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Change of microbial community composition in anaerobic digesters during the degradation of nonylphenol diethoxylate

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ABSTRACT

Nonylphenol polyethoxylate (NPnEO) surfactants, ubiquitous contaminants in sewage processing facilities, continue to receive attention due to their recalcitrance and diverse toxicity concerns. How the addition of nonylphenol diethoxylate (NP2EO) and its biodegradation products affect microbial community structure in anaerobic semi-continuous digesters was investigated by molecular techniques. Fluorescence *in situ* hybridization (FISH) and quantitative PCR (qPCR) were used in parallel to monitor total Bacteria, four classes of Proteobacteria, total Archaea, and two genera of acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*). Beta- and Gammaproteobacteria were the dominant phyla and the relative abundances of both groups were roughly doubled following NP2EO addition. While *Methanosaeta* was dominant over *Methanosarcina* throughout, NP2EO addition and accumulation of NP nearly doubled the relative abundance of *Methanosarcina*. While digester performance was not affected, this study revealed that relative abundances of the key players in anaerobic digestion were altered by the addition of NP2EO.

1. Introduction

Due to their diverse applications and widespread usage, surfactants are common organic pollutants entering domestic wastewater treatment facilities. Nonylphenol polyethoxylates (NPnEO) are used as nonionic surfactants in formulations of household, industrial and agricultural products (Birkett and Lester, 2002). The common use of NPnEO has led to continual input of these chemicals into industrial and domestic wastewater treatment plants, making the understanding of fate and effects of NPnEO critical.

Biodegradation of nonylphenol polyethoxylates starts by loss of ethoxy groups, leading to the formation of more toxic and persistent metabolites; nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), and the more water-soluble nonylphenoxy acetic acid (NP1EC) and nonylphenoxyethoxy acetic acid (NP2EC) (Ahel et al., 1994). Due to low solubility and high log K_{oc} values, these metabolites tend to accumulate on sewage sludge following anaerobic treatment.

Previous studies revealed that NPnEO and NP compounds have diverse toxic, carcinogenic and estrogenic effects (Liu et al., 2011; Soto et al., 1991). While the primary concern to date has been NP's estrogen-mimicking potential (Jobling and Sumpter, 1993; Rodgers-Gray et al., 2001), several studies have also shown that NPEOs and NP are acutely toxic to microorganisms (Soares et al., 2008; Nobels et al., 2011; Symsaris et al., 2015) and cause shifts in Bacterial communities in soil systems (Lozada et al., 2004; Wang et al., 2015) and aquatic systems (Zhang et al., 2008). However, it is not yet clear whether these observed community shifts are due to toxicity effects, utilization of the compounds for growth, or a combination of both.

Anaerobic digestion is a well-established technology for mass reduction of waste and sustainable energy generation via production of methane (Speece, 2008). However, anaerobic digesters are still somewhat regarded as a black box regarding the details of the microbial communities and the metabolic processes involved. Guo et al. (2015), utilizing a metagenomic approach to characterize microbial community structure of anaerobic digester sludge from a full-scale WWTP, reported that Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria accounted for 41.2%, 12.5%, 9.6%, and 5.2% of the total Bacterial

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community, respectively. Within the Proteobacteria, Alpha-made up 36%, Beta- 25%, Delta- 22%, and Gamma- 16%. Sidhu et al. (2017), characterizing the community of an Upflow Anaerobic Sludge Blanket reactor, described very similar relative abundances at the phylum level, with Proteobacteria making up 53.2% of the community. Proteobacteria are a crucial phylogenetic group in anaerobic digestion processes due to their involvement in glucose, propionate, butyrate, and acetate-consuming microbial communities (Ariesyady et al., 2007; Guo et al., 2015). Thus, this phylum might be one of the most important components in the degradation of organic molecules such as NP compounds in anaerobic digesters.

Acetoclastic methanogenesis is considered to account for most of the methane production (70%) in anaerobic digestion processes. *Methanosarcina* and *Methanosaeta* are considered the two most important genera of Archaeal acetoclastic methanogens involved in biogas production. It has been demonstrated that methanogens are vulnerable to changes in the loading rate, temperature, pH or presence of toxic organic compounds (De Vrieze et al., 2012; Guo et al., 2015). Overall, syntrophic community structure has been shown to be closely associated with anaerobic digester performance (Li et al., 2015).

NP compounds (NP, NP1EO, and NP2EO) have notable toxic effects on various microbes at low concentrations. These NP compounds are also ubiquitously detected in sewage treatment systems. While there has been some research into how NP compounds affect microbial communities under aerobic conditions, how anaerobic digester communities are affected by the presence and degradation of NP compounds is not well understood. The purpose of the present study was to investigate how NP compounds affect the distribution and relative abundances of microbial taxa that are key players in the syntrophic community of anaerobic digesters; four Proteobacteria classes (Alpha-, Beta-, Gamma- and Delta-) and the two major genera of acetoclastic methanogens, *Methanosarcina* and *Methanosaeta.* This was accomplished by application of fluorescent *in situ* hybridization (FISH) and qPCR assays. In order to approach real-world substrates and lower contaminant concentrations (Gu, 2016), these assays were performed in mixed-culture anaerobic digesters seeded with and fed from a municipal wastewater treatment plant (Central Wastewater Treatment Plant, Ankara, Turkey) and amended with low concentration of NP2EO (3 ppm mass/volume).

2. Materials and methods

2.1. Digester design and operation

In order to set anaerobic digesters, waste activated sludge (WAS) and anaerobically digested sludge (ADS) were collected from Ankara Central Wastewater Treatment Plant (Ankara, Turkey). The plant has a current flow rate of 765,000 m^3 day^{-1}. WAS taken from a return line of a secondary sedimentation tank was used as a feed source and ADS obtained from mesophilic digester was used as inoculum (seed) for the setup of the laboratory scale semi-continuous digesters. The food to microorganism ratio (F/M) was determined based on g-VS/g-VSS and set to 1 by mixing required volumes of WAS and ADS samples.

Two sets of duplicate lab-scale semi-continuous anaerobic digesters (biotic control and NP2EO spiked) were operated with 2 L-active sludge-volume. Digesters were mixed continuously by magnetic stirrers and operated at 35 °C in a temperature-controlled dark room. 3000 μg/L NP2EO (in acetone) was spiked into one set of digesters (spiked digesters) on the 63rd day following the digester stabilization period. The digesters were considered to be at steady state (stabilized) when the variability in concentrations of MLSS, MLVSS and NP compounds was less than 10%. Biotic control digesters were spiked with acetone (without NP2EO) to observe background effect (originated from WAS taken from Ankara WWTP) and to see the result when NP2EO was not added to the digesters. NP2EO and degradation products were monitored during operation time. Digesters were connected to graduated gas collection cylinders (4 L) to monitor gas production during operation. All digesters were operated with a sludge retention time (SRT) of 15 days for 147 days.

2.2. Analytical methods

Methane production was determined as previously reported (Kara Murdoch and Sanin, 2016). Briefly, methane was measured with a gas chromatograph equipped with a HP-Plot Q capillary column (30.0 m × 530 μm x 40.0 μm) connected to thermal conductivity detector (TCD) (Agilent Technologies 6890N, USA).

Extraction of NP compounds (NP2EO, NP1EO, NP and NP1EC) from solid and liquid phases of sludge samples was carried out as previously described in Ömeroğlu et al. (2015). NP compounds were extracted from solid phases with acetone by sonication-assisted extraction. Derivatized extracts were introduced into a GC/MS (7890A Agilent gas chromatograph coupled to a 5975C Agilent mass spectrometer with Triple-Axis) equipped with a HP-5MS 5% phenyl methyl siloxane (30 m × 0.25 mm I.D., 0.25 μm film) column with helium as a carrier gas. Details of the derivatization procedure and oven program are given in Ömeroğlu et al. (2015).

Chemical oxygen demand (COD) of the sludge samples was measured by Hach Method 8000 (Jirka and Carter, 1975). The pH measurements were performed three times a week according to Standard Method 4500H (American Public Health Association et al., 1992).

2.3. DNA extraction

0.5 g (wet weight) of sludge was first suspended in 1 mL lysis buffer (E.Z.N.A. Soil DNA extraction kit, Omega Bio-Tek, USA), added into bead-beating tubes and incubated at 70 °C for 10 min. The tubes were then placed on ice for 5 min. These steps were repeated following a brief vortexing. The sludge samples were then homogenized with glass beads by vortexing at maximum speed for 20 min. After this point, the E.Z.N.A. Soil DNA extraction kit manufacturer's instructions were followed. Extracted genomic DNA samples were quantified using a UV–Vis spectrophotometer (Thermo Scientific, DR, 2000 NanoDrop). The OD260/OD280 and OD260/OD230 values obtained for all total sludge DNA samples varied between 1.8-2.0 and 2.0–2.2, respectively. 1/100 dilutions of sludge DNA extracts in water were used as templates in qPCR assays.

2.4. qPCR assays

Quantitative PCR assays were performed using an ABI 7500 Real Time PCR System (Applied Biosystems, USA). The 25 μL qPCR mixture was composed of 12.5 μL of EvaGreen Ssofast Supermix with Low ROX (Biorad, USA), X μL of each primer (depending on target group, given in Table 1), 5.0 μL of template DNA and the remaining volume sterile dH_2O. The thermocycling protocol applied was as follows; initial denaturation for 3 min at 98 °C followed by 35 cycles of 15 s at 98 °C and 1 min at the appropriate annealing temperature, which also served as the amplification temperature (Table 1). After amplification, a melting curve analysis was carried out to confirm that the signal obtained in qPCR originated from specific target PCR products and not from artifacts. qPCR amplifications were performed in triplicate for each DNA sample and standard.

PCR products representing each target group (Table 1) were cloned into the pGEM®-T Easy vector system as described in the manufacturer's protocol (Promega, USA). Following ligation of each target gene product into pGEM®-T Easy, they were transformed into competent *E. coli* JM109 (Promega, USA). The recombinant plasmids were isolated using a Plasmid Miniprep Kit (Promega) following sub-culturing. Quality and quantity of isolated plasmid DNA was determined by UV–Vis spectrophotometer.

10-fold serial dilution series of the recombinant plasmids were