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Long-term operation of a partial nitritation pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process

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ABSTRACT

The goal of this work was to demonstrate the feasibility of treating leachate with high ammonium concentrations using the SBR technology, as a preparative step for the treatment in an anammox reactor. The cycle was based on a step-feed strategy, alternating anoxic and aerobic conditions. Results of the study verified the viability of this process, treating an influent with concentration up to 5000 mg N–NH_4^+ L^{-1}. An effluent with about 1500–2000 mg N–NH_4^+ L^{-1} and 2000–3000 mg N–NO_2^- L^{-1} was achieved, presenting a nitrite to ammonium molar ratio close to the 1.32 required by the anammox. Furthermore, taking advantage of the biodegradable organic matter, the operational strategy allowed denitrifying about 200 mg N–NO_2^- L^{-1}. The extreme operational conditions during the long-term resulted on the selection of a sole AOB phylotype, identified by molecular techniques as *Nitrosomonas* sp. IWT514.

1. Introduction

Leachate generated from landfill sites is highly contaminated with a wide range of chemical contaminants. Among them, urban landfill leachate is usually characterised by high ammonium concentrations and low biodegradable organic matter content (Kulikowska and Klimiuk, 2008). Because of this, treating leachate through conventional nitrification–denitrification processes is economically expensive due to the high oxygen demand and the requirement of a supplemental external carbon source.

Treatments based on anaerobic ammonium oxidation (anammox) metabolism may pose a more sustainable alternative to the treatment of such wastewater, due to the reduced aeration requirements and lower dosage of external organic carbon (Liang and Liu, 2008). Eq. (1) presents the anammox stoichiometry (Strous et al., 1998):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (1)$$

Prior to the anammox process, the ammonium present in wastewater must be partially oxidised to nitrite (Eq. (2)) by ammonium oxidising bacteria (AOB). Further nitrification to nitrate (Eq. (3)), carried out by nitrite oxidising bacteria (NOB), must be avoided in order to allow optimal N-removal by the anammox bacteria (Yamamoto et al., 2008). In addition, biodegradable organic matter should be removed to avoid its negative effects on the subsequent anammox process (Chamchoi et al., 2008; Ruscalleda et al., 2008)

$$NH_4^+ + 2HCO_3^- + 1.5O_2 \rightarrow NO_2^- + 3H_2O + 2CO_2 \quad (2)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (3)$$

According to the literature, nitrite build-up can be achieved successfully by oxygen limitation (Aslan et al., 2009), as well as by high temperatures coupled with low sludge residence times (Fux et al., 2002; Hellinga et al., 1998; van Dongen et al., 2001). It can also be accomplished by inhibiting NOB with free ammonia (FA) and/or free nitrous acid (FNA) (Ganigué et al., 2007; Lai et al., 2004). However, several studies (Fux et al., 2004; Villaverde et al., 2000) have reported problems in maintaining nitrite build-up over the long-term in such systems when the NOB becomes acclimatised to high concentrations of these inhibitory compounds.

The aim of a partial nitritation system is to oxidise about half the influent ammonium to nitrite. In the particular case of highly ammonium-loaded wastewater as the landfill leachate, the ammonium and nitrite concentrations inside a partial nitritation reactor could be very high. This overcomes an operational problem, since AOB can be inhibited by the unionised forms of their substrate

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and product, NH_3 and HNO_2 (widely described by Anthonisen et al., 1976; Ganigué et al., 2007; Vadivelu et al., 2007; Van Hulle et al., 2007). Partial nitritation systems have been used extensively to treat sludge digester supernatants (Fux et al., 2002; Gut et al., 2006; Vázquez-Padín et al., 2009; among others), which present ammonium concentrations between 500 and 1500 mg N–NH_4^+ L^{-1}. Nevertheless, inhibition can be a critical issue when dealing with landfill leachate, which can present concentrations up to 6000 mg N–NH_4^+ L^{-1} (Kurniawan et al., 2006). In this light, any reduction in total nitrogen during the partial nitritation reactor must be observed as an opportunity to reduce the inhibition factors and thus lower operational costs. Therefore, despite the low levels of biodegradable organic matter available in the leachate, the inclusion of anoxic phases during the feeding events may help reduce the inhibition of AOB by heterotrophic denitrification via nitrite.

The primary aim of this paper is to demonstrate the feasibility of treating urban landfill leachate with extremely high ammonium concentrations (up to 5000 mg N–NH_4^+ L^{-1}) by a partial nitritation-sequencing batch reactor (PN-SBR), as a step prior to an anammox reactor. Specifically, this study seeks to achieve the stable production of a suitable mixture of ammonium and nitrite, as well as to demonstrate the viability of long-term nitrite build-up in a biomass retention system. This study also focuses on harnessing the low levels of available biodegradable organic matter for denitrification purposes, and assesses the role of bicarbonate on the nitritation process. Furthermore, the microbial populations involved in the aerobic processes of N-compound oxidation (AOB and NOB) have been discerned by DNA-based molecular techniques to better understand the partial nitritation process under these extreme conditions.

2. Methods

2.1. PN-SBR pilot plant

This study was conducted in a PN-SBR pilot plant, located at the LEQUIA facilities of the University of Girona. The pilot plant was comprised of a 250 L pilot-scale SBR operated at a minimum volume of 111 L. The reactor was water jacketed, allowing temperature control by means of a thermostated water bath. Complete mixture was achieved by means of a mechanical stirrer, and aeration was carried out using air diffusers (Magnum, from OTT System GmbH & Co.) located at the bottom of the reactor. Raw leachate was stored in a 300 L storage tank prior to treatment. The pilot plant was also equipped with a monitoring and control system. On-line data provided by pH, ORP, DO, and temperature probes (CPF 81, CPF 82 and OXYMAX-W COS-41, from Endress-Hauser) were acquired by means of interface cards (PCI-1711 and PCLD-8710 from Advantech) and by our own software, which was developed using Lab-View®. Program commands were transmitted to the pilot plant through another interface card (PCI-885 from Advantech) and a relays output board, which controlled the switch on/off of all electrical devices and thus allowed the repetition of a previously defined operational cycle.

2.2. Operational conditions

Temperature was maintained at 36 ± 1 °C, and dissolved oxygen (DO) was controlled at a set-point concentration of 2 mg L^{-1} during the aerobic reaction phases. The pH was kept below a maximum set-point value of 8 through the addition of hydrochloric acid (1 M).

The reactor was operated according to an anoxic–aerobic step-feed strategy. The cycle, with a total length of 24 h, consisted of 14 feeding events under anoxic conditions, followed each by an aerobic reaction phase of 85 min. Total inflow was equally distributed among all feeding events. Therefore, the cycle could be divided into 14 identical sub-cycles of 100 min, each consisting on 15 min of anoxic phase (feeding between 4 and 14 min) followed by 85 min of aerobic reaction. The cycle ended with a 20 min settling phase followed by 20 min of draw.

Due to the high amount of suspended solids removed from the reactor by the outflow (393.7 ± 179.4 mg TSS L^{-1}), no purge phase was carried out. Despite this, the average concentration of mixed liquor suspended solids (MLSS) during the study was 666.0 ± 240.6 mg TSS L^{-1} with a volatile fraction of about 66–75%. On the other hand, due to significant oscillations in the raw leachate ammonium concentration, influent flow had to be adjusted to keep the nitrogen loading rate (NLR) within a suitable range of values. This caused significant hydraulic retention time (HRT) fluctuations, ranging from 3 to 6 days. Under these conditions the sludge retention time (SRT) was not a controlled system parameter, but was calculated considering reactor MLSS and effluent suspended solids concentrations; its average value was 6.44 ± 2.34 days (ranging from 3.1 to 12 days).

2.3. Analytical methods

Total suspended solids (TSS; APHA-2540D), volatile suspended solids (VSS; APHA-2540E), chemical oxygen demand (COD; APHA-5220B), total organic carbon (TOC; APHA-S310), inorganic carbon (IC; APHA-S310), ammonium (N–NH_4^+; APHA-4500-NH_3.B-C5220B), total kjeldahl nitrogen (TKN; APHA-4500-Norg.B), nitrites (N-NO_2^-; APHA-4110B), and nitrates (N–NO_2^-; APHA-4110B) were all measured according to Standard Methods (APHA, 2005). Biological oxygen demand (BOD) was measured using OxiTop® system (from WTW), based also on Standard Methods (APHA-5210D). Total nitrogen was calculated as the sum of N–TKN, nitrite, and nitrate concentrations as mg N–TN L^{-1}. Conductivity was measured by a conductimeter (EC-Meter Basic 30+ from Crison).

Concentrations of Free Ammonia (FA) and Free Nitrous Acid (FNA) were calculated as a function of pH, temperature, and Total Ammonium as Nitrogen (TAN) for FA, or Total Nitrite (TNO_2) for FNA, according to Anthonisen et al. (1976).

The specific oxygen uptake rate (OUR, mg O_2 g VSS^{-1} h^{-1}) was calculated according to Puig et al. (2005) from on-line measurement of the drop in dissolved oxygen when no air flow was supplied.

2.4. Molecular analysis of AOB and NOB

DNA isolation. DNA was isolated from the collected samples using DNeasy Blood & Tissue commercial kit (Qiagen, Venlo, The Netherlands), in accordance with the manufacturer's instructions for Gram-negative microorganisms. The DNA isolation efficiency was verified in a 0.8% (w/v) electrophoresis gel.

Polymerase chain reaction (PCR). PCR analyses were carried out with a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Perkin–Elmer, CA, USA), using the PCR programs as described in the respective references of the primer sets. AOB populations were detected through amplification of the 16S rDNA operon with the CTO primer set: CTO654R (Kowalchuk et al., 1997) coupled to a CTO189F mix, which is a 2:1 mixture of CTO189F A/B and CTO189F C. Detection of *Nitrobacter* and *Nitrospira* was also achieved by amplification of the 16S rDNA operon. The primer set FGPS872R coupled with FGPS1269F (Degrange and Bardin, 1995) was used for *Nitrobacter* species, while *Nitrospira* species were amplified with NSR1113F in combination with NSR1264R (Dionisi et al., 2002).

Cloning, sequencing and identification. For cloning procedures, PCR products were ligated to pGEM Teasy vectors (Promega) and transformed into Top10 *Escherichia coli* cells following manufacturer instructions. The vectors were then isolated from *E. coli* colonies growing in LB + Ampicillin medium using the Ultraclean 6 minute Mini Plasmid Prep Kit (MoBio). Cloning was only necessary to achieve DNA sequences from amplifications performed with CTO primers. PCR products obtained with *Nitrobacter* and *Nitrospira* primer sets were sequenced directly, since only one phylotype was detected in each product. 16S rDNA fragments were sequenced by the Macrogen Service (Macrogen Ltd., Seoul Korea, www.macrogen.com) and the partial sequences were compared with the National Center for Biotechnology Information (NCBI) database using the BLASTn algorithm tool (Altschul et al., 1990) to identify the phylotypes. The sequences were also submitted to the Greengenes (www.greengenes.lbl.gov) via the Bellerophon software to check for the presence of chimeras.

2.5. Raw leachate

The raw leachate treated in this study, which came from the Corsa urban landfill site (41° 6′ 28″ N, 1° 7′ 4″ E; Reus, Spain), presented a highly variable composition. The concentration range and mean values of the principal chemical compounds, along with the electrical conductivity (EC) and pH, are summarised in Table 1.

2.6. Experimental procedure

The SBR was inoculated with a mixture of nitrifying sludge from the Sils-Vidreres municipal WWTP (41° 47′ 58″ N, 2° 45′ 7″ E; Catalonia, Spain) and the Orís urban landfill leachate treatment plant (42° 03′ 28″ N, 2° 14′ 15″ E; Catalonia, Spain). After a brief start-up, the PN-SBR was operated under an anoxic–aerobic step-feed strategy.

From the process operation point of view, the 450-day study can be divided into three periods. First, the reactor was operated without any bicarbonate adjustment (Period I). Once the low percentage of ammonium being oxidised to nitrite was observed, bicarbonate dosage ($NaHCO_3$ 0.5 M) was implemented on day 59 to increase the conversion (Period II). This addition was made when pH decreased below a set-point value of 7.2. Due to the low reliability of this control, on day 220, solid bicarbonate began to be dosed at the influent. The amount of bicarbonate added was calculated based on the stoichiometrical requirements for achieving a suitable effluent to feed an anammox reactor. During Period III, the minimum pH was also controlled by the bicarbonate solution dosage.

Table 1
Leachate characteristics throughout the study.

Compound	Units	Range	Mean ± σ
Ammonium, NH_4^+	mg N–NH_4^+ L^{-1}	2237–4938	3772 ± 956
Nitrite, NO_2^-	mg N–NO_2^- L^{-1}	0.0–1.2	0.2 ± 0.5
Nitrate, NO_3^-	mg N–NO_3^- L^{-1}	0.0–8.0	1.4 ± 3.2
Alkalinity	mg HCO_3^- L^{-1}	2059–11,223	8638 ± 3314
Total Kjeldahl nitrogen, TKN	mg N L^{-1}	2494–5540	4058 ± 987
Chemical oxygen demand, COD	mg O_2 L^{-1}	2480–7040	4357 ± 692
Biological oxygen demand, BOD_5	mg O_2 L^{-1}	230–1025	810 ± 278
Total organic carbon, TOC	mg C L^{-1}	1509–2420	1946 ± 457
Total carbon, TC	mg C L^{-1}	2977–3812	3541 ± 385
Inorganic carbon, IC	mg C L^{-1}	1336–1904	1571 ± 296
Conductivity, EC	μS cm^{-1}	60,600–70,500	68,065 ± 1863
pH	–	7.48–8.56	8.11 ± 0.20

3. Results and discussion

A suitable nitrite to ammonium ratio in the influent of around 1.32 is crucial for proper operation of an anammox reactor. With this objective, partial nitritation was conducted in an industrial-scale PN-SBR to achieve the desired conversion, as well as to highlight the keys to this proper operation. In a previous study (Ganigué et al., 2008), the step-feed strategy was proven to be a good cycle design for achieving stable partial nitritation, since the distribution of the inflow across different feeding events avoided loading shocks and subsequent important pH variations in the reactor. Nevertheless, because of the high nitrogen concentrations in this study, anoxic phases were included during feeding events to promote heterotrophic denitrification.

3.1. PN-SBR performance

The reactor was successfully operated for 450 days treating raw urban landfill leachate, according to an anoxic–aerobic step-feed strategy. Fig. 1 presents the evolution of influent ammonium concentration and nitrogen loading rate (NLR) (Fig. 1a) and the concentration of effluent nitrogen compounds (Fig. 1b) and bicarbonate (Fig. 1c) along the whole study.

Fig. 1a clearly shows the notable oscillations in influent ammonium concentration throughout the study, ranging from 2000 to 5000 mg N–NH_4^+ L^{-1}. These variations were due to the raw leachate supplied by the landfill site. The initial NLR was around 0.85 kg N m^{-3} d^{-1}, ultimately reaching a value of 1.2 kg N m^{-3} d^{-1} by the end of the study.

Fig. 1b reveals that partial nitritation of influent ammonium was achieved over the 450 day period, preventing further nitrite oxidation to nitrate (NO_3^- concentration throughout the study was always below 25 mg N–NO_2^- L^{-1}).

A more in-depth analysis shows that during Period I (no external bicarbonate addition) the concentration of effluent ammonium was much higher than nitrite, with conversions around 25%. In this period, as presented in Fig. 1c, the available inorganic carbon in the raw leachate was much lower than the theoretical stoichiometric amount required for achieving the desired effluent composition. To overcome this, bicarbonate was added during Period II to enhance the oxidation of ammonium to nitrite. Thus, nitrite concentration substantially increased, achieving an effluent with about 1500 mg N–NO_2^- L^{-1} and 1200 mg N–NH_4^+ L^{-1}. Nevertheless, reactor conversion was quite unstable (ranging from 22% to 78%) due to the imprecise control of the bicarbonate dosing, and the nitrite to ammonium ratio fluctuated considerably. Furthermore, at the end of Period II (195th day) ammonium started to accumulate since the control system was unable to supply enough bicarbonate, reaching at the end of this period ammonium concentrations higher than 3000 mg N–NH_4^+ L^{-1}. The problem was solved by the addition of solid bicarbonate at the pre-treatment tank (Period III) resulting in an enhanced and stabilised nitrite production. This method resulted in the production of a suitable influent to feed an anammox reactor during Period III, with ammonium and nitrite concentrations reaching about 1800 mg N–NH_4^+ L^{-1} and 2600 mg N–NO_2^- L^{-1}.

Finally, it should also be mentioned that all bicarbonate supplied to the reactor was removed from the system, as presented in Fig. 1c, mainly by the AOB activity.

All biodegradable organic matter should be removed in the partial nitritation step. Fig. 2 presents the organic matter evolution of

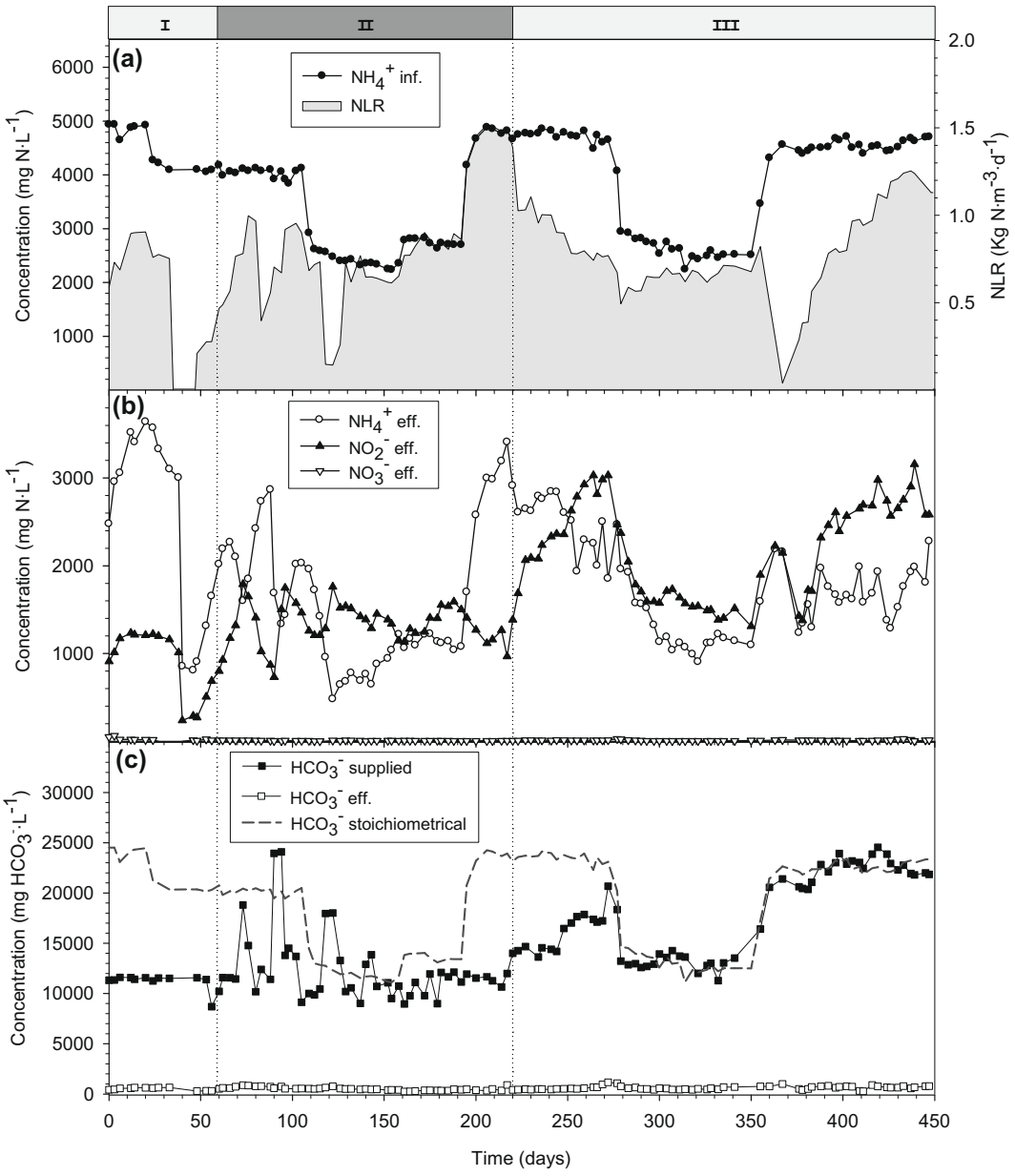

Fig. 1. Evolution of the PN-SBR over the course of the study (Periods I, II and III). (a) Influent ammonium concentration and NLR. (b) Effluent nitrogen compounds, (c) Supplied, effluent and stoichiometrical bicarbonate.

both influent and effluent in terms of TOC values and removal percentage.

The raw leachate presented influent TOC concentrations ranging from 1500 to 3000 mg C L^{-1}. The biodegradable fraction of this organic matter was removed in the PN-SBR process (either aerobically or anoxically). Nevertheless, except for the periods with operational problems, TOC concentrations at the effluent were higher than 1000 mg C L^{-1} throughout the study. The low TOC removal percentage (less than 50%) was related to the high refractory organic matter fraction in the raw leachate. This was corroborated by a mean BOD_u to COD ratio of 0.32 in the raw leachate and a soluble BOD_u of zero in the effluent.

3.2. Heterotrophic denitritation

The nitrogen balance and organic matter removal over a period of stable operation were assessed to estimate the nitrogen removal by denitritation. Based on this stability requirement the assessment was done between days 145th and 335th (Periods II and III), neglecting all data biased due to influent composition changes. The theoretical amount of COD necessary for denitrification was then calculated and plotted, based on Tchobanoglous et al. (2003), obtaining a theoretical ratio of 1.97 g COD per g N–NO_2^-. Results are depicted in Fig. 3.

The average amount of nitrogen eliminated in the system was only about 200–250 mg N L^{-1}. As can be observed in Fig. 3a, 15% to 20% of the influent nitrogen was removed by denitritation between days 145 and 225 (Period II), declining to 5% over the next 110 days (Period III). Fig. 3b presents the amount of COD eliminated in respect to the total organic matter at the influent. The COD removed from the system over this 190-day period was about 25–30%. During Period II, more than half of the biodegradable organic matter was used for denitrification purposes, and this value plummeted to less than 10% in Period III. It is important to high-

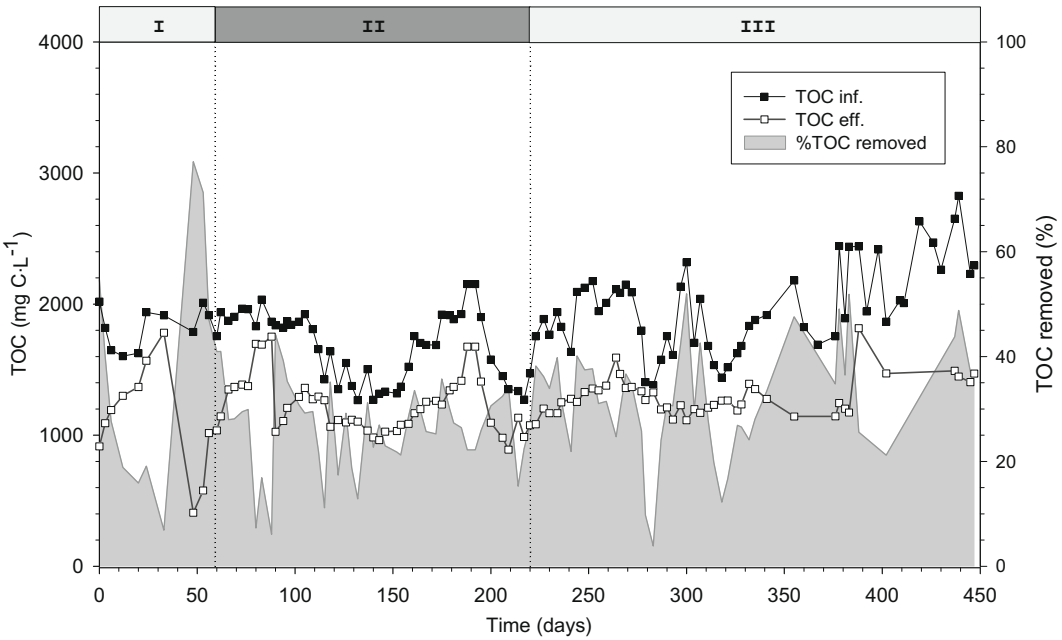

Fig. 2. Evolution of TOC influent/effluent and TOC removal.

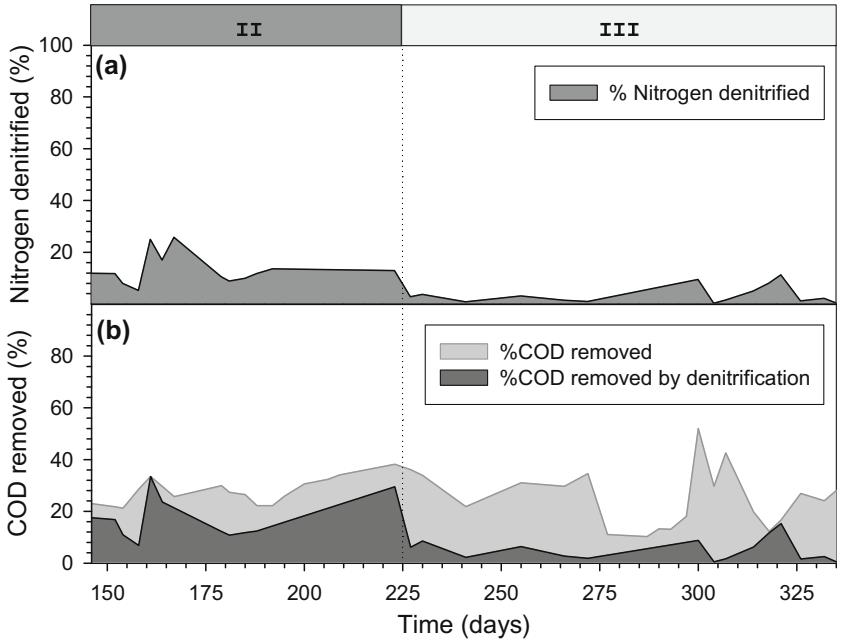

Fig. 3. Denitrification assessment. (a) Evolution of the percentage of nitrogen denitrified; (b) Evolution of the percentage of COD removed from the reactor, along with the theoretical amount of COD necessary to achieve this denitritation.

light that denitrification performance declined when solid bicarbonate began to be dosed to the influent (day 225). There is no literature regarding any direct harmful effect of $NaHCO_3$ on heterotrophic bacteria. However, indirect effects as the high increase in conductivity may have negatively affected heterotrophic bacteria.

3.3. Assessment of influent and effluent molar ratios

Fig. 4 shows the evolution of HCO_3^-:NH_4^+ influent molar ratio (Fig. 4a) and the NO_2^- to NH_4^+ effluent molar ratio (Fig. 4b). Note that bicarbonate supplied by the pH control in Period II has also been taken-up in the influent.

During Period I, the influent presented HCO_3^-:NH_4^+ molar ratios around 0.6 that lead to effluent molar ratios between 0.18 and 0.41, far from the stoichiometric requirements of the further anammox process (1.32 mol of NO_2^- per mole of NH_4^+). The external $NaHCO_3$ dosage during Period II resulted in an increase in HCO_3^-:NH_4^+ influent molar ratio, which manifested in an increased nitrite to ammonium effluent molar ratio. Nevertheless, the dosage strategy induced significant fluctuations, with values ranging from 0.3 to 3.7. Preconditioning the influent with solid $NaHCO_3$ addition (Per-

iod III) provided a more stable HCO_3^-:NH_4^+ influent molar ratio; this kept the effluent molar ratio within a suitable range over the 230 days, between 1 and 1.5 mol NO_2^- per mole of NH_4^+ with peaks up to 2.

With the aim of further studying the relationship between influent HCO_3^-:NH_4^+ molar ratio and effluent NO_2^-:NH_4^+ molar ratio, a data subset was selected wherein the reactor operated under stable conditions. Fig. 5 shows the experimental nitrite to ammonium effluent molar ratio versus influent bicarbonate to ammonium molar ratio. In addition the theoretical effluent NO_2^-:NH_4^+ molar ratio has been calculated based on the AOB stoichiometry (Eq. (2)).

As can be seen, experimental results fit quite well with the stoichiometric curve, validating bicarbonate as the key to control the conversion of ammonium to nitrite. Experimental points deviating from the theoretical behaviour gave information about the process performance and the ongoing phenomena. When the effluent molar ratio was lower than the theoretical, this could be attributed to a bias linked to the heterotrophic denitrification process and/or a bicarbonate loss by CO_2 stripping. On the other hand, a higher than theoretical effluent ratio could be related to ammonium removal from the system due to NH_3 stripping, and/or to additional CO_2 coming from the organic matter elimination, which may allow a higher conversion.

3.4. Determination of AOB and NOB populations

One of the aims of this study was to evaluate the initial AOB and NOB populations and analyze their evolution over the course of a

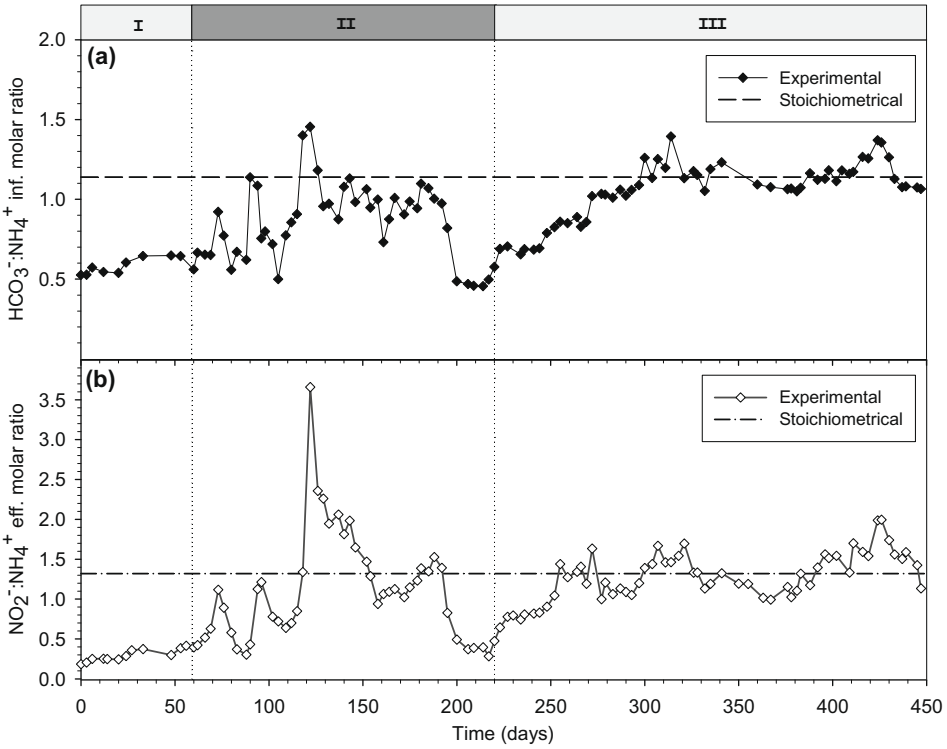

Fig. 4. (a) Evolution of HCO_3^-:NH_4^+ influent molar ratio; (b) Evolution of NO_2^-:NH_4^+ effluent molar ratio.

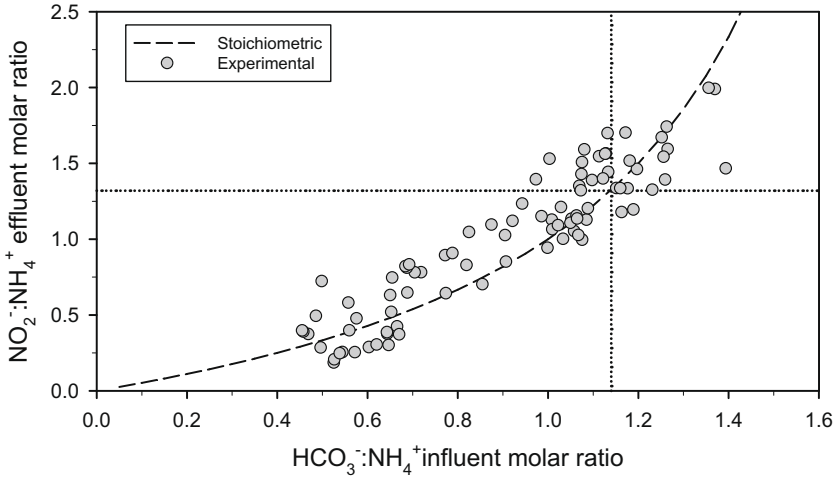

Fig. 5. Experimental and stoichiometrical nitrite to ammonium effluent molar ratio versus bicarbonate to ammonium influent molar ratio.

Table 2
OTUs obtained from the successfully identified AOB phylotypes throughout PCR with CTO primers.

	OTU	Closest BLASTn phylotype	NCBI accession number	% phylotypes
R0	1	Uncultured bacterium clone IIIEA1-rp-O2 nit	gi|161367780|gb|EU267435.1|	27
	2	*Nitrosomonas* sp. Is32	gi|40994846|emb|AJ621027.1|	27
	3	Uncultured bacterium clone S_1	gi|121592404|gb|EF175894.1|	20
	4	Uncultured bacterium clone 58	gi|89348071|gb|DQ413117.1|	10
	5	*Nitrosomonas* sp. IWT514	gi|13958147|gb|AF363293.1|AF363293	10
R450	5	*Nitrosomonas* sp. IWT514	gi|13958147|gb|AF363293.1|AF363293	100

long-term operation. Given the high ammonium and nitrite concentrations in the bulk media (both higher than 1000 mg N L^{-1}), the elevated salinity (always above 60,000 μS cm^{-1}) and high temperature (36 °C), identifying the AOB capable of resisting such extreme conditions would represent an important microbiological feature with potential environmental implications. The microbial community analysis also intended to determine whether or not NOB organisms were present in the community after long-term system operation. With these purposes, five samples were collected and the genomic DNA was isolated and processed. R0 was an aliquot from the initial sample from the mixture of nitrifying sludges used to inoculate the reactor, whereas R192, R288, R415 and R450 were obtained from the PN-SBR on days 192nd, 288th, 415th and 450th, respectively.

All DNA isolations were screened by PCR using different combinations of primers, each of them specific for a bacterial group. Positive PCR amplifications with the CTO primer sets confirmed the presence of AOB during the entire working period. Based on these results, only R0 and R450 were cloned since no changes were detected in the PCR products between days 192 and 450 (data not shown). 16S rDNA sequences created from the cloning procedure showed a high homology with known uncultured bacteria phylotypes, all of them related to *Nitrosomonas*-like species. Phylotypes detected in R0 were grouped into five Organism Taxonomic Units (OTU), while all R450 sequences clustered together, indicating that only one of the OTUs present in the inoculum became dominant in the reactor (Table 2). This OTU showed a high similarity (98–99%) with *Nitrosomonas* sp. IWT514, which was therefore positively selected by the severe operational conditions in the reactor.

Despite the stable nitrite build-up over the long-term, positive PCR amplifications were also obtained for NOB in all the DNA isolations using FGPS and NSR primer sets, which were chosen to search for the main NOB groups in wastewater treatment plants, respectively *Nitrobacter* and *Nitrospira*. All the sequences from each amplification belonged to the same phylotype, and no changes were detected between the inoculum and the reactor samples. Sequences for *Nitrobacter* showed high homology with *Nitrobacter winogradskyi* (99%), while *Nitrospira* sequences perfectly matched (100% homology) with *Candidatus* Nitrospira defluvii. It was initially expected that such extreme conditions would completely remove NOB from the reactor. However, results proved that both *Nitrobacter* and *Nitrospira* were still present in the system and coexisted after 450 days of operation. Therefore, despite being strongly inhibited, changes in environmental conditions may lead to the development of NOB populations and the expression of nitrite oxidation activity.

3.5. Cycle analysis: on-line parameters

On-line parameters, such as pH, DO and specific OUR, provide information about the biological activity and the process performance. Thus, in order to clearly understand the behaviour of the system a specific presentation of an aerobic–anoxic sub-cycle (100 min of the whole 1440 min cycle) is depicted in Fig. 6. The plot is comprised of three sections: aerobic reaction (white area), anoxic reaction (stripped area) and feeding in anoxic conditions (gray stripped area).

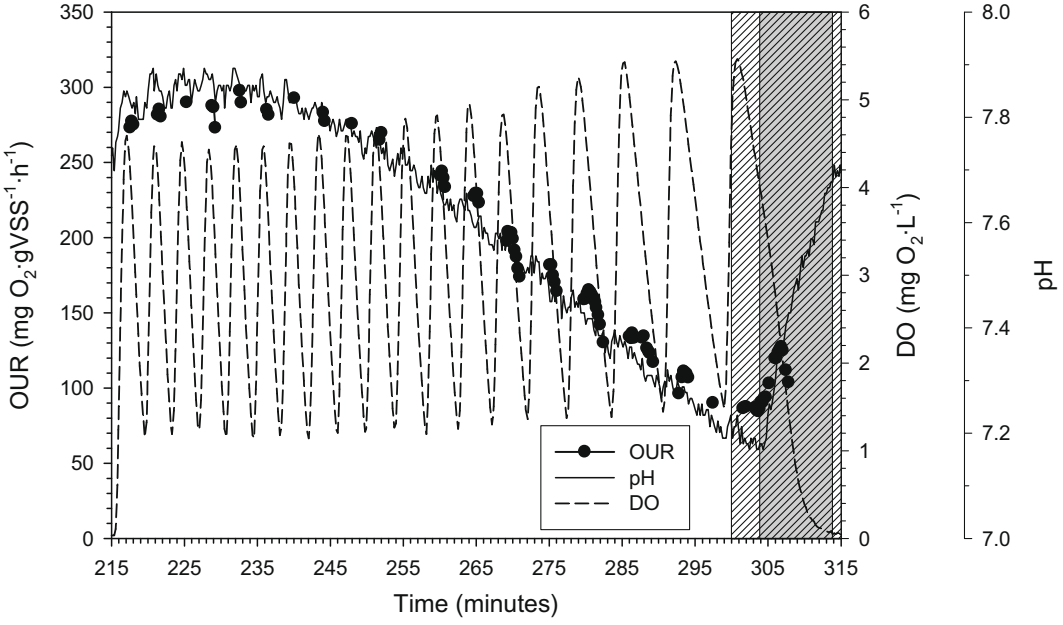

Fig. 6. Specific oxygen uptake rates (OUR), dissolved oxygen (DO) and pH, from 215 to 315 min of a 1440 min cycle.

At the beginning of the reaction phase pH presented values around 7.7–7.8, and declined during the aerobic reaction phase until 7.2, due to the proton production linked to AOB activity. On minute 305, pH sharply increased during the feeding event, since the high pH of the influent (about 8.3), but also due to the OH^- contribution by the denitritation process.

Concerning the specific OUR, it was initially about 260 mg O_2 $gVSS^{-1}$ h^{-1}, increasing to 300 mg O_2 $gVSS^{-1}$ h^{-1} after only 20 min of aeration. From this point, the specific OUR slightly decreased until the end of the aerobic phase. At the beginning of the anoxic phase the DO concentration was still around 5 mg O_2 L^{-1}, which meant that aerobic reactions could continue. At minute 304 the feeding event started, supplying raw leachate to the PN-SBR. This contribution induced an initial increase in specific OUR values until the DO declined to values near zero. By minute 312 the oxygen was completely depleted, and the strict anoxic conditions necessary for denitrification process were reached. Nevertheless, the presence of available oxygen during part of the anoxic feeding period enabled aerobic consumption of the biodegradable organic matter supplied in the influent, which may explain the poor denitrification performance. Denitritation could be then enhanced by extending the anoxic periods before and after the feeding events. However, this solution may negatively impact the aerobic ammonium oxidation process, since transient response phenomena (Vanrolleghem et al., 2004) could affect aerobic organism activity after the anoxic phases. The optimization of the DO control may also help improving the denitrification process.

It is important to point out the close relation between pH and specific OUR, since both parameters declined following the exact same trend. It can be thought that pH directly governs AOB activity. Nevertheless, AOB activity could have also been inhibited by FA and FNA. In addition, as reported by Guisasola et al. (2007) or Wett and Rauch (2003), bicarbonate substrate limitation can also reduce AOB activity. In this sense, further studies are needed to clarify this issue.

Finally, a cycle control could be proposed from the on-line parameter profiles based on Puig et al. (2005). In this way, when OUR decreased below a certain threshold, the control system would change to the next phase. The length of the phases would thus be optimised, and conditions stressful to the AOB would be avoided.

4. Conclusions

This work has proven the feasibility of long-term stable nitrite build-up in a PN-SBR treating raw urban landfill leachate with extremely high ammonium concentrations (up to 5000 mg N–NH_4^+ L^{-1}).

Bicarbonate has proven to be a key parameter for controlling the ammonium to nitrite effluent molar ratio.

The operational strategy, based on an anoxic–aerobic step-feed cycle, helped to reduce the amount of total nitrogen in the reactor, diminishing the inhibition of FA and FNA over AOB. However, denitritation process still requires upgrading.

Molecular techniques have enabled the identification of *Nitrosomonas* sp. IWT514, as the only AOB phylotype present in the reactor at the end of the study. Moreover, *Nitrobacter winogradskyi* and *Candidatus* Nitrospira defluvii were also detected, revealing that NOB were not completely removed from the system.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Pollut. Con. F 48, 835–852.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Aslan, S., Miller, L., Dahab, M., 2009. Ammonium oxidation via nitrite accumulation under limited oxygen concentration in sequencing batch reactors. Bioresour. Technol. 100, 659–664.

Chamchoi, N., Nitisoravut, S., Schmidt, J.E., 2008. Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation (ANAMMOX) and denitrification. Bioresour. Technol. 99, 3331–3336.

Degrange, V., Bardin, R., 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. Appl. Environ. Microbiol. 61, 2093–2098.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68, 245–253.

Fux, C., Boehler, M., Huber, P., Brunner, I., Siegrist, H., 2002. Biological treatment of ammonium-rich wastewater by partial nitritation and subsequent anaerobic ammonium oxidation (anammox) in a pilot plant. J. Biotechnol. 99, 295–306.

Fux, C., Huang, D., Monti, A., Siegrist, H., 2004. Difficulties in maintaining long-term partial nitritation of ammonium-rich sludge digester liquids in a moving-bed biofilm reactor (MBBR). Water Sci. Technol. 49, 53–60.

Ganigué, R., López, H., Balaguer, M.D., Colprim, J., 2007. Partial ammonium oxidation to nitrite of high ammonium content urban landfill leachates. Water Res. 41, 3317–3326.

Ganigué, R., López, H., Ruscalleda, M., Balaguer, M.D., Colprim, J., 2008. Operational strategy for a Partial Nitritation-SBR (PN-SBR) treating urban landfill leachate to achieve a stable influent for an anammox reactor. J. Chem. Technol. Biot. 83, 365–371.

Guisasola, A., Petzet, S., Baeza, J.A., Carrera, J., Lafuente, J., 2007. Inorganic carbon limitations on nitrification: experimental assessment and modelling. Water Res. 41, 277–286.

Gut, L., Płaza, E., Trela, J., Hultman, B., Bosander, J., 2006. Combined partial nitritation/Anammox system for treatment of digester supernatant. Water Sci. Technol. 53, 149–159.

Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., van Loosdrecht, M.C.M., Heijnen, J.J., 1998. The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. Water Sci. Technol. 37, 135–142.

Kowalchuk, G.A., Stephen, J.R., Boer, W.D., Prosser, J.I., Embley, T.M., Woldendorp, J.W., 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. 63, 1489–1497.

Kulikowska, D., Klimiuk, E., 2008. The effect of landfill age on municipal leachate composition. Bioresour. Technol. 99, 5981–5985.

Kurniawan, T.A., Lo, W.H., Chan, G.Y.S., 2006. Physico-chemical treatments for removal of recalcitrant contaminants from landfill leachate. J. Hazard. Mater. 129, 80–100.

Lai, E., Senkpiel, S., Solley, D., Keller, J., 2004. Nitrogen removal of high strength wastewater via nitritation/denitritation using a sequencing batch reactor. Water Sci. Technol. 50, 27–33.

Liang, Z., Liu, J., 2008. Landfill leachate treatment with a novel process: anaerobic ammonium oxidation (Anammox) combined with soil infiltration system. J. Hazard. Mater. 151, 202–212.

Puig, S., Corominas, Ll., Vives, M.T., Balaguer, M.D., Colprim, J., 2005. Development and implementation of a real-time control system for nitrogen removal using OUR and ORP as end points. Ind. Eng. Chem. Res. 44, 3367–3373.

Ruscalleda, M., López, H., Ganigué, R., Puig, S., Balaguer, M.D., Colprim, J., 2008. Heterotrophic denitrification on granular anammox SBR treating urban landfill leachate. Water Sci. Technol. 58, 1749–1755.

Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M., 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50, 589–596.

Tchobanoglous, G., Burton, F.B., Stensel, H.D., 2003. Wastewater Engineering Treatment and Reuse, forth ed. Metcalf and Eddy, Inc., USA.

Vadivelu, V.M., Keller, J., Yuan, Z., 2007. Free ammonia and free nitrous acid inhibition on the anabolic and catabolic processes of *Nitrosomonas* and *Nitrobacter*. Water Sci. Technol. 56, 89–97.

Van Dongen, L.G.J.M., Jetten M.S.M., van Loosdrecht, M.C.M., 2001. The Combined Sharon/Anammox Process. STOWA Report, IWA Publishing, London, UK.

Van Hulle, S.W.H., Volcke, E.I.P., López-Teruel, J., Donckels, B., van Loosdrecht, M.C.M., Vanrolleghem, P.A., 2007. Influence of temperature and pH on the kinetics of the SHARON nitritation process. J. Chem. Technol. Biot. 82, 471–480.

Vanrolleghem, P.A., Sin, G., Gernaey, K.V., 2004. Transient response of aerobic and anoxic activated sludge activities to sudden substrate concentration changes. Biotechnol. Bioeng. 88, 277–290.

Vázquez-Padín, J., Fernádez, I., Figueroa, M., Mosquera-Corral, A., Campos, J.L., Méndez, R., 2009. Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature. Bioresour. Technol. 100, 2988–2994.

Villaverde, S., Fdz-Polanco, F., Garcia, P.A., 2000. Nitrifying biofilm acclimation to free ammonia in submerged biofilters. Start-up influence. Water Res. 34, 602–610.

Wett, B., Rauch, W., 2003. The role of inorganic carbon limitation in biological nitrogen removal of extremely ammonia concentrated wastewater. Water Res. 37, 1100–1110.

Yamamoto, T., Takaki, K., Koyama, T., Furukawa, K., 2008. Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox. Bioresour. Technol. 99, 6419–6425.