Leadership in Sustainable Infrastructure
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Vancouver, Canada
May 31 – June 3, 2017/ *Mai 31 – Juin 3, 2017*

AN INNOVATIVE APPROACH TO TREAT AMMONIA-RICH WASTEWATER BY PARTIAL NITRIFICATION/ANAMMOX IN BIOCAST REACTOR

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Abstract: The release of untreated or improperly treated ammonium-rich wastewater to the environment has deleteriously impacted human health and aquatic life. Biologically-based technologies are extensively used to transform nitrogenous pollutants to non-polluting forms (e.g. N_2). These technologies have proven to be effective, but obtaining an efficient removal of nitrogenous pollutants in a one-vessel treatment system that can simultaneously provide a balance in the environmental conditions (e.g. dissolved oxygen) for the nitrifiers and anaerobic ammonia oxidizers (“Anammox”), that require aerobic and anaerobic conditions, is extremely challenging. The main objectives of this study were to investigate the potential application of a biological reactor (BioCAST) for the treatment of an ammonium-rich wastewater under the nitrite-limited feeding conditions (NO_2: 0 mg-N/L) through providing the favorable conditions for both nitrifying and anammox communities in a single bioreactor and to identify the anammox communities developed in the system. The bioreactor was initially seeded with a sludge containing nitrifiers and anammox bacteria and was fed with an ammonium-rich synthetic wastewater (NH_3-N: 350 mg/L) and operated at a hydraulic retention time of 2 days at 35°C. The results showed that under favorable conditions, nearly 80% and 98% of total nitrogen (TN) and NH_3-N were removed, respectively. Sequencing analyses revealed that *Candidatus brocadia* genus was the most abundant in the bioreactor. High ammonium removal and existence of anammox bacteria imply that the suitable conditions for the activity of nitrifying and anammox communities were provided in this system.

1 INTRODUCTION

The “Anammox” process is the oxidation of ammonium under anoxic conditions by ammonia oxidizers, at which nitrite (NO_2) is used as the electron acceptor and hydroxylamine (NH_2OH) and hydrazine (N_2H_4) and N_2 are produced as the intermediates and final products, respectively (Kuenen 2008). This process in combination with nitrification has been successfully used to treat ammonium-rich wastewater with a low carbon to nitrogen ratio (Dijkmall and Strous 2002; Duan et al. 2012; Mulder et al. 1995) since the autotrophic anammox bacteria do not need organic carbon sources (Li et al. 2008; van-Dongen et al. 2001). However, providing an environmental balance for the aerobic nitrifying and anoxic ammonia oxidizers in a single vessel system has proven to be extremely challenging.

Studies showed the formation of microbial granules in the anammox reactors (Lu et al. 2012; Tang et al. 2011). Microbial granules are formed when suspended biomass aggregates as a result of physicochemical and microbiological interactions. Those studies have showed that the anammox bacteria form granules (composed of the granule, subunit, microbial cell cluster and single cell) and excrete extracellular polymeric substrates (EPSs) (Lu et al. 2012; Tang et al. 2011) composed of mainly polysaccharides and proteins (Liu et al. 2009). Studies on the anammox granule characteristics such as size, settling property, density, morphology, color, and specific anammox activity revealed that the properties of anammox granules are influenced by several factors, including reactor configuration, hydrodynamic shear force, and nitrogen loading rate (Liu et al. 2009; Zhang et al. 2015). Furthermore, it

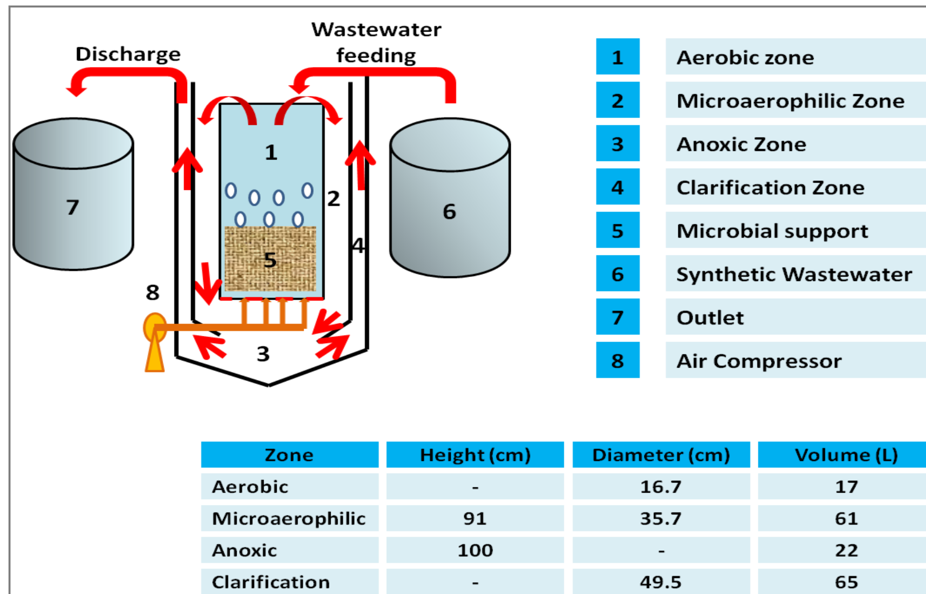
was proposed that the granule characteristics can be used as an indicator of the anammox reactor operation (Lu et al. 2012, 2013; Zhang et al. 2015).

The BioCAST technology is an airlift biological wastewater treatment system, proven to effectively provide multi-environment conditions for the treatment of wastewater containing organic materials, nitrogen, and phosphorous (Alimahmoodi et al. 2012; Yerushalmi et al., 2013). In this study, the multi-environment BioCAST bioreactor was modified by placing geotextile material in the microaerophilic zone as the medium for biofilm growth and attachment to enhance the retention of microorganisms. The nonwoven geotextile material was selected because it has been shown effective in biofilm formation in a previous study (Alimahmoodi et al. 2012). The main objective of the present study was to investigate the feasibility of this technology for the removal of ammonium from a synthetic wastewater with partial nitrification/anammox processes, by providing environmental conditions (e.g. dissolved oxygen and temperature) that are suitable for both the nitrifiers in the aerobic zone (which produce the nitrite required for the anammox microorganisms) and for the anammox bacteria in the anoxic zone (which can convert the remaining ammonia and the produced nitrite to nitrogen gas). Moreover, the granule microbial and physicochemical characteristics and the presence of anammox bacteria developed in this system were monitored to better overcome operating problems and optimize the BioCAST technology. This technology is promising due to providing stable and reliable operation conditions for a long period (more than one year), a homogeneous distribution of ammonium and biomass in the reactor, and successful and relatively rapid anammox start-up.

2 MATERIALS AND METHODS

2.1 BioCAST Reactor Design

The BioCAST reactor (Figure 1) is an airlift treatment system comprised of three main compartments/zones, namely aerobic, microaerophilic, and anoxic for biological treatment, and a clarification zone for solid-liquid separation. The aerobic zone is connected to the microaerophilic zone through eight adjustable opening structures (e.g. 1/4 - 1 inch) to the interconnected microaerophilic and anoxic zones. A cylindrical microbial support made of stainless steel and covered with a non-woven geotextile (estimated porosity of 0.88 (Rowe, 2001), density of 950 kg/m^3, specific surface area of 1500 m^2/m^3, Texel Inc., Quebec (Alimahmoodi et al. 2012)) was placed in the aerobic zone and strips of geotextile were placed in the microaerophilic zone as support materials for the bacterial attachment.



Zone	Height (cm)	Diameter (cm)	Volume (L)
Aerobic	-	16.7	17
Microaerophilic	91	35.7	61
Anoxic	100	-	22
Clarification	-	49.5	65

Figure 1: Schematic diagram of the BioCAST reactor

2.2 BioCAST Start-Up and Operation

The system start-up was carried out by using a sludge seed containing nitrifying and anammox bacteria obtained from Hampton Roads Sanitation District (USA). The bioreactor was operated continuously for over 500 days and it was fed with a synthetic wastewater prepared with the addition of $(NH_3)_2CO_3$ (different concentrations), and trace elements to a feeding tank (100 L) containing tap water, following the Van de Graaf et al. (1996) and Strous et al. (1997) procedures with modifications. The process was started with an NH_4-N concentration of 10 mg/L which was kept constant for a few days until ammonium removal efficiency reached above 80%. Subsequently, the concentration of NH_4-N was increased stepwise to 250 mg/L at a hydraulic retention time (HRT) of 4 days. When the nitrogen removal efficiency reached above 80% the HRT was reduced to 2 days. At this stage, the NH_4-N concentration increased stepwise to 400 ± 25 mg/L and operation was continued at this HRT using a peristaltic pump to maintain a flow rate of 2.1 L/h for over a year. Furthermore, the aeration was provided through air diffusers placed at the bottom of the aerobic zone and air at 13 kPa entered the reactor to supply oxygen for the aerobic microorganisms (final concentration of <2 mg/L) and to support the circulation of liquid between the aerobic, microaerophilic and anoxic zones. The microbial granules/sludge was wasted from the bottom of the anoxic zone under gravity flow using clear vinyl tubing. The temperature was maintained at 35°C using a 1000 W titanium heating system with a digital controller (JBJ TRUE TEMP) and pH was monitored by a portable pH meter (OAKTON PD-650). The bioreactor was wrapped with a black plastic sheet/aluminum foil to prevent the bacteria from exposure to light. The operating period and conditions of the BioCAST technology are shown in Figure 2.

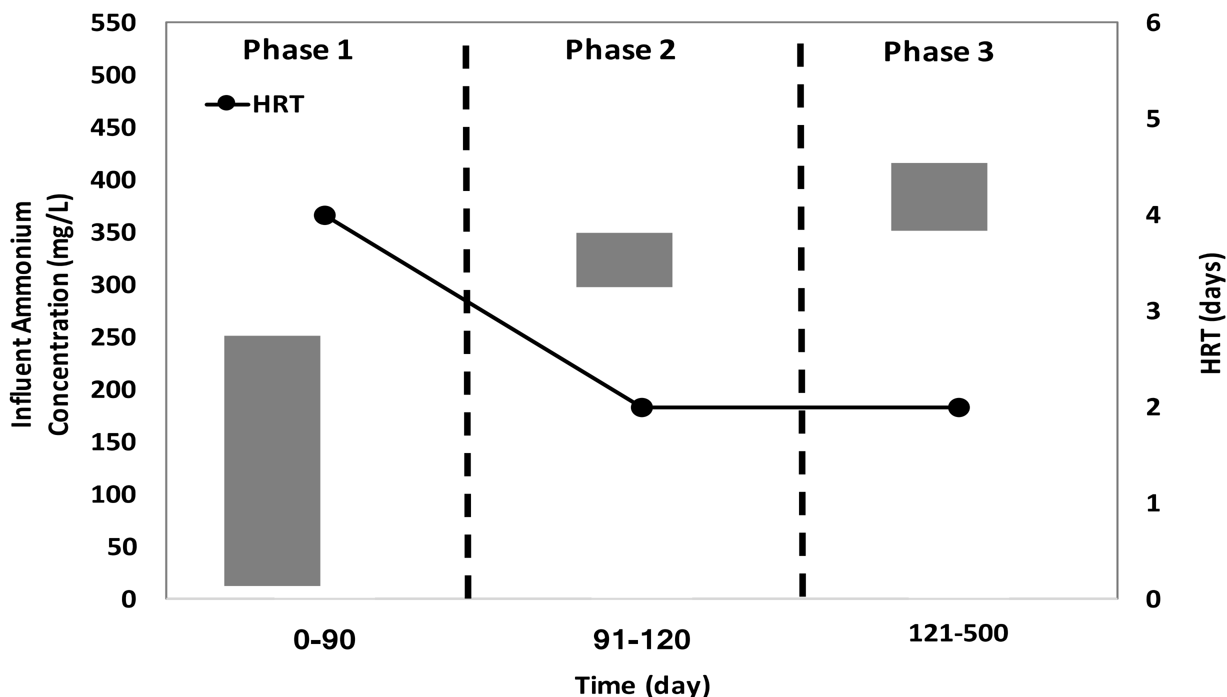


Figure 2: Influent ammonium concentration and bioreactor hydraulic retention time (HRT; right y-axis) from days 0 to 500. The operating period is divided into three phases defined by the influent ammonium concentration and HRT

2.3 Sample Collection and Analysis

Three sets of samples including liquid, granules, and biomass were collected for chemical, microbial, and molecular characterization and all measurements and tests were carried out at room temperature. The liquid sampling was conducted at least three times a week and samples were taken from the influent as well as the aerobic, microaerophilic, and anoxic zones to determine the total alkalinity, NH_4-N, NO_2-N, and NO_3-N concentrations (colorimetric methods TNT 870, 832, 840, 836; HACH Canada).

The granule sampling was conducted biweekly or monthly to determine granule morphological and settleability properties. Settling tests were performed by transferring one liter of granules from the anoxic zone of the bioreactor to an Imhoff cone filled up to the 1-L mark and the granule settling was recorded (as mL/L) every 5 min for 30 min. Total suspended solids (TSS) were determined by filtration (WhatmanTM, No.1, 42.5 mm) of 1 ml of wet granules from the anoxic zone using a vacuum filtration. Then the filter was placed in an Al dish and dried at 105°C (Blue M oven, USA) for 30 minutes and cooled in a desiccator and the filter was weighed (Denver Instruments Balance, 0.1 mg). The TSS was calculated from the difference in weight of empty filter and dried filter at 105°C (mg/L of the sample). Then the filter was placed at 550°C (Blue M oven, USA) for 20 minutes to remove the organic matter in the suspended solids and was weighed again. The VSS was calculated from the difference in weight between the dried (VSS) and ignited (550°C) matter (mg/L of the sample) (Standard Methods 2540 D/E, APHA, 2005).

Granule size distribution and morphological properties were determined following Lu et al. (2013) with modifications. Known amounts of granules were placed in a series of clean Petri dishes (e.g. 1 mL, n = 3) and on clean glass microscope slides (e.g. 0.1 mL, n = 3) using a disposable pipet. The Petri dishes and slides were air-dried for 10 min and an optical microscope (OMAX), a USB digital camera and ScopeImage 9.0 professional imaging software were used to capture and analyze the images (Lu et al. 2013).

Volume and density of the granules were measured as follows: first, the granules were dried in an oven with 103°C for 1 h and the dry mass of the granules was determined until nearly 2 g of mass were obtained (Mu et al. 2008). Then the volume of granules was measured by merging the granules in a known volume of deionized water (e.g. 5 mL) and the volume of the granules was calculated from the difference between the initial and final volumes of water. The density was determined by dividing the mass of washed granules (e.g. 2 g) by the volume of the granules.

2.4 DNA Extraction and PCR Amplification

The 16S rRNA gene sequencing method was used to identify the developed bacteria in the BioCAST reactor. First, the liquid containing biomass was taken from different zones of the reactor. The biomass from the microbial support and geotextile carriers were collected by scratching the surface of the geotextile biofilm. Then the biomass was transferred to the sterile centrifuge tubes (2 mL, autoclaved at 121°C, 20 min) and was centrifuged (12000 rpm, Fisher Scientific™ High-Speed Mini Centrifuge) until a 1 g of biomass was recovered and the biomass was kept in the freezer until the DNA extraction step (Environmental Engineering laboratory, Concordia University, BCEE).

The PowerLyzed Power Soil DNA Isolation kit was used for the genomic DNA extraction without modifications. The total volume of the recovered genomic DNA was around 100 µL and the DNA was preserved in a 10 mM Tris solution. The possible contaminants in the genomic DNA were removed using an OneStep™ PCR Inhibitor Removal Kit (Cedarlane, Canada). The purity of the DNA was determined by running the DNA on a 0.8% agarose gel. The extracted DNA was quantified and qualified (ratio (260/280)) using the Qubit R ds DNA BR assay (Qubit@ 2 Fluorometer, Invitrogen) and Infinite® 200 PRO NanoQuant (TECAN), respectively. The genomic DNA was amplified according to a protocol developed in our laboratory (Department of Biology, Concordia University, Canada). Two anammox-specific primer combinations including 519F/805R and 533/805R (Integrated DNA Technologies, Inc.) from published data (Sonthiphand and Neufeld 2013) and one universal primer set were used in this study. The specifications of the primers used in this study are summarized in Table 1. Nuclease-free water (Ambion) and the swamp water DNA were used as the negative and positive controls, respectively. The master mix per each reaction (total volume of 25 µl) contained a 10X buffer (2.5 µl), dNTP (0.5 µl), forward primers (1.25 µl), reverse primer (1.25 µl), taq polymerase (0.5 µl), genomic DNA template (either 0.5 or 1 µl, dilutions of 10^0-10^{-3}), and nuclease-free water (18 or 18.5 µL). The amplification was started with one cycle of a hot start at 94ºC for 3 min and followed the conditions in Table 1 and terminated by one cycle at 72ºC for 10 min. The PCR products were run on an agarose gel (0.8%, Agarose A (BIOTECH), run time of 100 min, 60 V) and the gel was visualized by a UV (UltraCam digital imaging) to check the quality of PCR products. The genomic DNA was submitted to the MR DNA molecular research laboratory (Shallowater, TX, USA) for sequencing.

Table 1: Primers specifications and PCR conditions used in this study

Primers	Sequences 5’-3’	PCR conditions						
		Denaturation		Annealing		Extension		
		Temp (ºC)	Time (sec)	Temp (ºC)	Time (sec)	Temp (ºC)	Time (sec)	Max cycle #
Universal bacteria								
27F	AGAGTTTGATCMTGGCTCAG	94	45	55	30	72	90	30
1492R	TACGGYTACCTTGTTACGACTT							

Anammox bacteria								
513F	CAGCMGCCGCGGTAA	94	45	50	30	72	90	30
Bakt_805R	GACTACHVGGGTATCTAATCC							
533F	TGCCAGCAGCCGCGGTAA							
Bakt_805R	GACTACHVGGGTATCTAATCC							

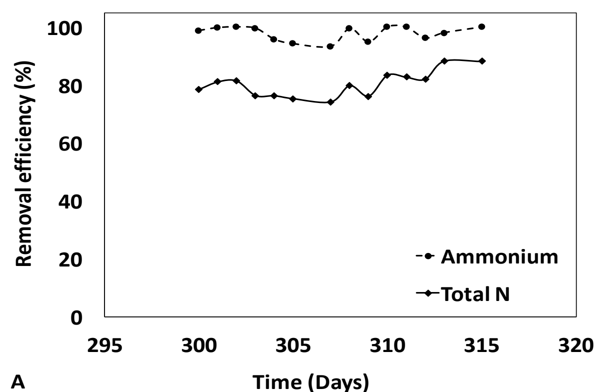
3 RESULTS AND DISCUSSION

3.1 BioCAST Performance

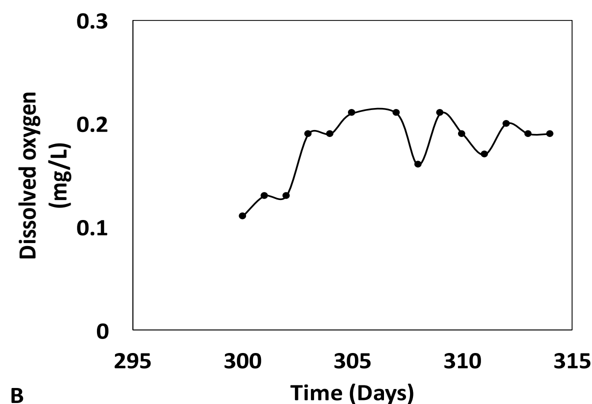
The overall performance of the BioCAST technology at HRT of 2 days and at 35°C is summarized in Table 2. Measurements of NH_4^+, NO_2^- and NO_3^- in the influent and different zones of the bioreactor showed that a maximum removal of 98% and 80% for ammonium and total nitrogen (Figure 3 A) were achieved in this system under the selected operating conditions (Figures 3 B and C). The 16S rRNA sequence data indicated the existence of Planctomycetes phyla in this system. High ammonium removal and the dominancy of *Candidatus brocadia* species (Figure 3 D) in the system imply the success of the bioreactor in providing favorable environmental conditions for the effective coexistence of nitrifying and anammox bacteria in this system.

Table 2: Ammonium removal from synthetic wastewater with BioCAST technology in Phase-3

Parameter	Value
Influent NH_4^+ concentration (mg NH_4^+-N/L)	350.71 ± 6.61
Effluent NH_4^+ concentration (mg NH_4^+-N/L)	8.21 ± 7.9
Influent NO_2^- concentration (mg NO_2^--N/L)	0.0
Ammonium removal (%)	97.8 ± 2.29
Total nitrogen removal (%)	80.21 ± 4.23



A



B

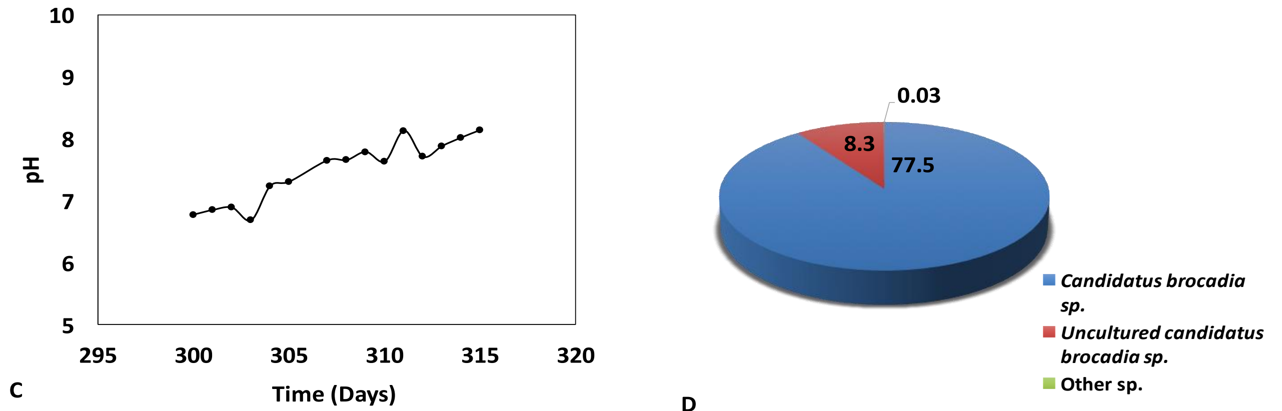


Figure 3: Performance of the BioCAST reactor in Phase-3 (NH_4-N: 350.7 ± 6.6 mg/L, NO_2: 0 mg/L, HRT: 2 days, temperature 35ºC): (A) NH_4-N and TN removal efficiency; (B) level of dissolved oxygen (DO) in the anoxic zone; (C) variations of pH in the bioreactor; (D) percentage of anammox species developed in the BioCAST as determined by 16S rRNA gene sequencing

3.2 Biofilm Development

Microbial biofilm development can be used as an indicator of overall biological reactor performance. Microbial biofilms can be comprised of a single heterotrophic and/or autotrophic species or mixed-species and developed through several steps including cell-surface and cell-cell interactions, and mature biofilm development as an adaptive strategy in response to changes in environmental conditions such as the availability of nutrients to microbial species, temperature, osmolarity, pH, iron, and oxygen (O`Toole et al. 2000).

Figures 4 and 5 show the gradual development of biofilm on the microbial support in the aerobic zone and the geotextile carriers in the microaerophilic zone following the start-up of the BioCAST system. The biofilm development suggests the positive effect of the microbial support and geotextile structure on the attachment and growth of the biomass and the appropriate environmental conditions including dissolved oxygen, pH, temperature, and nutrients for their growth (Figure 4).

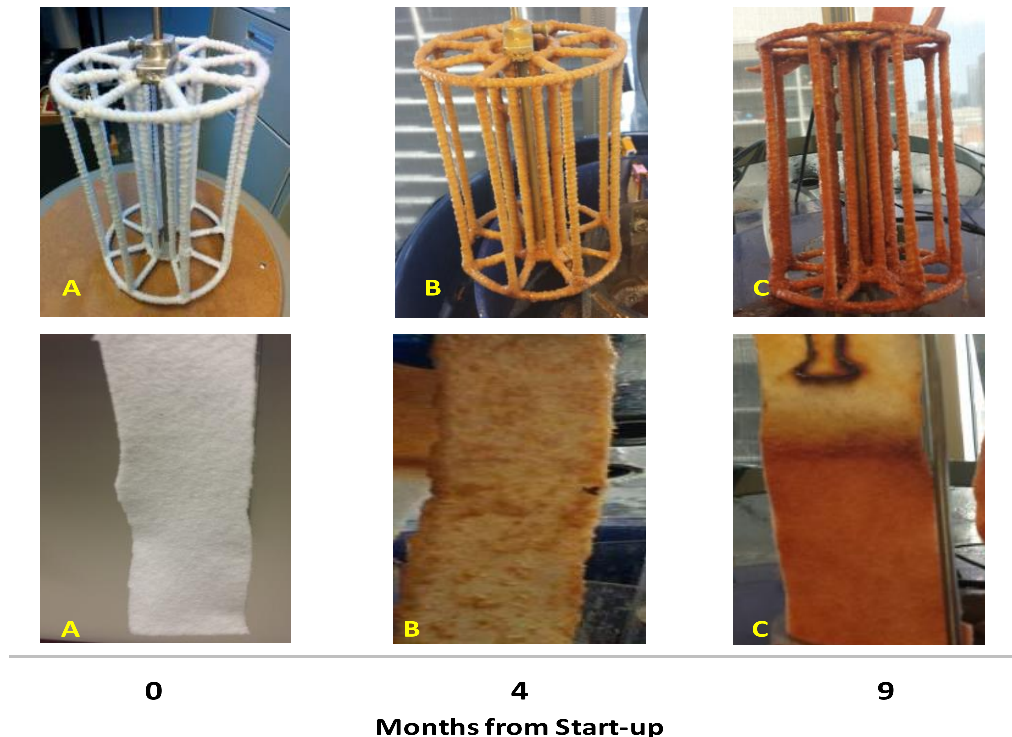


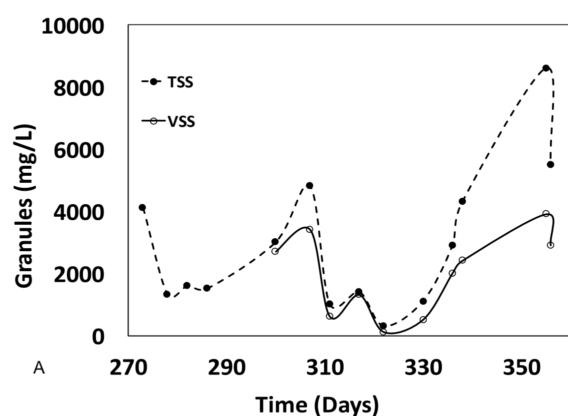
Figure 4: Growth of microbial communities on the microbial support installed in aerobic zone (top) and geotextile strips in the microaerophilic zone (bottom); (A) day 0, (B) day 120, (C) day 270

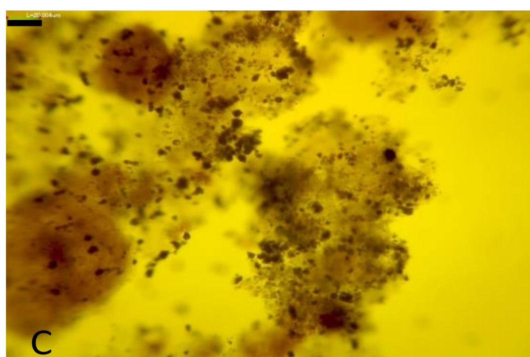
3.3 Granule Formation and Morphology

In the present study, various tests were performed to determine the physicochemical characteristics of anammox granules developed in the bioreactor. The granules were monitored because it can show the overall performance of bioreactors, the sludge-liquid separation, and consequently the effluent quality (Pereboom 1997). The microbial granule properties usually depend on the granule size and concentration (Liu et al. 2009).

The VSS measurements (Figure 5 A) showed that under stable operating conditions, when no excess sludge was withdrawn, the cell concentration increased from an initial 487 mg/L to over 1500 mg/L (e.g. 780 mg/L at day-30 after the start-up period) and the anammox granules exhibited an excellent settling capacity, so that following a 5-min settling time, the granules were settled and a clear supernatant was observed.

Visual observation (Figure 5 B) and light microscopic (Figure 5 C and D) of the granules confirmed the presence of single and clusters of brown and red granules with irregular shapes and sizes. Analysis showed that the single granules had an average surface area of 26.5 μm^2 (n = 24) with a range of 3.82 - 156.5 μm^2 and the clusters of granules had an average surface area of 2366.2 μm^2 (n =3). The granule density was 24.6 ± 4.3 kg/m^3 (n = 6).





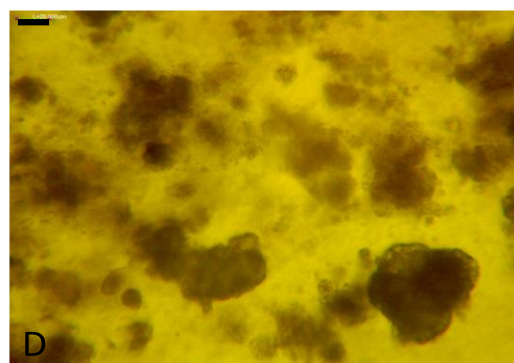


Figure 5: (A) TSS and VSS in the bioreactor (B) photographs of the granular sludge (C and D) light microscopic of granular sludge (magnification :4X and 40X, respectively, scale bar: 20 μm)

4 CONCLUSIONS

The potential application of a biological reactor (BioCAST) for the treatment of ammonium-rich synthetic wastewater by nitrification and anammox processes was investigated. The removal of ammonium coupled with the development of anammox granular sludge with high settleability and gradual growth of distinctly red biomass on microbial support covered with the geotextile in the aerobic zone and geotextile carriers in the microaerophilic zone highlighted the following points. First, the appropriate environmental conditions for partial nitrification and anammox processes were effectively established in this bioreactor. Second, the geotextile facilitated rapid growth of biofilm due to the creation of favorable conditions in the interior spaces of the geotextile for anaerobic bacteria and anoxic ammonia oxidizers and aerobic condition in the exterior part of the biofilm for aerobic nitrifying bacteria. In the next steps of this study, the environmental conditions that trigger the formation of anammox biofilm and distribution and activity of anammox and other coexisting bacteria in the biofilm and suspended medium to better understand the overall BioCAST performance and to further optimize this technology will be investigated.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council (NSERC), BIOCAST and Concordia University.

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