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The microbial community in a high-temperature enhanced biological phosphorus removal (EBPR) process

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ABSTRACT

An enhanced biological phosphorus removal (EBPR) process operated at a relatively high temperature, 28 °C, removed 85% carbon and 99% phosphorus from wastewater over a period of two years. This study investigated its microbial community through fluorescent *in situ* hybridization (FISH) and clone library generation. Through FISH, considerably more *Candidatus* "Accumulibacter phosphatis" (Accumulibacter)-polyphosphate accumulating organisms (PAOs) than *Candidatus* 'Competibacter phosphatis' (Competibacter)-glycogen accumulating organisms were detected in the reactor, at 36 and 7% of total bacterial population, respectively. A low ratio of Glycogen/Volatile Fatty Acid of 0.69 further indicated the dominance of PAOs in the reactor. From clone library generated, 26 operational taxonomy units were retrieved from the sludge and a diverse population was shown, comprising Proteobacteria (69.6%), Actinobacteria (13.7%), Bacteroidetes (9.8%), Firmicutes (2.94%), Planctomycetes (1.96%), and Acidobacteria (1.47%). Accumulibacter are the only recognized PAOs revealed by the clone library. Both the clone library and FISH results strongly suggest that Accumulibacter are the major PAOs responsible for the phosphorus removal in this long-term EBPR at relatively high temperature.

1. Introduction

Enhanced biological phosphorus removal (EBPR) process is an activated sludge process tailored for phosphorus removal. This process is based on the enrichment of polyphosphate accumulating organisms (PAOs) in the activated sludge community [1]. Till date, *Candidatus* "Accumulibacter phosphatis", hereafter abbreviated as Accumulibacter, are the best known PAOs present in EBPR process. These organisms are able to store phosphate as intracellular polyphosphate via alternating anaerobic-aerobic conditions. Carbon sources, particularly volatile fatty acids (VFAs) are taken up by PAOs anaerobically and stored as polyhydroxyalkanoates (PHAs) using energy generated mostly from hydrolysis of polyphosphate and partly from degradation of glycogen. In the subsequent aerobic condition, a greater amount of phosphorus is taken up to replenish polyphosphate reserve and accompanied by intracellular organic matter degradation for biomass growth. Phosphorus removal is achieved via removal of PAO biomass from the waste activated sludge.

As in the case of many biological wastewater treatment processes, microbial activity in EBPR process is affected by climates, particularly temperature. Temperature appears to be one of the key factors influencing the PAOs-GAOs (glycogen accumulating organisms) competition in the EBPR process [2–4]. Like PAOs, GAOs take up VFAs anaerobically without performing anaerobic phosphorus release or aerobic phosphorus uptake. When GAOs are present in significant numbers, they will compete with PAOs for carbon sources, which in turn limit the potential of PAOs for aerobic phosphorus uptake [4]. A group of GAOs named *Candidatus* 'Competibacter phosphatis', hereafter named Competibacter, has been commonly found in laboratory- and full-scale EBPR processes [5].

Successful EBPR operation has been observed at very low temperatures, as low as 5 °C [6], though a higher sludge age was necessary due to the decrease in process kinetics at low temperatures. Low temperatures in the range of 10–20 °C, have been found

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to favor the growth of PAOs and thus improve EBPR performance [2,7]. Lopez-Vazquez [8] claimed that at 10 °C, the anaerobic metabolism of GAOs, in particular the anaerobic glycogen hydrolysis, was inhibited and limiting the substrate uptake rate, thus, the growth of GAOs. At higher temperatures (> 20 °C), caused by seasonal variations [9,10] or geographical location [11] deterioration of EBPR capacity was observed. It is hypothesized that PAOs are less competitive than GAOs at higher temperatures. At the laboratory scale, researchers have studied the temperature effects on EBPR and the PAOs-GAOs competition [2,3,7,8,12]. In general, these reports agree that at temperatures higher than 20 °C, the EPBR activity tends to deteriorate and GAOs become dominant. These experimental evidences indicate that the operation of EBPR process in tropical climates could be challenging.

Nevertheless, there have been successful cases of EBPR process operated at 30 °C by Freitas et al. [13] with short sequencing batch reactor (SBR) cycle, and Winkler et al. [14] through selective sludge removal in a segregated aerobic granular biomass system. In our previous study on the long term performance evaluation of a lab scale EBPR process at 28 °C, stable phosphorus removal efficiency of 99% was observed over a period of two years [15]. From these studies, doubts are casted over the predominance of GAOs in high-temperature EBPR systems. Curiosity of the microbial population present that contributes to EBPR is also raised. Although much knowledge of EBPR microbiology has been accumulated over the years, microbial study of high-temperature EBPR system still lacking. Following the success in operating a lab-scale EBPR reactor at 28 °C [15], we further examine the microbial community involved. Firstly, the most recognized Accumulibacter-PAOs and Competibacter-GAOs were examined through fluorescent *in situ* hybridization (FISH). Secondly, a clone library was generated to assess the microbial community structure of the aforementioned 28 °C EBPR reactor. This work aimed to shed some lights on the bacterial groups that drive the high-temperature EBPR.

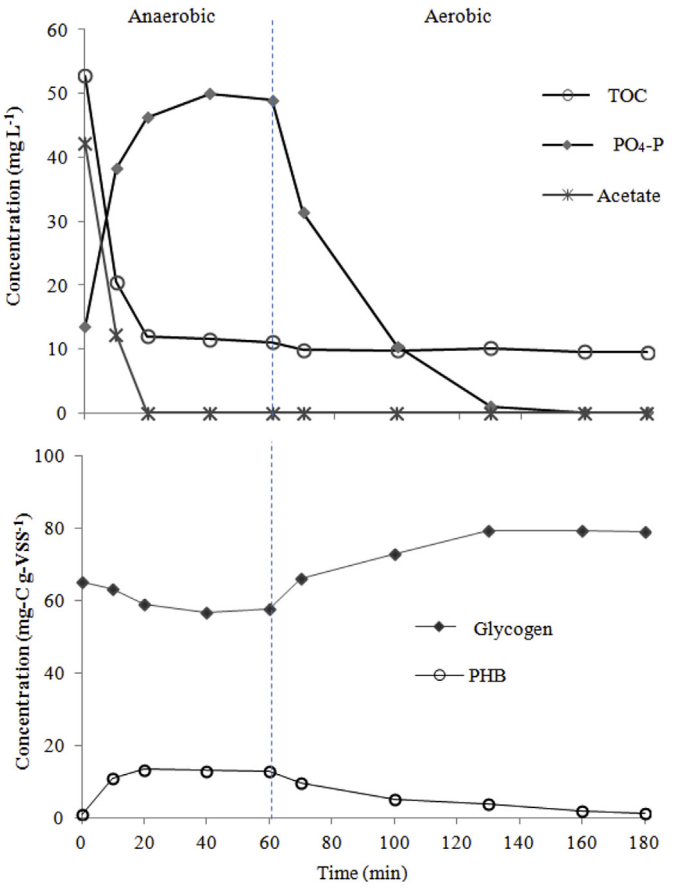

Fig. 1. Typical cyclic concentration profiles of phosphorus, TOC and acetate; and intracellular carbohydrate and PHB in a SBR cycle of day 700 during steady state of reactor operation.

2. Materials and methods

2.1. Long term EBPR reactor operation at 28 °C

The EBPR process was operated over a two-year period in a SBR at 28 °C using synthetic wastewater, with solids retention time (SRT) and hydraulic retention time (HRT) set at 10 d and 10 h respectively, as detailed in Ong et al. [15]. The key features were that acetate, yeast extract and peptone were the main carbon sources, constitute to 50 mg C L^{-1} in the feed. The carbon to phosphorus ratio (C:P) was 3:1.

Under steady state conditions, the process removed over 85% of total organic carbon and 99% phosphate, with phosphorus concentration in the effluent below 1.0 mg L^{-1}. Fig. 1 illustrates the biochemical transformations of a SBR cycle at 28 °C during the two-year operation. Sludge sample was collected from the reactor during steady state for the subsequent chemical and microbial analysis.

2.2. Microbial characterisation with fluorescent in situ hybridization (FISH)

Sludge samples were collected periodically and fixed in 4% paraformaldehyde. FISH was performed according to [16] to study the relative abundance of PAOs and GAOs in the microbial community. FISH samples were observed using a fluorescence microscope (Model DM 2500, Leica, German) and images were captured with a cooled charged-coupled device camera (Model DFC 310 FX, Leica, German). The oligonucleotide probes used in this study included 5′ FITC labeled EUBmix (i.e., EUB338, EUB338-II, EUB338-III) that targets most of the bacteria, 5′ Cy3 labeled PAOmix (i.e., PAO462, PAO651, PAO846) that targets most of the PAOs members in Accumulibacter [17] and 5′ Cy3 labeled GB probe that targets most of the GAO members in Competibacter [17,18]. A minimum of 20 microscopic fields were captured randomly for each sample. FISH quantification of the PAOs and GAOs was done by image analysis software VideoTesT- Morphology 5.1. The relative abundance of PAOs or GAOs was determined respectively as the ratio of the mean image areas with a positive signal for PAOmix or GB to the area with a positive signal for EUBmix.

2.3. Microbial characterization through DNA extraction and polymerase chain reaction (PCR) amplification

The sludge was extracted for total genomic DNA using ZR Soil Microbe DNA Micro Preps (Zymo Research, USA) according to the manufacturer's instructions. The DNA template was subjected to PCR by using bacterium specific 11f (5′-GTTTGATCCTGGCTCAG-3′) and 1512r (5′-GGYTACCTTGTTACGACTT- 3′) primers. The PCR mixture contained 4 μL of DNA template, 3 μL of each primer (10 μM), 37.5 μL GoTaq® Green Master Mix 2X (Promega, USA), and 27.5 μL of sterile ultra pure water. PCR amplification was carried out in a thermocycler (MyCycler, Biorad, USA) with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min, followed by a final extension at 72 °C for 15 min. The PCR products were then purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA).