Study of bacterial population dynamics in sequence batch reactors under different operating conditions in the presence of a metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS)

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Abstract. The process of uncoupled metabolism has been studied since the ´90s for the reduction of sludge production. Under normal growth conditions, the catabolism and anabolism of bacteria are coupled, however under uncoupled conditions (brought on by chemical uncouplers), the catabolism is undisturbed leading to unchanged substrate consumption, while the ATP synthesis and anabolism slow down causing reduction in biomass yield. The 3,3',4',5-tetrachlorosalicylanilide (TCS) has been widely adopted as an environmentally-benign uncoupler to reduce yield of activated sludge. However, its potential impact, in the microbial community of SBR activated sludge, is unknown yet. Four parallel sequence batch reactors (SBR) with and without TCS addition were operated under different conditions, two F/M ratios, to research the microbial population dynamics by uncouplers. The 16S rRNA gene amplicon sequencing (NGS) was performed to reveal the microbial community. During the 41 days operation, the TCS of the SBR was 1 mg/L. Specific comparisons down to the family, class and genus level were done from Illumina-MiSeq amplicons. This study provides a general view of the composition of microbial communities in activated sludge of SBR operated under different conditions with TCS

Keywords: NGS, TCS, Metabolic uncoupler, Microbial communities, sludge reduction

1. Introduction

The process of uncoupled metabolism has been studied since the ´90s for the reduction of sludge production. Russel and Cook (1995) introduced the term “uncoupling” to define the inability of oxidative phosphorilation to generate the maximum theoretical quantity of metabolic energy in the form of ATP. Under normal growth conditions, the catabolism and anabolism of bacteria are coupled, however under uncoupled conditions (brought on by chemical uncouplers), the catabolism is undisturbed leading to unchanged substrate consumption, while the ATP synthesis and anabolism slow down causing reduction in biomass yield. The phenomenon of uncoupled metabolism may be carried out under abnormal conditions such as the presence of inhibitory compounds or some heavy metals (Zn, Ni, Cu, Cr), not optimal temperatures, nutrient limitations, oxic-anoxic (or oxic-anaerobic) cycling conditions and chemical uncouplers (Foladori *et al* 2010, Velho *et al* 2016). The commonly used chemical uncouplers include DNP (2,4-dinitrophenol), TCP (2,4,6-trichlorophenol), pNP (para-dinitrophenol), mNP (m-nitrohenol), oNP (o-nitrophenol), mCP (m-chlorophenol), pCP (p-chlorophenol), TCS (3,3',4',5-tetrachlorosalicylanilide) and THPS (tetrakis (hydroxymethyl) phosphonium sulfate) (Wang *et al*. 2017). The TCS has been widely adopted as an environmentally-benign uncoupler to reduce yield of activated sludge. However, its potential impact, in the microbial community of SBR activated sludge, is unknown yet.

In recent years, high-throughput sequencing has become a popular method because it provides sufficient sequencing depth to cover complex microbial communities (Ye and Zhang 2013, Ju and Zhang 2015).The 16S rRNA next generation sequencing involves PCR amplification of taxonomically informative regions of the 16S ribosomal rRNA (rRNA) gene by using mixtures of primers corresponding to conserved regions flanking the informative variable regions (Conrad and Vlassov 2015). These are then subjected to next-generation DNA sequencing (NGS), enabling the classification of individual reads to specific taxa at various levels from phylum to genus (Conrad and Vlassov 2015). The activated sludge process has a very high phylogenetic diversity (Yang *et al* 2011).

Different bacteria with variable cell structures in activated sludge might show different responses to TCS treatment. In the present study, NGS was used to analyze in detail the bacterial community structure of AS samples with TCS addition at different organic loading rates (F/M).

2. Methods

2.1. Continuous operation of four sequence batch reactors

In order to identify the effect of TCS at different (F/M), batch tests were conducted (Table 2). First, Two 5-L sequence batch reactors (SBR) were operated in batch mode in parallel.

Reactor (R1) was operated with 1.0 mg/L TCS. Reactor 2 (R2) was operated as the control reactor without TCS addition. The two SBR were operated under F/M of 0.18 kg CODs/kg MLVSS d F/M for 42 days at room temperature in 8 h cycle with 15 min for filling phase, 6 h for aeration phase, 1.5 h for settling phase and 15 min for drawing phase. The experiment was repeated at different F/M (0.35 kg CODs/kg MLVSS d) with two SBR, reactor 3 (R3) with 1.0 mg/L TCS addition and reactor 4 (R4) without TCS addition.

2.2. DNA extraction and PCR-based Illumina sequencing

Total DNA of 1 ml activated sludge sample was extracted in duplicate. Lysis was performed with the FastPrep® -24 instrument at 6 m/sec for 40 sec and the DNA was extracted using the FastDNA® SPIN kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. OneStep™ PCR Inhibitor Removal Kit (Zymo Research, CA, USA) was used in order to remove sample inhibitors which can affect downstream enzymatic reactions such as PCR. For Illumina amplicon sequencing of the hypervariable V3–V4 region of bacterial 16S rRNA gene, the primers PRO341F and PRO805R were used (Takahashi *et al*., 2014) by Fundación FISABIO sequencing service (Valencia, Spain). Libraries were sequenced using 250 bp paired end sequencing chemistry on an Illumina MiSeq platform by Fundación FISABIO sequencing service (Valencia, Spain) using a 2 × 300 nucleotide paired reads protocol.

2.3. Bioinformatics analysis

Processing of reads was carried out using QIIME pipeline version 1.8.0 (http://qiime.org/). Operational Taxonomic Units (OTUs) were picked using the open reference OTU clustering script, in which the representative sequence of each OTU were assigned taxonomy using EzBioCloud database clustered at 97% identity using default parameters.

2.4. Membrane integrity analysis

The Film Tracer™ LIVE/DEAD® Biofilml viability kit (Molecular Probes Eugene, OR, USA) was used for the estimation of the membrane integrity of bacteria (viable/intact plus dead/damaged bacteria). It contains two nucleic acid stains. The green fluorochrome (SYTO9) is a small molecule that can penetrate intact plasma membranes while the larger red fluorochrome (propidium iodide) penetrates only compromised membranes. One milliliter of activated sludge sample was mixed with 3 µL of the dye mixture (SYTO9/Propidium iodide, 1:1) and incubated for 15 minutes at room temperature (Ramírez *et al* 2000). The stained samples were evaluated with a BX50F microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury lamp. Viable cells result in green colour while damaged cells result in red colour. The image analysis software used to analysis of the stained samples was BioImageL™ v. 2.1 (Chávez de Paz 2009). The number of images taken for every sample was 20.

3. Results and Discussion

Membrane integrity is one of the most reliable criteria for differentiating viable and dead bacteria cells (Kramer *et al*. 2009). As shown in Figures 1A and 1B, with the addition of TCS, the viable biomass decreased at the two F/M ratio assayed (0.18 and 0.35 kg CODs/kg MLVSS d). The results indicated that after the sludge was exposed to TCS at 1 mg/L the viable biomass displayed a substantial decrease, with a reduction rate reaching 59% at 0.35 F/M and 43% at 0.18 F/M. The TCS addition drove to a diminution of the proton gradient across cell membrane, which decreased the ATP generation (Liu 2003). It implied that the biomass growth was lower in the reactors where TCS was added. Addition of chemical metabolic un-couplers would uncouple bacterial catabolism from their anabolism which has become an effective technology to achieve sludge reduction in the wastewater treatment line (Wang *et al* 2017).

The differences in global composition of bacterial communities of SBR 1 (with TCS) and SBR2 (without TCS) operated under F/M of 0.35. The differences in composition of bacterial communities of SBR 3 (with TCS) and SBR4 (without TCS) operated under 0.18 F/M are showed in Figure 2. The most abundant phyla in samples SBR1, SBR 2, SBR3 and SBR 4 were: *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Planctomycetes*, *Bacteroidetes* and *Verrumicrobia* (Figure 2A). The SBR1, SBR 2, SBR3 and SBR 4 samples showed microbial communities where most of the member belonged to the phylum *Proteobacteria* (>40% abundance relative) (Figure 2A). The relative abundance of the phylum *Proteobacteria* increased, while those of the phylum *Chloroflexi* decreased during TCS addition. Members of *Chloroflexi* are metabolic versatile (Miura *et al*. 2007). *Chloroflexi* excrete hydrolytic enzymes involved in the conversion of proteins, polysaccharides and dead cell debris (Nielsen *et al*. 2010; Yamada *et al*. 2005). Therefore, a lower abundance of *Chloroflexi* phylotypes can interfere in the biodegradation processes of organic compounds.

The change pattern of viable bacterial population during the TCS addition suggested that there are some TCS-tolerant bacteria in activated sludge, which require more TCS to break their membranes. *Escherichia coli* (*Gammaproteobacteria*) was found to adapt to the uncoupler of oxidative phosphorilation DNP (Gage and Neidhardt, 1993). The rates of synthesis of 53 proteins were increased following exposure to DNP (Gage and Neidhardt, 1993). *Gammaproteobacteria* increased the relative abundance during TCS treatment under the two F/M ratio assayed (Figure 2B).

The study of adaptation to TCS at two F/M ratio may shed some light on how a bacterial community modifies the structure in order to survive and grow under stressful conditions.

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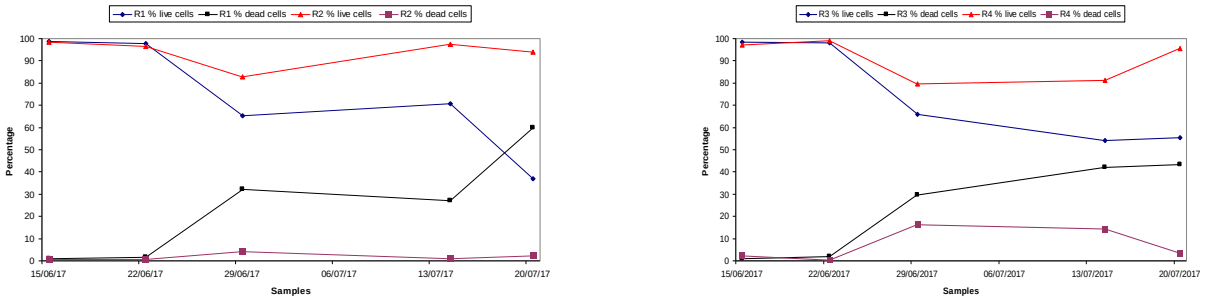
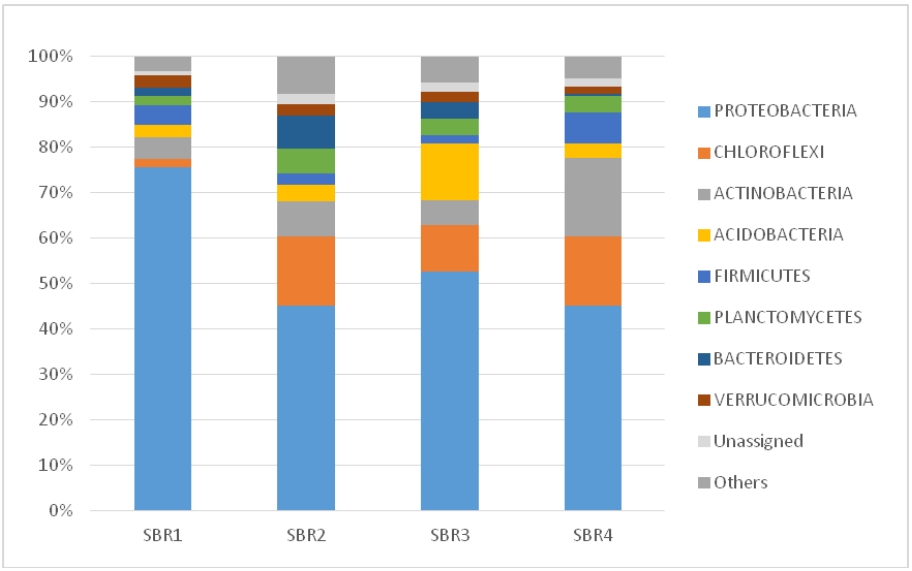
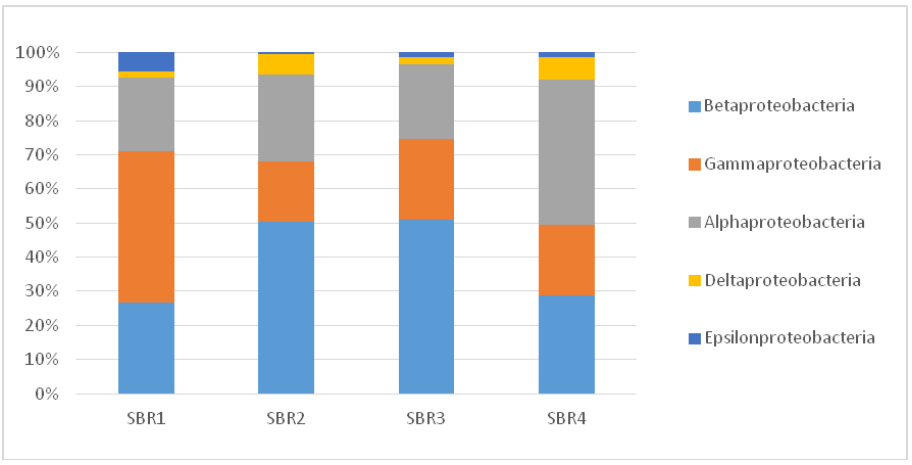


Figure 1. A) Percentage of live and dead cells. Reactor (R1) was operated with 1.0 mg/L TCS. Reactor 2 (R2) was operated as the control reactor without TCS addition. The two SBRs were operated under F/M of 0.18 kg CODs/kg MLVSS d F/M. 1B) (R3) was operated with 1.0 mg/L TCS Reactor 4 (R4) was operated as the control reactor without TCS addition. The two SBRs were operated under F/M of 0.35 kg CODs/kg MLVSS d F/M.



(A)



(B)

Figure 2. A) Composition of SBR samples at phyla level. B) Composition of SBR samples at *Proteobacteria* class level.

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