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An Innovative Approach to Remove Nitrogen from Wastewater Using a Biological ANaerobic AMMonium OXidation (ANAMMOX) Process

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Nitrogen (N) is essential for life because it is one of the main constituents of organic molecules such as amino acids and proteins. However, at the same time it is a threat to both the environment quality and human health. To avoid the negative effects of N, recent worldwide regulations have been promulgated to limit the use of nitrogen based fertilizers and the amount of nitrogen discharged into water bodies from wastewater treatment plant (WWTP). An aerobic oxidation of reduced N either followed or preceded by an anoxic reduction of oxidized N are the most common systems used in WWTP to convert the reactive N (ammonium, nitrite and nitrate) to the harmless nitrogen gas (N_2). An alternative to the traditional systems is represented by the innovative biological process named Anammox that anaerobically converts ammonium into N_2. Compared to the traditional processes, this new process requires lower amount of oxygen, has no need of organic carbon supply and is highly efficient with any concentration of ammonium. However, a wide use of this process at full scale is hampered by the extremely slow growth rate of bacteria operating Anammox process and by the difficulty in the control of critical co-existence of different bacterial strains that compete for the same substrate and thrive in opposite environmental conditions. This work highlights the strategies to improve the enrichment of Anammox operating bacteria from two different biological sludge (activated and anaerobic) and to optimize the efficiency of two Anammox biological sequential batch reactors (SBR) by changing the operational conditions. The main results have shown that in an activated sludge the Anammox biomass grows faster than in anaerobic sludge and the performance of Anammox one-stage process can be regulated by controlling the stirring system as well as the oxygen and inorganic carbon (IC) concentrations in the system even if no one of the previous operating parameters has resulted to be decisive.

1. Introduction

The intensive use of nitrogen(N)-based fertilizers for increasing the productivity of cultivated fields (Pindozzi et al., 2013) as well as the discharge of wastewaters rich in N-based compounds into surface waters have distorted the equilibrium of the natural N cycle (Infascelli et al., 2010). To prevent further excess introduction of N-based compounds into the environment, it is therefore mandatory to intervene in limiting the main sources of N (i.e. agricultural fertilizer use and wastewaters discharge). Regarding the wastewaters, most of the currently operating treatment plants have been recently upgraded by adding a tertiary biological treatment with the aim of removing residual N-based compounds. The conventional processes used in wastewater treatment plants (WWTP) for this purpose achieve the complete oxidation of ammonium to nitrate in the presence of

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oxygen in the first step (nitrification) and the conversion of nitrate into the inert N_2 under anoxic conditions in presence of a sufficient amount of biodegradable organic material (already present in the wastewater or added from an external source if required) in the second step (denitrification). However, if ammonium (NH_4^+) is present in high concentrations in water, it becomes toxic and thus inhibits the activity of the microorganisms involved in its removal. Therefore, the conventional processes based on biological nitrification and denitrification of nitrogen compounds appear to be not suitable for high NH_4^+ load wastewaters. The operating limits shown by the conventional N-based compounds removal can be overcome by using a novel alternative biological process (Hu et al., 2013) which is furthermore able to outcompete them in terms of cost as well as removal efficiency. This process is named Anammox as performs an anaerobic ammonium oxidation by using special anaerobic ammonium oxidizing bacteria, capable of using nitrite as electron acceptor to directly convert NH_4^+ into N_2 gas in the absence of dissolved O_2 (Isaka et al., 2006) according to the following chemical reaction Eq. (1):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- \rightarrow 1.02N_2 + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} + 0.26NO_3^- \quad \text{Eq. (1)}$$

Bacteria performing the Anammox process are autotrophic and use CO_2 as carbon source. The O_2 demand for converting NH_4^+ to N_2 through partial nitritation/Anammox pathway is much lower than that through complete nitrification/denitrification pathway, since the O_2 is necessary only for a partial oxidation of the total NH_4^+ to nitrite (NO_2^-) (partial nitritation) conducted by autotrophic and aerobic ammonium oxidizing bacteria (AOB), whereas the remaining part of NH_4^+ reacts with NO_2^- to form N_2 gas by Anammox bacteria (Fux et al., 2002). Compared with the conventional biological ammonium removal processes, the Anammox process has no need of an external carbon source (Panepinto et al., 2013), furthermore shows a low yield of biomass and consequently a lower sludge production. These aspects, in addition to a lower O_2 demand, keep low the operation costs to perform an Anammox process. Moreover the emissions of CO_2 in the environment are lower than conventional treatments, thus this process results also in low environmental impact. This work presents preliminary results of experimental activities conducted on Anammox bacteria with the aim of accelerating the Anammox bacteria enrichment process from two different biomasses as well as improving the performance of a previously enriched Anammox biomass in a lab scale completely autotrophic nitrogen removal over nitrite (CANON) type reactor designed to remove NH_4^+ (Third et al., 2001). All the experiments were conducted in sequencing batch reactors (SBR) systems (Backburne et al., 2008). The most critical points of the Anammox process faced in this work were the slow growth rate of Anammox bacteria (Li et al., 2012) that requires long time of start-up and the antagonism (Mattei et al., 2015) between Anammox bacteria and AOB that compete for the same substrate (inorganic carbon and ammonium) and need opposite operating conditions (i.e. anoxic for Anammox bacteria, aerobic for AOB).

2. Materials and methods

The Anammox process was performed in 4 discontinuously fed SBRs, where the processes of biological oxidation and settling took place alternatively. Purified effluents were discontinuously discharged just before the feeding operation, whereas the excess sludge was recycled when necessary. All these processes and operations were performed according to a specific and cyclic time sequence.

2.1 Biomass collection and preparation

Two different biomasses were selected as seeding sludge to develop Anammox bacteria in two identical SBRs: the first reactor (AcS) was fed with 2 L activated sludge (1.2% in total solids-TS content, v/v) taken from the denitrification tank of a municipal WWTP, located in Nola (NA), southern Italy; the second reactor (AnS) was filled with 0.9 L sludge (2.7% in TS content, v/v) taken from an anaerobic digester treating buffalo manure and milk serum located in Albanella (SA), southern Italy. The remaining two SBRs (BRS1 and BRS2) were filled with 0.1 L sludge (1.4% in TS, v/v) taken from a full-scale partial nitritation-Anammox process at the municipal WWTP of Brunico (BZ), Italy.

2.2 Synthetic Wastewater preparation

The composition of the mineral medium used to feed all SBRs has been prepared following the recipe of van de Graaf et al. (1996). In detail, 0.5 g $KHCO_3$, 0.5 g $NaHCO_3$, 0.054 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.136 g $CaCl_2 \cdot 2H_2O$, 1 mL of trace element solution I and 1 mL of trace element solution II were added to 1L of demineralized water (Elga, PURELAB Option Q-series, Italy). Trace element solutions I was composed of 5 g/L EDTA and 5 g/L $FeSO_4 \cdot 7H_2O$. Trace element solution II was composed of 5 g/L EDTA, 0.43 g/L $ZnSO_4 \cdot 7H_2O$, 0.24 g/L $CoCl_2 \cdot 6H_2O$, 0.99 g/L $MnCl_2 \cdot 4H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, 0.22 g/L $Na_2MoO_4 \cdot 2H_2O$,

0.19 g/L $NiCl_2 \cdot 6H_2O$, 0.21 g/L $Na_2SeO_4 \cdot 10H_2O$, and 0.014 g/L H_3BO_3. Synthetic wastewater was prepared by adding to the mineral medium required amounts of $(NH_4)_2SO_4$ and $NaNO_2$ for Anammox biomass enrichment SBRs (i.e. AcS and AnS) and only $(NH_4)_2SO_4$ for the other two SBRs (i.e. BRS1 and BRS2) where Anammox process was performed.

2.3 Anammox biomass enrichment reactors

Two identical 5L cylindrical glass SBRs with working volume of 4L were used to perform the Anammox biomass enrichment. The operating anoxic conditions were achieved and maintained by hermetically closing the reactors and temporarily flushing them by inert Argon gas. A temperature of 34°C was constantly maintained inside the reactors by an external thermostatic bath heated by thermostat heaters (Odyssea, heat pro 100, Canada). The hydraulic retention time (HRT) was set to 4 days. The cyclic exchanging volume was set to 1 L (25%). The working sequence lasted 24 hours and it was composed of 4 phases as follows: feeding time (80 minutes); reaction time (16 hours and 40 minutes); sedimentation time (4 hours); discharge time (20 minutes). A magnetic stirring system (Ika, C-MAG MS 10, Germany) was used to maintain the biomass suspended. Feeding and discharging operations were performed by peristaltic pumps (Watson Marlow, 520 Du, UK) to achieve desired flow according to the feeding and discharging scheme of different reactors described above. The on/off operations of all devices used in the experiment were regulated by an electronic timer (GIB, HWD EG01, Germany). All the reactors were fed with synthetic wastewater (see sub-section 2.2) prepared every 2 days. The molar ratio of NO_2^--N/NH_4^+-N shifted between 0.76 and 1.5, in response of nitrite accumulation. Changes (i.e. amounts of nitrogen based compounds and their molar ratio) in the feeding solution were operated when the reactors experienced acceptable nitrogen removal efficiency (positive indication) or accumulation of undesirable compounds, i.e. nitrite and nitrate (negative indication). Before feeding, the synthetic wastewater was appropriately degassed by a vacuum pump (KNF Laboport, N 820 FT.18, Germany) to completely eliminate oxygen.

2.4 Partial nitritation-Anammox (CANON type) system reactors

The partial nitritation-Anammox (single stage) process was conducted in two SBRs with working volume of 2L, constituted by a 2L Schott bottles (Duran, Germany). The biomass was maintained in suspension by a mechanical stirrer (Guangzhou, mod. AM300S-H, China). The degradation of substrate (i.e. synthetic wastewater) was performed in alternating aerobic/anoxic conditions. A temperature of 34°C was maintained constantly by an external thermostatic bath heated by thermostat heaters (Odyssea, heat pro 100, Canada). HRT was set equal to 4 days. The cyclic exchanging volume was set to 0.5 L (25%). The working sequence lasted 24 hours and it was composed of 4 phases as follows: feeding time (30 minutes); reaction time (22 hours); sedimentation time (1 hour); discharge time (30 minutes). Feeding (Watson Marlow, mod. Varmeca, UK) and discharging pumps (Watson Marlow, 302s, UK) were set with a hydraulic flow equal to 1.25 mL min^{-1}. The oxygen in the system was discontinuously (30 minutes for cycle) supplied by micro pore air diffusers (Ecoplus, aquarium cylinder, UK) with a air flow of 0.5 L min^{-1}. The on/off operations of all devices used in the experiment were regulated by an electronic timer (GIB, HWD EG01, Germany). The reactors used to perform partial nitritation-Anammox system were fed with synthetic wastewater (see subsection 2.2) where ammonium was added in concentration that was increased during the investigation time according to the efficiency level in nitrogen removal achieved by the system. To limit nitrate accumulation the composition of synthetic wastewater was changed during the experiment as well as the operating conditions.

2.5 Analytical methods

The ammonium concentration (mg NH_4^+-N/L) was measured by Nessler method using a spectrophotomer (WTW, PhotoLab 6600 UV-VIS series, Germany) as well as by distillation equipment (Velp Scientifica, UDK 132 Semiautomatic Distillation Unit, Italy) when concentration is higher than 3 mgNH_4^+-N/L (APHA standard methods, 2005). Nitrite and nitrate concentration (mg NO_2^--N/L, mg NO_3^--N /L, respectively) were measured by ionic chromatography device (Metrohm, 761 Compact IC, Switzerland) and spectrophotometric equipment (WTW, PhotoLab 6600 UV-VIS series, Germany). The pH was measured by a portable pH meter (WTW, inolab, Germany). Total and carbonate alkalinity were determined by titration according to Anderson and Yang (1992). Titrations were performed by using an automated titrator (Radiometer Copenhagen, TT80, France).

3. Results and discussions

3.1 Anammox biomass enrichment

Both reactors were initially fed with a total nitrogen loading rate (NLR) of 0.01 kgN m^3 d^{-1} by pumping into the reactors a solution containing 26 mgNO_2^--N /L of $NaNO_2$and 20 mgNH_4^+-N/L of $(NH_4)_2SO_4$ according to a stoichiometric molar ratio of 1.32 (Eq. (1)). After day 8, the NO_2^--N/NH_4^+-N ratio was modified to 1.50 by

increasing the NO_2^--N concentration to 30 mgNO_2^--N /L, with the aim of preventing a lack of NO_2^--N for Anammox bacteria (Figure 1). In this time interval the concentration of NH_4^+-N ranged in the effluent between 1.0 and 8.8 mg/L whereas the concentration of NO_2^--N between 2.1 and 18.2 mg/L. From day 26 to day 44 the concentration of NH_4^+-N in the influent was increased up to 23 mg/L, with the aim of establishing again the NO_2^--N/NH_4^+-N molar ratio to 1.32 and at day 45 the concentrations of NO_2^--N and NH_4^+-N were doubled (60 mg/L NO_2^--N and 46 mg/L NH_4^+-N). From day 40 the reactor AcS experienced an increase of nitrite concentration up to 65 mg/L NO_2^--N on day 78. This occurrence led to the decision to feed the reactor for the next 7 days with a solution containing only NH_4^+-N at concentration of 46 mg/L since nitrites in high concentrations are considered toxic for Anammox (Lotti et al., 2012). Nitrite accumulation was probably due to presence in the sludge of different bacterial strains that competing with Anammox bacteria for NH_4^+-N caused a greater consumption of NH_4^+-N than NO_2^--N. Feeding the reactors with only NH_4^+-N resulted in a progressive reduction of NO_2^--N concentration due to a dilution effect as well as consumption by Anammox bacteria. Subsequently, to prevent further NO_2^--N accumulation, from day 87 the molar ratio between NO_2^--N and NH_4^+-N was changed from 1.32 to 0.76, by adding in the influent solution 35 mg/L of NO_2^--N instead of 60 mg/L. In AcS reactor the concentrations of NH_4^+-N in the effluent fluctuated between 2 to 30 mg/L for the first 117 days, whereas, from day 117 it showed a decreasing trend (with values ranged between 0.1 and 5.1 mg/L) that was explained as an effect of an increase of the number and consequently the activity of Anammox bacteria. As a consequence of the good performance of the system, from day 128 the concentration of NH_4^+-N in the influent was furthermore increased from 46 to 58 mg/L and accordingly to a molar ratio of 0.86 was also increased the concentration of NO_2^--N up to 50 mg/L Since the AcS reactor at this new operating conditions showed a removal efficiency approximately of 90%, the molar ratio between NO_2^--N and NH_4^+-N at day 141 was increased from 0.86 to 1 by adding to the influent solution, 58 mg/L of NO_2^--N instead of 50 mg/L, with the aim of making the molar ratio between the nitrogen based compounds progressively equal to the stoichiometric value of 1.32. From day 141, the nitrogen removal efficiency decreased to approximately 70%, presumably due to the increased NLR (0.03 kgN m^3 d^{-1}) and the short time given to the Anammox bacteria to get adapted to this new high load level. The nitrate production was relatively low during the whole experiment fluctuating between 2 and 26 mg/L. In the last 50 d it was noticed a significant decrease with values lower than 15 mg/L. This result was a proof of the successful enrichment process for the Anammox biomass in the sludge collected from the WWTP of Nola.

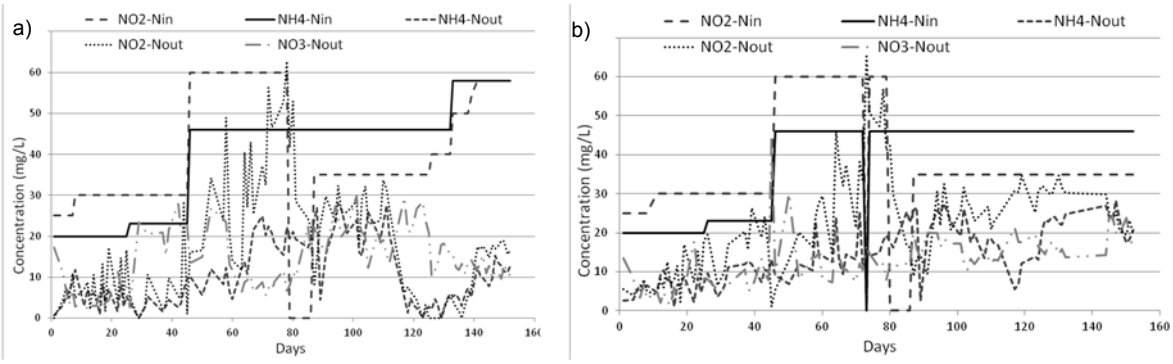

Figure 1: Evolution of N-based compounds during Anammox biomass enrichment: a) AcS reactor; b) AnS reactor

Reactor AnS did not show the same experimental results. From day 120, the reactor showed an excessive accumulation of NH_4^+-N, NO_2^--N and NO_3^--N, and therefore, in contrast to what was made with AcS reactor, the concentrations of NH_4^+-N and NO_2^--N in the feeding solution were not changed and the molar ratio of 0.76 was maintained. From day 144 the concentration in the effluent of N-based compounds started to show a decreasing trend that was a proof of a good level of the Anammox activity, that was achieved almost 30 days after the AcS. This result proves that activated sludge from a denitrification tank is better than anaerobic sludge to develop Anammox biomass.

3.2 Partial nitritation-Anammox (CANON type) system reactors handling and performance

Both reactors were initially fed with an ammonium concentration in the influent of 5 mg/L NH_4^+-N that was gradually increased up to 20 mg/L NH_4^+-N on day 40. In the first period of operation, the reactors performed an ammonium removal of around 50% with a production of nitrates around 40% of the ammonium consumed in BRS1 and 30% in BRS2. In both reactors the nitrates production was actually higher but not much than the

theoretical production that is 26% of the ammonium consumed. Feeding the reactors with a higher ammonium concentration (i.e. 30 mg/L) from day 50 with the aim of setting the condition for the removal of a high concentration of ammonium, it was noticed an accumulation of nitrate in the system up to a concentration of 60 mg/L NO_3^--N at day 86. This event was likely due to a faster growth of the nitrite-oxidizing bacteria (NOB) than Anammox biomass. To prevent the excessive production of nitrates the operating conditions were changed with the aim of limiting the growth of NOB and simultaneously increasing the number of Anammox. To achieve this purpose the oxygenation of reactors was previously reduced by turning off the micro pore air diffusers on day 90 because oxygen inhibits Anammox bacteria and a low concentration of O_2 limits the nitrate production. This operation resulted in a slight decrease of nitrate concentration but not enough compared to the efficiency level expected from Eq. (1). Therefore the mixing system was changed from a magnetic stirrer to a mechanical stirrer from day 107. This change in the mixing system favours the formation of granules of sludge, which usually contain more Anammox bacteria in the inner part whereas the external part is mainly composed of AOB and NOB due to the oxygen gradient (Kindaichi et al., 2007). With this configuration the O_2 dissolved in the bulk inhibits less the Anammox activity because the bacteria living in the inner part of the granule have limited contact with O_2. To further moderate the nitrate production, the concentration of alkalinity in the feeding solution was reduced from 1 g/L to 177 mg/L from day 130 because AOB and NOB bacteria need a higher amount (e.g. 1.98 mol of HCO_3^- per mol of $NH4^+$ converted for AOB and 0.5 mol of HCO_3^- per mol of NO_2^- converted for NOB) of inorganic carbon (IC) compared with Anammox bacteria that require only 0.066 mol of HCO_3^- per mol of NH_4^+-N consumed (Kimura et al., 2011). From day 163, the alkalinity was further lowered to 150 mg/L where a positive decrease of NO_3^--N concentration up to 20 mg/L was observed, presumably resulted from the granular structure as well as the decreased alkalinity. Simultaneously the reactors experienced an unexpected decrease in ammonium removal. This may be due to the granular configuration of Anammox sludge, which hampers the availability of IC to Anammox bacteria as IC has to diffuse into the granule to be used by Anammox bacteria. Therefore AOB and NOB, which compete for IC with Anammox bacteria, are favoured as they are located on the surface of the granules and have easier access to IC. To study this effect the two reactors were fed from day 200 with a different alkalinity concentration: in BRS1 the alkalinity was gradually increased, initially up to a value of 69 mg/L at day 204, and then up to 200 mg/L at day 207 whereas in BRS2 it was gradually decreased up to 36 mg/L at day 187. The higher level of alkalinity in BRS1 resulted in an increase of the ammonium removal efficiency as well as nitrate production, whereas the lower level of alkalinity in BRS2 resulted in a further reduction of ammonium removal efficiency and the presence of nitrite in the effluent. This result proves that a low amount of IC affects more the activity of Anammox bacteria than AOB and NOB, contrary to the stoichiometry of the reactions, i.e. ammonium oxidation by AOB, nitrite oxidation by NOB and anaerobic ammonium oxidation by Anammox bacteria.

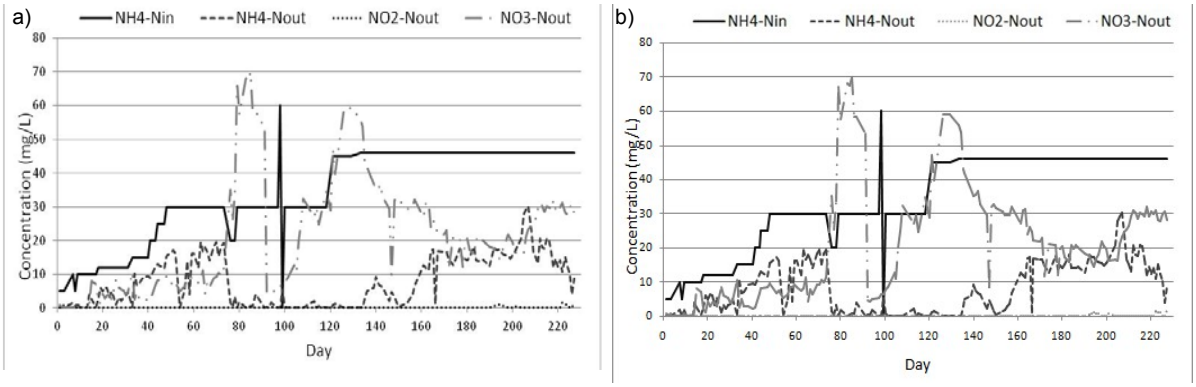

Figure 2: Performance of the Partial nitritation-Anammox: a) BRS1 reactor; b) BRS2 reactor

4. Conclusions

The research presented in this report has showed that the growth of Anammox biomass from wastewater treatment sludge as well as from the sludge of an anaerobic digester treating organic solids is possible even if very slow: actually after 160 days the enrichment process resulted in a partial growth of the Anammox biomass. In terms of enrichment performances the sludge from the denitrification tank of a WWTP was superior to the sludge from an anaerobic digester since the development of Anammox biomass was faster. Moreover the research showed the critical aspects concerning the management of the combined process of partial nitritation-Anammox (CANON-type system). Several attempts by changing the operating conditions

were made with the aim of favoring the growth of Anammox rather than AOB and NOB, but no one gave the expected results. This outcome proves that it is difficult to manage in the same reactor the coexistence of different biomasses that compete for the same substrate and need different environmental conditions to grow.

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