtration tank (2 m^3) equipped with three ultrafiltration hollow-fiber membrane modules (0.034 μm nominal pore size) made of polyvinylidenefluoride (PVDF) (GE Water & Process Technologies, Fairfield, USA). The MBR was fed urban wastewater taken from the pretreatment of the nearby "Estación Depuradora Sur"-WWT plant (Granada, Spain), managed by the company EMASAGRA S.A. It was operated in the pre-denitrification mode with fixed hydraulic retention time (HRT = 38 h) and solids retention time (SRT = 20 days). The inflow and outflow rates were 1 m^3/h, and the recirculation rate between the BRs was seven times the influent flow-rate (700%). Air was supplied to the aerated BR with a fine bubble membrane diffuser, automatically keeping the dissolved oxygen (DO) concentration in the range of 0.5–1.6 mg/L. The filtration tank was also aerated to control membrane fouling and clogging.

The temperature inside the activated sludge of the MBR system was measured automatically every second, and activated sludge daily medium internal temperature (IT) was calculated by the software Active Factory v.9.2 (Wonderware, Spain). Concentrations of fixed and volatile suspended solids (iFSS and iVSS), total nitrogen (tN), NH_4^+, total chemical oxygen demand (COD), and total biological oxygen demand at five days (BOD_5) were analyzed in influent and effluent (permeate) water. Fixed and volatile suspended solids (FSS and VSS) concentrations in the activated sludge of both BRs were also measured. The quality of the effluent was in agreement with the EU regulation for discharges to the environment (Directive 91/271/EEC, European Council, 1991) (Table S1).

2.2. Activated sludge sampling for qPCR assays

The biological tests in the activated sludge from the MBR plant were initiated when the system reached steady state conditions. The study spanned from April to December, including three different seasons of the year: spring (April and May), summer (June, July, August, and September) and autumn (October, November, and December), which encompassed the standard annual range of temperatures of the city of Granada (Gómez-Silván et al., 2013). Samples (50 ml) of activated sludge from both BRs (aerobic, A, and anoxic, X) were collected in sterile plastic containers once a month.

Two replicates were prepared for each DNA and RNA extraction, centrifuging 4 ml of the activated sludge samples (1 min, 14*g*) in a MiniSpin Plus table centrifuge (Eppendorf, Hamburg, Germany). After discarding the supernatant, the pellets intended for DNA extraction were carried at 4 °C from the experimental plant to the laboratory and stored at −20 °C. The pellets intended for RNA extraction were resuspended in 1 ml of RNAprotect® Bacteria Reagent (Qiagen, Hamburg, Germany) and carried unrefrigerated from the experimental plant to the laboratory, then precipitated again by centrifuging (1 min, 14*g*) in a MiniSpin Plus table centrifuge (Eppendorf, Hamburg, Germany), discarding the supernatant and storing the pellets at −20 °C.

2.3. Nucleic acids extraction and purification

Genomic DNA and RNA were extracted from the preserved samples using the FastDNA® SPIN kit for Soil and the FastRNA® PRO BLUE kit, respectively, following the manufacturer's indications. The FastPrep®24-Instrument (MP-Bio, Santa Ana, CA, USA) was used for all extractions.

Traces of DNA in the RNA samples were digested by the rigorous procedure of TURBO DNA-*free*™ kit (Ambion®, Life Technologies Corporation, Carlsbad, CA, USA), following the manufacturer's indications, and were subsequently purified using the RNA Cleanup protocol from the RNeasy Mini Kit (Qiagen, Hamburg, Germany).

The concentrations and quality of DNA and RNA extractions were measured with a BioPhotometer Plus (Eppendorf, Hamburg, Germany).