Chemical Engineering Journal 325 (2017) 257–269

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Performance and bacterial community structure of a granular autotrophic nitrogen removal bioreactor amended with high antibiotic concentrations

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HIGHLIGHTS

- A CANON bioreactor was subjected to a mix of antibiotics at high concentrations.
- Nitrogen removal performance was irreversibly affected by the antibiotics.
- Granular biomass changed its structure when exposed to antibiotics.
- The fungus *Scedosporium boydii* proliferated under the antibiotics pressure.
- The bacterial community structure shifted to antibiotics-resistant phylotypes.

ARTICLE INFO

Article history:
Received 22 November 2016
Received in revised form 4 April 2017
Accepted 13 May 2017
Available online 15 May 2017

Keywords:
Anammox
Antibiotic
CANON
Granular biomass
Autotrophic nitrogen removal
Microbial population

ABSTRACT

An autotrophic nitrogen removal bioreactor with granular biomass was exposed to high antibiotics concentration in order to evaluate its impact over the performance and the biomass of this bioprocess. A mixture of azithromycin, norfloxacin, trimethoprim and sulfamethoxazole caused loss of autotrophic nitrogen removal performance, coupled to a deep change in the bacterial community diversity and structure of the granular biomass. Azithromycin, norfloxacin and trimethoprim were efficiently removed in the CANON bioreactor, reducing its concentration 77.9 ± 11.2%, 51.7 ± 10.7% and 57.8 ± 8.1%, respectively. The granular biomass changed significantly with the addition of the antibiotics, decreasing in settling velocity but increasing in compactness, losing its inner porous structure but developing a protective outer layer build of cell material. Prolonged operation under the antibiotics loading promoted the adaptation of multi-drug resistant fungus *Scedosporium boydii* fungal species and of *Acidovorax ebreus TPSY*, *Alcaligenes aquatilis*, *Paracoccus versutus* or *Ochrobactrum antropii*, which have been identified as human, animal and/or plant pathogens.

1. Introduction

The resistance to antibiotics of environmental bacteria is a human and environmental health problem at global scale in this era [1,2]. In terms of human health, antibiotic-resistant bacteria and fungi cause infections difficult to treat, with increasing mortality rates, longer hospital stays, and higher medical costs [3]. As well, ecological health could be endangered by the microbial evolution caused by antibiotics exposure in the environment [2]. It has been reported that the spread of antimicrobial resistance genes in the environment is completed through animal manure and human waste [2,4]. In this sense, wastewater treatment systems play a key role in the promotion of antibiotics-resistance microorganisms [5,6].

Many different animal and human wastes with high antibiotic concentrations are treated through the anaerobic digestion process. Among these, the most important are livestock manure and pharmaceutical industry effluents. The anaerobic digestion process has been regarded as an efficient treatment for livestock manure due to the reduction of its environmental hazard and its economic efficiency due to biogas production [7]. In addition, these processes offer several advantages over other biological treatments for

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http://dx.doi.org/10.1016/j.cej.2017.05.078
1385-8947/

pharmaceutical wastewater, such as less energy input, production of methane as valuable by product and less sludge generation, among others [8]. The reported antibiotics concentrations in these effluents are usually in the 10^0–10^2 mg L^{-1} order of magnitude [9–11]. On the other hand, the inefficiency of the anaerobic digestion process to remove antibiotics has been reported, showing low removal percentages for some of these [10] and effluent concentrations in the order of magnitude of 10^0–10^1 mg L^{-1} [11].

Novel technologies based on the metabolism of autotrophic bacteria have been developed in the last years for the treatment of high nitrogen-low carbon effluents, such as anaerobic digestion supernatant [12]. These technologies are based on a two-step process: first, a partial-nitritation, or oxidation of half of the influent ammonium to nitrite, followed by a subsequent anaerobic ammonium oxidation using nitrite as terminal electron acceptor, step which is developed by anammox bacteria [13]. These two steps can be achieved in two different bioreactors (partial nitritation/anammox technology) or inside the same bioreactor (Completely Autotrophic Nitrogen-removal Over Nitrate (CANON), aerobic/anoxic DEamMONification process (DEMON) technologies [14]. The main difference between the single bioreactor processes consists in the aeration strategy and the biomass structure. For the DEMON system, the bioreactor is subjected to intermittent aeration to achieve ammonium oxidation and elimination of nitrogen via anammox reaction. On the other hand, the granular biomass developed in the CANON bioreactor makes possible the existence of a gradient of nutrients and oxygen within the granule, and thus promotes the syntrophic coexistence of microbial communities with very different metabolic requirements, such as ammonium oxidizing bacteria and anammox. In this sense, the CANON bioreactor can be operated under limited, continuous aeration.

In this research, the effect of an antibiotic mix simulating anaerobic digestion supernatant of high-antibiotic wastes, such as livestock manure or pharmaceutical industry, over a CANON system was analyzed in terms of ammonium oxidation performance, antibiotics removal capacity and bacterial community structure using molecular biology techniques and electron microscopy. The results obtained were of importance for the operation of this technology treating effluents with high antibiotics concentrations. As well, the potential proliferations of antibiotic resistant microorganisms within the bioprocess were discussed.

2. Material and methods

2.1. CANON bioreactor configuration and operation

Two CANON bioreactors were set-up for the experiment (Fig. S1). Both were of 2.2 L of total volume. Both bioreactors were inoculated with the same granular biomass from a CANON bioreactor located in Polytechnic of Milano, Italy, and were operated under synthetic wastewater feeding during four months.

After inoculation, one of the CANON bioreactor was used as control experiment (Control CANON bioreactor) while the other (Experimental CANON bioreactor). The control CANON bioreactor was continuously fed with wastewater #1 (without antibiotics) from day 1 to day 120 (Table S1). On the other hand, the experimental CANON bioreactor was continuously fed with wastewater #1 from day 1 to day 60 but from this day the influent wastewater composition was changed to wastewater #2 (with a high antibiotics concentration) containing azithromycin, norfloxacin, sulfamethoxazole and trimethoprim (Table 1), which was maintained until the end of the experiment. The antibiotics azithromycin, norfloxacin and sulfamethoxazole were chosen due to their affiliation with a common antibiotic group frequently found in wastewater: azithromycin for macrolides, norfloxacin for quinolones, and sulfamethoxazole for sulfonamides. The presence of trimethoprim was derived from its combined use with sulfamethoxazole in commercial antibiotics products. Sulfonamides and macrolides have shown a very poor removal in wastewater treatment processes, and quinolones have been found to have the highest concentration among all antibiotics groups in wastewater [6]. The synthetic wastewater used in our study simulated the leachate from an anaerobic digester from pharmaceutical wastewater treatment plants. In this way, the high antibiotics concentrations used in this study has been reported in different high concentrations influents, such as livestock manure and pharmaceutical sludge, by several authors [10,11,15,16]. Both CANON bioreactors operated under the same operational conditions with a hydraulic retention time (HRT) of 4 h, a dissolved oxygen concentration (DO) of 1.0 ± 0.1 mg O_2 L^{-1}, a pH of 7.5 ± 0.04 and a temperature of 35 °C in accordance with Gonzalez-Martinez et al. [17]. During the operation of the bioreactors no purge of granular biomass was done. Therefore, the wasting of biomass in the effluent was limited to the washout of eventual small biomass aggregates.

2.2. Determination of inorganic nitrogen ions: ammonium, nitrite and nitrate

The inorganic forms of nitrogen ammonium, nitrite and nitrate were measured daily in the influents and the effluents of the two CANON used in this experiment. The ions were determined through ionic chromatography using a Metrosep C 2-150 and a Metrosep A supp-4-250 columns (Metrohm) to measure concentrations of cationic and anionic nitrogen ions, respectively, followed by electrochemical detectors. Calibration curves of known solutions of 100, 500 and 1000 mg L^{-1} of ammonium, nitrite and nitrate were used to determine concentrations of these ions in the samples measured.

2.3. Determination of azithromycin, norfloxacin, sulfamethoxazole and trimethoprim antibiotics

Azithromycin, norfloxacin, sulfamethoxazole and trimethoprim, were purchased from Sigma Aldrich (Seelze, Germany). Internal standard azithromycin-d_3, enrofloxacin-d_5, sulfamethazine-d_4 and trimethoprim-d_3 were provided by Toronto Research Chemicals (Ontario, Canada). All the standards were 98% purity or higher.

Water, methanol (MeOH), acetone and acetonitrile were of HPLC-grade; formic acid was of MS-grade. They all were purchased from Merck (Darmstadt, Germany). High quality nitrogen (N_2) and argon were supplied by Abelló Linde (Barcelona, Spain).

Individual antibiotic stock standard solutions were prepared at 100 μg L^{-1} by dissolving 10 mg of the individual drug in 10 mL of MeOH and subsequently 10-folds diluted. A 5 μg mL^{-1} solution of the internal standards and a stock standard solution of the mixture of all compounds at 5 and 1 μg mL^{-1}, respectively, were prepared. Working standard solutions of the mixture were freshly made by appropriate dilution of the stock standard mixture in MeOH. Solutions were transferred to amber bottles and stored in the dark at 4 °C to minimize potential analyte degradation.

Quantification of target antibiotics was performed by the internal standard calibration method using the best suited labelled compound for each analyte. A series of 10 freshly antibiotic mixture standard solutions were prepared at concentrations ranging from 10 ng L^{-1} to 1500 ng L^{-1} for calibration. The analyzed water samples were conveniently diluted, spiked with the internal standard mixture at a concentration of 500 ng L^{-1} and pH adjusted to fit in the calibration range curves before their analysis.

The analysis of the selected antibiotics was carried out by online solid phase extraction coupled to high performance liquid