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Bacterial community dynamics within an aerobic granular sludge reactor treating wastewater loaded with pharmaceuticals

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ABSTRACT

Pharmaceuticals are micropollutants often present in wastewater treatment systems. In this study, the potential impact of such micropollutants on the bacterial population within aerobic granular sludge (AGS) bioreactor was investigated. The AGS bacterial community structure and composition were accessed combining DGGE fingerprinting and barcoded pyrosequencing analysis. Both revealed the existence of a dynamic bacterial community, independently of the pharmaceuticals presence. The AGS microbiome at both phylum and class levels varied over time and, after stopping pharmaceuticals feeding, the bacterial community did not return to its initial composition. Nevertheless, most of the assigned OTUs were present throughout the different operational phases. This core microbiome, represented by over 72% of the total sequences in each phase, probably played an important role in biological removal processes, avoiding their failure during the disturbance period. Quantitative-PCR revealed that pharmaceuticals load led to gradual changes on the abundance of ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and polyphosphate-accumulating organisms (PAO) but their persistence during that phase demonstrated the resilience of such bacterial groups. AGS microbiome changed over time but a core community was maintained, probably ensuring the accomplishment of the main biological removal processes.

1. Introduction

The aerobic granular sludge (AGS) technology has been successfully applied for the treatment of industrial and domestic wastewaters (Adav et al., 2008). In the last years, the implementation of AGS at full-scale wastewater treatment plants has been growing worldwide (Li et al., 2014; Pronk et al., 2015). Aerobic granules are spherical sludge aggregates composed by bacteria embedded in a matrix of extracellular polymeric substances (Adav et al., 2008). The compact structure of AGS, leads to the formation of an oxygen diffusion gradient within these aggregates, thus creating a layered structure with aerobic, anaerobic and anoxic zones (de Kreuk et al., 2005). The presence of these different zones inside granules will allow the coexistence of different bacterial groups, which in turn are involved in the main biological removal processes. In this way, through application of a fill-and-draw system operation mode, concomitant removal of carbon, nitrogen and phosphorous can be accomplished inside granules. Hence, wastewater treatment can be carried out in a single unit, which is one of the attractive AGS technology features since it greatly reduces the plant footprint (de Kreuk et al., 2005). However, the capacity of the system to eliminate carbon, nitrogen and phosphorus compounds could be compromised due to the presence of environmental contaminants (e.g. pharmaceuticals) in influent wastewaters (Amorim et al., 2016; Moreira et al., 2015; Shi et al., 2013). In fact, pharmaceuticals are a class of emerging environmental contaminants that have been often detected both in domestic and industrial wastewaters (Santos et al., 2013). Even at low levels, they can affect the composition of the microbial communities, and hence disturb the established metabolic networks (Caracciolo et al., 2015).

Presently, there is a tighter legislation on effluent water quality standards, thus systems should be able to properly remove nutrients from wastewater before its discharge into natural water bodies. The excess of nutrients in wastewater effluents can cause negative ecological effects, namely harmful algal blooms and fish mortality (Carey and Migliaccio, 2009). As bacteria play a key role in carbon, nitrogen and phosphorous removal, a better understanding on the ecology and microbiology in the AGS system is necessary to understand treatment performance. Up to date, most studies on AGS technology treating recalcitrant wastewaters have mainly focused on treatment performance and operation conditions and do not properly assess impact on the

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bacterial community (Moreira et al., 2015; Shi et al., 2013; Xia et al., 2014; Zhao et al., 2015).

For this reason, understanding the effect of the environmental contaminants frequently present in wastewaters, such as pharmaceuticals, on the AGS microbial community is crucial for this technology implementation. The removal of pharmaceuticals present in wastewater and the effect on COD, nitrogen and phosphorous removal performance have been assessed demonstrating that AGS was able to remove pharmaceuticals by biotic and abiotic processes, but the main biological removal processes were temporally affected by the presence of such micropollutants (Amorim et al., 2016). Therefore, in the present study, a combination of different molecular biology methods was applied to follow the bacterial population inhabiting granules when exposed to that scenario. The bacterial community structure and dynamics were evaluated firstly using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and secondly by 454-pyrosequencing of 16S rRNA gene amplicons. DGGE analysis gave a first overview on the compositional patterns and dynamics while pyrosequencing allowed deeper insights into the structure of bacterial community within AGS. Additionally, quantitative PCR (*q*PCR) was performed to infer about the abundance of bacterial phylogenetic groups involved in the main biological removal processes.

2. Material and methods

2.1. AGS-SBR set-up and operation

Detailed set-up and operational information of the lab-scale AGS-SBR was given elsewhere (Amorim et al., 2016). Briefly, the reactor was operated in successive 6 h cycles comprising 60 min of anaerobic feeding, 292 min of aeration, 3 min of settling and 5 min of effluent withdrawal. The SBR operation lasted for 86 days and was split into three phases: phase I (day-0 to day-28), phase II (day-29 to day-57) and phase III (day-58 to day-86). The SBR was fed with a synthetic wastewater which composition was similar to the one used by de Kreuk et al. (2005). During phase II, the influent wastewater was amended with pharmaceuticals from different therapeutic classes, namely alprenolol, bisoprolol, metoprolol, propranolol, venlafaxine, salbutamol, fluoxetine and its metabolite norfluoxetine (Amorim et al., 2016). The selection of these pharmaceuticals was mainly based on their frequency in real wastewater influents and the applied concentrations were similar to those reported in monitoring studies (Santos et al., 2013).

2.2. Bacterial community analysis

2.2.1. Genomic DNA extraction

AGS mixed liquor samples were collected from the reactor during the aeration phase, on different operational days. The collected biomass was aseptically crushed, using a pottering tube and a pestle. The resulting bacterial suspension was then used for genomic DNA extraction using the UltraClean Microbial DNA Isolation Kit (MoBio, USA) according to manufacturer's instructions. The DNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). The extracted DNA was stored at − 20 °C for further use.

2.2.2. DGGE analysis of PCR-amplified 16S rRNA gene fragments

The V3 region of bacterial 16S rRNA gene was amplified using the primers set 338F and 518R with a GC clamp attached to the forward primer (Muyzer et al., 1993). Amplification was performed in a Bio-Rad iCycler (Bio-Rad, USA) and the PCR reaction mixture and temperature profile were as described by Amorim et al. (2014). The PCR-amplified fragments were separated by DGGE using DCode Universal Mutation Detection System (Bio-Rad, USA) and a denaturing gradient ranging from 35% to 70%. The DGGE banding patterns were analyzed with Bionumerics software (Applied Maths, Belgium). A dendrogram was constructed using Pearson similarity coefficient with 1% tolerance and clustered according to the unweight pair group mean average (UPGMA) algorithm. Bacterial diversity was estimated by the Shannon diversity index (H) (Shannon and Weaver, 1963) and the evenness of the community was estimated by an equitability index (E) (Pielou, 1975).

2.2.3. Barcoded 454 pyrosequencing and data analysis

Total DNA from AGS mixed liquor samples was prepared for 454 pyrosequencing by PCR amplification of the V3–V4 hypervariable region using the forward primer 5′- ACTCCTACGGGAGGCAG-3′ and the reverse primer 5′- TACNVRRGTHTCTAATYC -3′. These primers were ligated to the Roche-454 A and B sequencing adapters and to an eight-base barcode sequence. Fragments were amplified in 40 μL reactions with Advantage Taq (Clontech, USA) using 0.2 μM of each primer, 0.2 mM dNTPs, 1X polymerase mix and 6% DMSO. PCR conditions were 94 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 44 °C for 45 s and 68 °C for 60 s and a final elongation step at 68 °C for 10 min. The amplicons were quantified by fluorometry with PicoGreen (Invitrogen, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, accordingly to manufacturer's instructions (Roche, 454 Life Sciences, USA) at Biocant (Cantanhede, Portugal). Each DNA sequence was afterwards traced back to its original sample through barcode analysis.

Sequences were processed using both UPARSE and QIIME pipelines on Linux© operating system as previously described by Alves et al. (2016). Briefly, in the UPARSE workflow, barcodes were striped and reads were quality filtered setting the maximum expected number of errors to 1.0, trimmed to 350 bp and dereplicated (identical reads were merged with the selection of unique sequences). Finally, Operational Taxonomic Units (OTUs) were defined at 97% similarity using UPARSE-OTU algorithm that simultaneously identifies and discards chimeras. Taxonomy assignment was made through QIIME using Uclust as assignment method and Greengenes as reference database. Concerning alpha-diversity, different diversity indexes were calculated using *vegan* package from R software (www.r-project.org) through the functions *diversity* and *diversity/log(specnumber)* for Shannon's diversity and Pielou's eveness, respectively, and *estimateR* function for Chao1 and ACE indexes. A membership Venn diagram was computed using the MetaComet web platform to determine the specific and shared OTUs across the bioreactor phases. The heat map of the top 10 most abundant OTUs was constructed using *hclust, vegdist*, *colorRampPalette* and *heatmap.2* functions from *stats, vegan* , *RcolorBrewer* and *gplots* packages, respectively, on R software. All nucleotide sequences obtained are available in the NCBI platform under BioProject accession number PRJNA318174.

2.2.4. Quantification of targeted bacteria by real-time polymerase chain reaction (qPCR)

The abundance of total bacteria, ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and polyphosphate-accumulating organisms (PAO) was determined by qPCR using primer sets 338F/518R, amoA-1F/amoA-2R, nxrB-169F/nxrB-638R and 518F/PAO846R, respectively (He et al., 2007; Muyzer et al., 1993; Pester et al., 2014; Rotthauwe et al., 1997). The PCR mixture (20 μL) consists of 10 μL SsoAdvanced™ Universal SYBR® Green Supermix (2×) (Bio-Rad, USA), 0.5 μL of each forward and reverse primers (10 μM), 5 μL of DNA template and 4 μL of nuclease free water. For unknown samples, each DNA amplification reaction was performed with approximately 1 ng of total genomic DNA and triplicate PCR reactions were performed for each sample. PCR amplifications were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) according to the following conditions: 98 °C for 3 min; followed by 40 cycles consisting of 98 °C for 30 s, annealing at the given temperatures for each primer set for 45 s and 60 °C for 45 s; and a final melting curve with temperature ramping from 65 to 95 °C. The annealing temperature for the total bacteria, AOB, NOB and PAO-targeting primers were 55, 51, 52 and 65 °C, respectively. Serial 10-fold dilutions of plasmid DNA harboring