Treatment of nitrogen-rich wastewater using partial nitrification and Anammox in the CANON process

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Abstract Partial nitrification combined with Anammox in a single reactor (the CANON process) is an energy-efficient N-removal technology that could substantially lower the N-load of a WWTP by separate treatment of nitrogen-rich side streams, preventing the need for extensive expansion and reducing the total energy requirement. This study looks at the enrichment of Anammox from activated sludge and its application in the CANON process on lab-scale. The aim was to identify the critical process control parameters necessary for successful operation of CANON. An Anammox culture capable of removing 0.6 kg $N/m^3/d$ was enriched in 14 weeks in a sequencing batch reactor. Nitrifying biomass was inoculated into the Anammox reactor (10% v/v) together with limited oxygen supply ($<$ 8 mL/min) to initiate the CANON process in continuous culture. The small flocs formed by the biomass ($<$ 1000 μm) were sensitive to low O_2 concentrations ($<$ 0.1 mg/L) which prevented simultaneous nitrification and Anammox. Operation with 20 min aerobiosis and 30 min anaerobiosis was necessary to achieve sustained, completely autotrophic N-removal for an extended period at a rate of 0.08 kg $N/m^3/d$. Essential process control parameters for stable CANON operation were the nitrite concentration, oxygen concentration, pH and the temperature.

Keywords Anammox; autotrophic N-removal; CANON; partial nitrification; SBR

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Introduction

Due to increasingly severe environmental laws governing the release of nutrients from wastewater treatment plants, a large number of existing wastewater treatment plants need to be upgraded. The CANON process is an innovative, sustainable nitrogen-removal technology for treatment of wastewater flows containing high concentrations of ammonium-nitrogen, such as wastewater side streams from dewatered digested primary sludge and waste activated biosolids, wastewater flows from sludge dryers and incinerators, as well as nitrogen-rich industrial wastewater. The method has been applied successfully to N-rich wastewater at laboratory scale (Sliekers *et al.*, 2002, 2003; Third *et al.*, 2001); however, the system has yet to be applied on large scale as the critical process control and design parameters have yet to be clearly defined. The reduction of the nitrogen load of a WWTP through side stream treatment results in a significant saving in aeration energy through reduced aeration required for nitrification in the main process, as well as reduced aeration for endogenous respiration, due to the application of a lower sludge concentration in the aeration basins. The name given to the treatment of wastewater by simultaneous partial nitrification and Anammox in a single reactor is CANON (Completely Autotrophic Nitrogen-removal Over Nitrite) and was first developed in Delft in 2000 (Strous, 2000). It targets wastewater streams high in ammonium ($>$ 0.1 g/l) and low in organic carbon (C:N ratio lower than 0.15). The process relies on the interaction of two groups of autotrophic bacteria under oxygen-limiting conditions that perform two sequential reactions, simultaneously. Under oxygen limitation, ammonium is oxidised to nitrite

by aerobic ammonium oxidisers, such as *Nitrosomonas* and *Nitrosospira* (Equation 1). The nitrite produced in this reaction can be used by Anammox bacteria, which anaerobically oxidise ammonium using nitrite as the electron acceptor (Equation 2). The combination of the above two reactions results in overall nitrogen removal according to equation 3:

$$\text{Partial nitrification} \quad 1NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{1}$$

$$\text{Anammox} \quad 1NH_4^+ + 1.3NO_2^- \rightarrow 1\,N_2 + 0.26\,NO_3^- + 2H_2O \tag{2}$$

$$\text{The CANON process} \quad 1NH_4^+ + 0.85O_2 \rightarrow 0.4N_2 + 0.13NO_3^- + 1.3H_2O + 1.4H^+ \tag{3}$$

This is a promising new principle for wastewater treatment as only a single oxygen-limited step is required to remove ammonium from wastewater. Compared to conventional nitrification and denitrification, this method saves 100% of a required carbon source (e.g. methanol) and 63% of the required oxygen (Kuai and Verstraete, 1998). This leads to a decrease in CO_2 emissions of more than 100% (the process actually consumes CO_2) and a decrease in energy demand. In addition, sludge production is low in the CANON process due to the low yield of Anammox bacteria (± 0.05 kg ds/kg N) and nitrifying bacteria (± 0.1 kg ds/kg N) (Strous, 2000). A current limitation to the widespread application of autotrophic nitrogen removal processes is the difficulty associated with growing large quantities of Anammox biomass due to the very low growth rate ($0.003\,h^{-1}$, doubling time 14 days) and biomass yield of Anammox bacteria (Strous *et al.*, 1999). Few cultivation techniques are designed to deal with slow-growing microorganisms such as Anammox. The sequencing batch reactor (SBR) has proven to be a successful experimental setup for Anammox enrichment due to its very efficient biomass retention ($>$ 90%) (Strous *et al.*, 1998). The persisting stable and strongly selective conditions of the SBR can lead to a high degree of enrichment of Anammox activity. In this study, it was aimed to apply the SBR for the enrichment of an Anammox culture from local activated sludge (Perth, Western Australia). As wastewater streams generally need to be treated on a continuous basis, the second aim was to establish a continuous flow, completely autotrophic nitrogen-removal (CANON) reactor system in the laboratory. Through optimisation of the CANON reactor it was aimed to establish the critical process control parameters for successful operation of the CANON process, in order to take the process one step closer to large-scale application.

Materials and methods

Reactor system for Anammox enrichment

A glass, fully automated 1 L sequencing batch reactor (SBR) was used for enrichment of Anammox from activated sludge. Temperature was maintained constant at 30°C and the pH was controlled to 7.8. Anaerobiosis was maintained by continuously bubbling an N_2/CO_2 (95%/5%) gas mixture through the liquid (4 mL/min). The stirring speed was maintained as low as possible to keep the biomass suspended (50 rpm) and a stirring paddle designed for minimum shear was used. The reactor was operated as a continuously fed SBR with a 12 hour cycle. It was filled continuously with fresh medium over 11.5 hours at a constant flow rate of $0.72\,mL\,min^{-1}$ (resulting in the addition of 0.5 L feed over 11.5 hours). The minimum volume in the reactor was 0.5 L and at the end of the cycle the final volume was 1 L. At the end of the fill period the biomass was allowed to settle for 15 minutes (settle period). In the following 15 minutes, 0.5 L of liquid was decanted (decant period). The resulting hydraulic retention time (HRT) was 1 day. The N-loading rate was

increased by varying the concentration of NH_4^+ and NO_2^- in the feed vessel (as specified in the Results section).

Reactor system for CANON

For CANON operation, the same reactor set-up (1 L) was changed from an SBR to a continuous flow reactor by removing the settle phase and maintaining constant volume through synchronised operation of the inflow and outflow pumps. Both pumps were turned on for 13 seconds (100 mL/min) in every 50 minutes, resulting in a dilution rate of $0.025\,h^{-1}$. It was ensured that there was always an excess of ammonium present ($>$ 30 mg/L) such that the culture never became ammonium-limited, causing nitrite accumulation. Full biomass retention was achieved by fitting the outflow tube with a biomass-settling device as described by Third *et al.* (2001). Dissolved oxygen and pH were monitored and controlled continuously on-line. During CANON operation, the gas supply was automatically alternated between air and the N_2/CO_2 mixture.

Activated sludge from a conventional continuous wastewater treatment plant (Subiaco, Western Australia) was used as inoculum for the Anammox enrichment. The inoculum sludge had a total sludge age of 15 days and contained an active denitrifying population (7 mg NO_3^--N/g biomass/h) and nitrifying population (4 mg NH_4^+-N/g biomass/h). FISH analysis of the original inoculum showed no visible Anammox presence in the biomass samples tested. The same activated sludge was used as a source of aerobic nitrifiers, which was added to the enriched Anammox culture to start the CANON process (10% v/v inoculum).

To prepare the synthetic wastewater ammonium and nitrite were added to a mineral medium in the required amounts in the form of $NaNO_2$ and $(NH_4)_2SO_4$, as specified in the Results section. The composition of the mineral medium was (g/L): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and $1.25\,mL\,L^{-1}$ of trace elements solution. The trace element solution is described in Strous (2000). Nitrate, nitrite and ammonium were measured colorimetrically as described by Third *et al.* (2001). Cell fixation and FISH analysis were performed as described by Third *et al.* (2001).

Results and discussion

Enrichment of Anammox bacteria from activated sludge

The SBR was inoculated with concentrated activated sludge biomass (0.5 L) from a conventional continuous wastewater treatment plant and the reactor was filled to 1 L during an SBR cycle with synthetic wastewater (initial biomass concentration 5.4 g/L dry weight). To avoid possible toxicity of nitrite accumulation at any stage, the feed initially contained ammonium as the electron donor (14 mgN/L) and nitrate (140 mgN/L) as the electron acceptor (Van de Graaf *et al.*, 1996). The idea of supplying nitrate was that, since the inoculum contained an active denitrifying population, nitrite would be produced in the culture in low amounts by nitrate reduction, using remaining organic compounds in the inoculum. The low amount of nitrite production should be sufficient for any Anammox bacteria present in the sludge. Small spikes of hydrazine (N_2H_4) and hydroxylamine (NH_2OH) were added to the reactor daily (2 mg/L final reactor concentration) in attempt to kick-start the Anammox reaction. Due to the cyclic nature of the Anammox mechanism, cells need to invest reducing power to start their catabolism by producing NH_2OH from nitrite. This initial energy barrier can be overcome by the direct addition of NH_2OH or N_2H_4 (Strous *et al.*, 1999). The reactor was monitored daily to test for ammonium consumption. After 47 days of feeding with NH_4^+ and NO_3^-, the biomass showed the first signs of ammonium consumption. At this point, the feed composition

was changed to contain 30 mg/L NH_4^+-N and NO_2^--N. The feed flow rate was initially dropped to half its value (0.36 mL/min) to prevent possible nitrite accumulation. Anammox activity becomes completely inhibited at nitrite concentrations of 0.1 g NO_2^--N/L (Strous *et al.*, 1999; Strous, 2000). As long as nitrite was undetectable in the liquid, the flow rate was increased gradually each day. When the feed flow rate reached its maximum value of 1 L/day (0.72 mL/min), the feed concentrations were increased. This resulted in a step-wise increase in the nitrogen load over time (Figure 1). The total nitrogen consumption rate increased from 0.03 to 0.06 kg N/m^3/day within 45 days (92 days after start-up).

As biomass measurements were compromised by attached wall growth in the reactor, the doubling time was estimated from the increase in their activity. A doubling of the nitrogen consumption rate was achieved over approximately 10–12 days (Figure 1). However, as the N-loading rate was similar to the N-consumption rate it is possible that the culture was substrate-limited and not growing at maximum rate, resulting in an under-estimation of their growth rate. From the data it can be inferred that the Anammox doubling time was not longer than 12 days (growth rate $< 0.004\,h^{-1}$). After 100 days, the nitrogen load was increased suddenly to 0.75 kg N/m^3/day (Figure 1). This caused a temporary accumulation of nitrite (> 70 mg NO_2^--N/L) for more than 12 hours and loss of all Anammox activity. Anammox activity did not resume again until all nitrite had been removed from the reactor and a small amount of hydrazine (2 mg/L final concentration) was added. Resumption of activity was detected by formation of gaseous bubbles in the liquid together with combined ammonium and nitrite consumption. However, the nitrogen removal capacity after the nitrite accumulation was reduced by about half to 0.3 kg N/m^3/day, indicating a significant portion of the biomass had died or become inactive. It took several weeks to regain the same maximum N-consumption as occurred before the nitrite accumulation (0.6 kg N/m^3/day).

The presence of Anammox bacteria in the enrichment culture was verified by qualitative FISH analysis. Positive hybridisation of the biomass with the probe Pla46 confirmed the presence of bacteria of the Order *Planctomycetales* and the culture also reacted positively with an oligonucleotide probe found to specifically detect *Candidatus B. anammoxidans* (Amx820). The enriched bacteria were therefore the same species as all the other already discovered Anammox strains in Europe. Several other specific oligonucleotide probes did not react with the enriched organism, indicating that the Anammox organisms are phylogenetically related to *Brocadia anammoxidans*, but are at least different in the target sites for the more specific probes for these organisms and may therefore

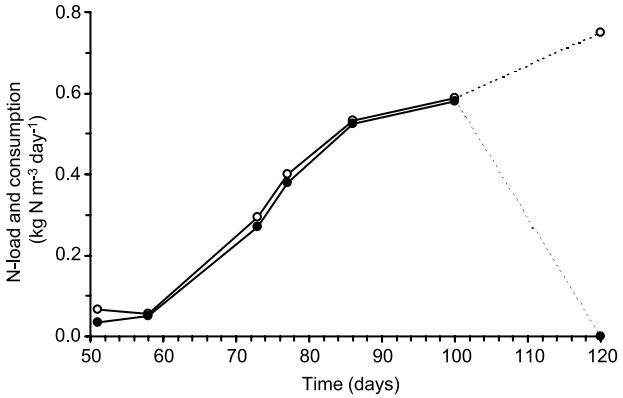

Figure 1 Increase in nitrogen load (◦) and nitrogen consumption (•) during enrichment of Anammox bacteria from activated sludge

represent a novel strain. In comparison to other Anammox enrichment studies, the time required for enrichment was relatively short (14 weeks). Strous *et al.* (1998) enriched a high-purity Anammox culture with a nitrogen consumption rate of 0.9 kg N/m^3/day within 21 weeks (compared to 0.6 kg N/m^3/day in this study), although from an already enriched Anammox culture. Egli *et al.* (2001) required 6 months to enrich an Anammox culture of 88% purity from a rotating biological contactor, which had also shown significant Anammox activity before the start of the enrichment period. Toh *et al.* (2002) reported biofilm growth after one year in a fixed bed reactor that demonstrated Anammox activity, although at quite low N-removal rates (0.06 kg N/m^3/day). In comparison to these studies, the final activity achieved in this study was high in the short enrichment time frame. The difference may have been due to the use of the SBR for enrichment, as opposed to a rotating biological contactor or fixed bed reactor, although Strous *et al.* (1998) also used the SBR for enrichment. The enrichment time may have been shortened by the daily addition of hydrazine and hydroxylamine during the initial enrichment period; however, the exact effect of these intermediates on Anammox growth still needs to be quantified. As reported in other studies, the enriched Anammox culture in this study was severely affected by an accumulation of nitrite for more than 12 hours and required several weeks to recover from the disturbance. This highlights one important requirement of process control for both CANON and Anammox: the prevention of nitrite accumulation.

Initiation of the CANON process

By supplying limited oxygen to an established Anammox culture, aerobic oxidation of ammonium to nitrite can provide the substrates required for simultaneous occurrence of the Anammox reaction (Equations 2, 3). The process control requirements of the CANON process are not yet well established. Previous studies on the CANON system have used granular Anammox biomass (granule diameter $>$ 2000 μm) enriched in Delft, the Netherlands (Sliekers *et al.*, 2002, 2003; Third *et al.*, 2001). In those studies, effective cooperation between the aerobic and ammonium oxidisers was enabled at constant, low airflow rates (8 mL/min), due to oxygen diffusion limitation imposed by the biomass granules. The smaller flocs formed in this study required a more careful DO supply to prevent oxygen diffusion inside the floc, as preliminary results showed that the application of constant low airflow rates ($<$ 8 mL/min) resulted in complete (reversible) inactivation of the Anammox biomass (data not shown). The objective was to set up a fully automated CANON process that could achieve sustained autotrophic ammonium removal using non-granular biomass over a long period.

The SBR was converted into a continuous flow reactor and the reactor was inoculated with a small amount of activated sludge (10% v/v) to provide rapid start-up of aerobic ammonium oxidising activity. The feed was changed to contain ammonium only (140 mg NH_4^+-N/L) and was fed at a rate of 3.5 mg NH_4^+-N/L/h. The reactor was programmed to stop the feed inflow and the aeration if the nitrite concentration increased to higher than 15 mg NO_2^--N/L. This process control proved to be critical in preventing irreversible inhibition of the Anammox biomass, as the bacteria become inhibited at nitrite concentrations higher than 30–50 mg NO_2^--N/L and irreversibly damaged at concentrations of 0.1 g NO_2^--N/L and above (Strous, 2000). Similarly, the air supply was controlled with an analog flow controller, such that the dissolved oxygen concentration remained below a setpoint of 0.1 mg O_2/L. The presence of a higher concentration of oxygen in the CANON reactor, together with the presence of nitrite, can allow the growth of aerobic nitrite oxidisers (eg. *Nitrospira, Nitrobacter* etc), leading to outcompetition of the Anammox bacteria and complete disruption of the CANON process (Third *et al.*, 2001). The pH was controlled at 7.8 and the temperature was controlled at 30°C.

It was initially unclear how much aeration would be required to obtain sufficient rates of both aerobic and anaerobic ammonium oxidising activity. A continuous, low airflow supply to the CANON bioreactor was found to completely inactivate the Anammox biomass due to penetration of oxygen into the small flocs. Therefore, the effect of intermittent air supply (as opposed to continuous air supply) on ammonium oxidising activity was investigated. Air was supplied (8 mL/min) for 10 minutes in every 90 minutes (Figure 2). The supply of air enabled aerobic ammonium oxidation (1.4 mg NH_4^+-N/g/h) for 20–30 minutes. Ammonium measurements showed that anaerobic ammonium oxidation was minimal during aerobic periods and only began 40 minutes after the aeration supply was turned off, when the oxygen concentration had fallen below 0.05 mg/L. Anammox occurred at a reduced rate (0.4 mg NH_4^+-N/g/h), compared to the anaerobic rate in the original enrichment culture (3.6 mg NH_4^+-N/g/h). The nitrite concentration in the reactor increased over time (Figure 2), which showed that the complete autotrophic nitrogen removal process (nitrification and Anammox combined) was limited by the Anammox reaction. The overall specific ammonium removal rate during intermittent aeration was 0.6 mg NH_4^+-N/g/h (equivalent to 0.04 kg N_{total}/m/day). At 260 minutes, the culture was flushed with N_2/CO_2, which caused an immediate increase in the rate of anaerobic ammonium oxidation to 1.1 mg NH_4^+-N/g/h. The results showed clearly that Anammox activity was significantly slowed down during intermittent aeration and experienced long lag times after the air supply was turned off. Flushing with N_2/CO_2 caused an immediate increase in Anammox activity. It was therefore decided to automatically run the reactor with alternating periods of aeration and N_2/CO_2 flushing.

The reactor was subsequently operated with 20 min aerobiosis (8 mL/min air) and anaerobic periods of 30 minutes (8 mL/min N_2/CO_2). In this manner it was attempted to achieve increased rates of aerobic and anaerobic ammonium oxidation in a sequential fashion, as opposed to simultaneous fashion as in previously reported CANON systems (Sliekers *et al.*, 2002; Third *et al.*, 2001). During aerobic periods, nitrite was produced 1.1 mg NH_4^+-N/g/h and it was again consumed during anaerobic periods, at a similar rate (Figure 3). This resulted in small oscillations in the nitrite level in the reactor over time, and resulted in completely autotrophic ammonium removal at an overall rate of around 1 mg NH_4^+-N/g/h. This was improved from the rate achieved without nitrogen flushing (0.5 mg NH_4^+-N/g/h), but was still around three times lower than the specific ammonium removal rate achieved in the anaerobic Anammox enrichment culture (3.6 mg NH_4^+-N/g/h). This ammonium removal rate was sustained for an extended period (> 4 weeks) and

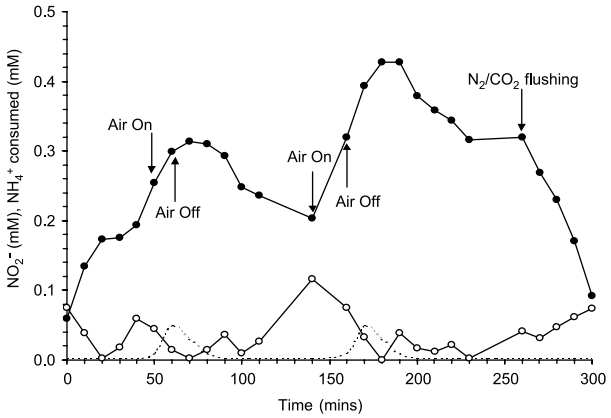

Figure 2 Operation of the CANON reactor with intermittent aeration, showing NO_2^- (•), NH_4^+ consumed (○) and DO (dotted line)

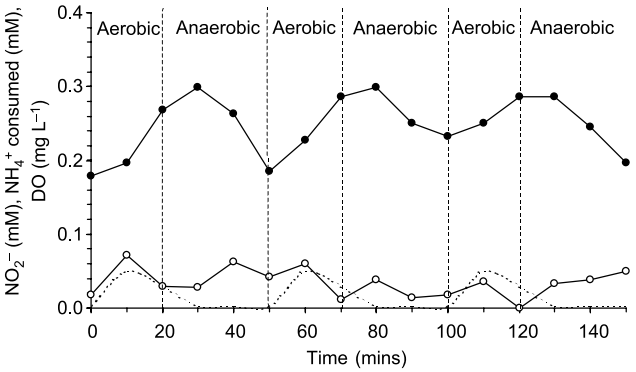

Figure 3 Changes in NO_2^- (•), NH_4^+ consumed (◦) and DO (dotted line) during operation of the CANON reactor with 20 minute aerobic and 30 minute anaerobic intervals

showed that both the aerobic and anaerobic ammonium oxidising activity could occur during intermittent aeration.

Nitrogen was removed at a relatively constant rate during intermittent aeration at 0.08 kg N/m^3/day. However, this study highlights the importance of efficient floc formation for the CANON process. The lower rate of NH_4^+-removal observed in this study in comparison to other CANON reactors (Table 1) was probably due to the lack of granular biomass formation characteristic of other systems. This prevented the possibility of supplying a constant, low airflow rate as in previous studies (Sliekers *et al.*, 2002, 2003; Third *et al.*, 2001). When using granular biomass, oxygen consumption by the aerobic nitrifiers in the outer layer of the granule prevents oxygen diffusion inwards, protecting Anammox cells from exposure to oxygen. In this manner, both aerobic and anaerobic ammonium oxidation can occur simultaneously, close to maximum rate. In contrast to granular systems, the operation of CANON with intermittent aeration results in significant downtime for both the aerobic and anaerobic ammonium oxidisers and only one group of bacteria is active at any one time, resulting in oscillations of the key intermediate, nitrite, over time (Figure 3). Instead of relying on oxygen diffusion limitation by granules, the reactor needed to be intermittently flushed with nitrogen gas in order for Anammox to proceed at a sufficient rate. However, this would be too expensive to achieve in large-scale reactors.

Conclusions

Results in this study highlight the importance of large floc formation($> 1000\,\mu m$) for the CANON process in order to establish oxygen diffusion limitation in the floc. In systems

Table 1 Overview of total nitrogen conversions and specific ammonium consumption rates in various completely autotrophic nitrogen removal reactor set-ups

Process	Reactor type	Total N-conversion (kg N/m^3/day)	Specific NH_4^+-consumption (g/kg biomass/h)	Reference
SHARON[1]-Anammox	CSTR-SBR	1.2	5.5	Van Dongen *et al.* (2001)
OLAND[2]	SBR	0.05	0.5	Kuai and Verstraete (1998)
CANON	CSTR	0.08	0.7	This study
CANON	SBR	0.16	3.1	Sliekers *et al.* (2002)
CANON	Gas-lift	1.5	11.3	Sliekers *et al.* (2003)
CANON	SBR	0.12	1.2	Third *et al.* (2001)

[1]Single reactor high activity ammonia-removal over nitrite
[2]Oxygen-limited autotrophic nitrification-denitrification

where floc formation is a problem it may be needed to use support material for biofilm growth, together with high biomass concentrations (> 8 g/L) to achieve high rates. Without efficient floc formation the nitrogen removal rates are significantly lower than in granular biomass systems (Table 1). While it is apparent this system still requires optimisation, this study has highlighted the minimum necessary process control requirements for successful operation of the CANON process. The process requirements are summarised below.

Process requirements of the CANON process:

- Large floc formation (> 2000 μm) or use of biomass carrier material;
- Full biomass retention;
- On-line control of the nitrite concentration (< 15 mg/L), with feedback control to the feed and air flow. Feed and air inflow must be stopped if the nitrite increases above the setpoint to allow Anammox to consume the accumulated nitrite;
- On-line control of the oxygen concentration (0.1 mg O_2/L), with coupling to nitrite measurement. Air supply must be stopped immediately if the nitrite concentration reaches above the setpoint to provide anaerobic conditions for Anammox;
- Continuous presence of ammonium (> 30 mg/L) to prevent ammonium limitation of the aerobic nitrifiers, resulting in an oxygen concentration increase, accumulation of nitrite and inhibition of Anammox bacteria;
- Control of the pH at 7.8 and the temperature at 30°C if possible.

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