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Effects of organic carbon source, chemical oxygen demand/N ratio and temperature on autotrophic nitrogen removal

J. A. Sánchez Guillén, Y. Yimman, C. M. Lopez Vazquez, D. Brdjanovic and J. B. van Lier

ABSTRACT

To assess the feasibility of the Anammox process as a cost-effective post-treatment step for anaerobic sewage treatment, the simultaneous effects of organic carbon source, chemical oxygen demand (COD)/N ratio, and temperature on autotrophic nitrogen removal was studied. In batch experiments, three operating conditions were evaluated at 14, 22 and 30 °C, and at COD/N ratios of 2 and 6. For each operating condition, containing 32 ± 2 mg NH_4^+-N/L and 25 ± 2 mg NO_2^--N/L, three different substrate combinations were tested to simulate the presence of readily biodegradable and slowly biodegradable organic matter (RBCOD and SBCOD, respectively): (i) acetate (RBCOD); (ii) starch (SBCOD); and (iii) acetate + starch. The observed stoichiometric NO_2^--N/NH_4^+-N conversion ratios were in the range of 1.19–1.43, and the single or simultaneous presence of acetate and starch did not affect the Anammox metabolism. High Anammox nitrogen removal was observed at 22 °C (77–84%) and 30 °C (73–79%), whereas there was no nitrogen removal at 14 °C; the Anammox activity was strongly influenced by temperature, in spite of the COD source and COD/N ratios applied. These results suggest that the Anammox process could be applied as a nitrogen removal post-treatment for anaerobic sewage systems in warm climates.

Key words | anaerobic post-treatment, Anammox, COD/N ratio, mainstream nitrogen removal, organic carbon source, temperature

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INTRODUCTION

Sewage is the main cause of point-source nitrogen (N) pollution. The dissolved inorganic nitrogen (DIN) is the most abundant N-compound, representing up to 75% of the total nitrogen content (Henze & Comeau 2008). The negative effects caused by the discharge of N-compounds on the environment mainly include eutrophication and hypoxia in water bodies. If the current removal efficiencies of nitrogen from sewage remain, the discharge of DIN will continue to increase sharply until 2030 (Seitzinger & Harrison 2008). This scenario has increased the need to have more efficient DIN removal processes in sewage treatment plants and to develop and implement cost-effective N-removal technologies, such as the Anammox process that requires less aeration, no organic carbon source and generates fewer greenhouse gas emissions.

So far, the Anammox process has been applied successfully for N-removal in side-stream treatment lines at sewage treatment plants, e.g. in reject water from sludge handling facilities. But, its potential application as a post-treatment technology for N removal from effluents of an anaerobic sewage treatment process, e.g. effluents from upflow anaerobic sludge bed (UASB) reactors, depends on the environmental and operational conditions, and sewage characteristics. These conditions and characteristics commonly differ from those found in side-stream lines, i.e. the presence of organic carbon, lower N-concentrations, and lower temperatures.

Certain Anammox bacteria are able to carry out the oxidation of the organic acid formate, acetate, and propionate coupled with the reduction of nitrate or nitrite. This biological pathway consists of nitrate reduction to nitrite and ammonium, which combine to produce dinitrogen gas

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(Kartal *et al.* 2007, 2008). Similarly, it has been proposed that Anammox bacteria enriched in reactors fed with propionate and acetate are able to out-compete heterotrophic denitrifiers based on the mechanism of the substrate affinities (Kartal *et al.* 2012). On the other hand, Veys *et al.* (2010) have applied an extension of the Activated Sludge Model (ASM) for the study of the Anammox process. The simulation study was performed at temperatures between 15 and 40 °C. From the model-based analysis, they concluded that, provided there are optimum conditions for Anammox growth, a simultaneous removal of chemical oxygen demand (COD) and N can be achieved by the cooperation between the ammonium oxidizing bacteria, Anammox and heterotrophic bacteria, particularly at low COD loads.

Taking into consideration the biodegradable COD fractions and the DIN concentration in sewage of medium strength (Henze & Comeau 2008), as well as the removal efficiencies of these compounds using large scale UASB reactors (Heffernan *et al.* 2011), it is expected that the effluent of the UASB reactors could contain approximately 60 mg of DIN /L and COD/N ratios between 2 and 6 (80 and 50% removal efficiency of biodegradable COD, respectively). As such, further research is necessary to elucidate the effects of key wastewater characteristics present in the UASB effluents, such as the organic carbon fractions in terms of their biodegradability (readily biodegradable COD: RBCOD; slowly biodegradable COD: SBCOD) and their relationship with the nitrogen concentrations and sewage temperature. For instance, Pontes & Chernicharo (2011) have used an UASB-trickling filter system for the treatment of sewage. They have reported carbohydrates (SBCOD) in the effluent of the UASB in the range of 19.7 mg/L (±1.9) and 12.2 (±4.8) mg/L.

The temperature influence on ANAMMOX activity has been investigated by several authors. The optimum temperature for Anammox growth has been established between 30 and 40 °C (Strous *et al.* 1999). Dosta *et al.* (2008) executed short- and long-term experiments to assess the influence of temperature on Anammox activity. Short-term experiments showed maximum activity at 35–40 °C. The long-term experiments were performed from 30 to 15 °C and the system was successfully operated up to 18 °C; the stability of the system was lost when the temperature was decreased to 15 °C. In order to analyze the effect of temperature on nitrogen removal by Anammox, it is necessary to consider physiological key aspects about the growth temperature of the Anammox strain used in the research. In this sense, the Anammox group *Brocadia* is one of the five known genera of Anammox bacteria (Jetten *et al.* 2010). So far, three species comprise the genus *Brocadia*: *Candidatus Brocadia fulgida*, *Candidatus Brocadia anammoxidans* and *Candidatus Brocadia sinica*. The growth temperature of the last two species has been reported to range between 20 to 43 °C (Strous *et al.* 1999) and 25 to 45 °C (Oshiki *et al.* 2011), respectively. For the *Candidatus Brocadia fulgida*, the growth temperature has been tested within different ranges ranging from as low as 16 up to 35 °C (Kartal *et al.* 2008; Park *et al.* 2010; Rattray *et al.* 2010; Figueroa *et al.* 2012; Winkler *et al.* 2012; Liu *et al.* 2013 and Puyol *et al.* 2013).

It is vital to study the combined effects of the features previously described to understand the interaction and competition among microbial communities, e.g. denitrifying ordinary heterotrophs and Anammox bacteria, and to promote the ones that favor Anammox metabolism. Thus, the objective of this research is to perform preliminary tests to assess the simultaneous effects of organic carbon sources, COD/N ratio and temperature on autotrophic nitrogen conversion by executing batch tests based on the stoichiometric NO_2^--N/NH_4^+-N conversion ratios and nitrogen removal efficiencies. This will contribute toward assessing, as a first approach, feasibility for the potential implementation of the Anammox process in the post-treatment-stream of the UASB reactors treating sewage, as a function of the effluent characteristics expected in different climates.

MATERIALS AND METHODS

Inoculum and substrates

The Anammox sludge used as inoculum to perform the batch activity tests was obtained from the Dokhaven–Sluisjesdijk wastewater treatment plant (Rotterdam, The Netherlands). This inoculum has the same origin and characteristics as the biomass used by Lotti *et al.* (2012) with a maximum specific Anammox activity (MSAA) of 0.458 g N_2-N/g VSS d and a granule diameter of 1.1 ± 0.2 mm (94% of the granules). Based on molecular techniques, the dominant Anammox microorganisms were *Candidatus Brocadia fulgida* (Lotti 2013). The synthetic autotrophic substrate applied in the experiments was modified from the substrate used by van de Graaf *et al.* (1997) as presented in Table 1.

The COD source for acetate was supplied as $CH_3COONa{\cdot}3H_2O$ and for starch as $C_6H_{10}O_5$; their

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Table 1 | Synthetic autotrophic substrate

Autotrophic substrate		Trace solution	
Compound	Concentration (mg/L)	Compound	Concentration (mg/L)
NH_4^+-N	30	Mg EDTA	1,500
NO_2^--N	30	$ZnSO_4{\cdot}7H_2O$	430
		$CoCl_2{\cdot}6H_2O$	240
Na EDTA	11.43	$MnCl_2{\cdot}4H_2O$	990
		$CuSO_4{\cdot}5H_2O$	250
$FeSO_4{\cdot}7H_2O$	11.43	$Na_2MoO_4{\cdot}2H_2O$	220
		$NiCl_2{\cdot}6H_2O$	190
$NaHCO_3$	11.88	$Na_2SeO_4{\cdot}10H_2O$	210
		H_3BO_3	14
Trace solution	1.25 mL/L	$NaWO_4{\cdot}2H_2O$	50

concentrations were adjusted according to the COD/N ratio required (either 2 or 6).

Anammox batch tests

The nitrogen removal efficiencies and the NO_2^--N/NH_4^+-N conversion ratios were estimated under the operating conditions of interest through the execution of short-term batch tests in 310 mL vials. The Anammox sludge, the synthetic autotrophic substrate, the COD solutions and a washing buffer solution (0.14 g/L of KH_2PO_4 and 0.75 g/L of K_2HPO_4) were left overnight at the corresponding temperature being studied. Prior to the execution of the tests, the Anammox sludge was washed three times with the buffer solution with the aim of removing any residual organic carbon or nitrogen compounds. Afterwards, the supernatant was discarded. The synthetic autotrophic substrate containing 32 ± 2 mg NH_4^+-N /L and 25 ± 2 mg NO_2^--N/L was used as the nitrogen source and different concentrations of acetate and starch were added to simulate the presence of RBCOD and SBCOD. Tests were carried out in duplicate under four different combinations as follows. (i) Control: only N-compounds without any organic substrate; (ii) acetate: N-compounds plus acetate (as RBCOD source); (iii) acetate + starch: N-compounds plus acetate and starch (25 and 75% on a COD basis as RBCOD and SBCOD sources, respectively); and (iv) starch: N-compounds plus starch (as SBCOD). Three different experimental conditions were tested at 14, 22 and 30 °C, at COD/N ratios of 2 and 6. Before the experiments, the pH level in the vials was adjusted to 7.8–8.01 using 0.1M HCl and/or 0.1 M NaOH. Then, samples were collected to analyze the initial concentration of NH_4^+-N, NO_2^--N, NO_3^--N, COD and the mixed liquor volatile suspended solids (MLVSS). After the addition of the Anammox biomass and substrates, the average MLVSS concentration in the vials was approximately 1.5 g/L with a final working volume of 200 mL. The vials were closed with a gas-tight coated septum to avoid oxygen intrusion, and the headspace was sparged with Helium gas for 5 min at a flow pressure of 0.2 bars to remove any remaining oxygen from the gas phase. Later on, the vials were incubated at the selected temperature and continuously stirred for 6 h. At the end of the experiments, the final pH was determined and samples were taken to determine the final concentrations of NH_4^+-N, NO_2^--N and NO_3^--N.

Analytical methods

Prior to the execution of the analyses, samples collected from the vials were centrifuged at 3,600 rpm for a period of 20 min to separate the solid particles. For COD determination, 5 mL of the supernatant were taken and the rest of the volume was filtered using 0.45 μm ion chromatography Acrodisc® Syringe Filters (Pall Corporation, The Netherlands). Filtered samples were used for the determination of NH_4^+-N, NO_2^--N and NO_3^--N. The concentration of NH_4^+-N was determined spectrophotometrically according to NEN 6472 (NEN 1983). MLVSS, NO_2^--N (spectrophotometric method) and COD (closed reflux colorimetric method) were analyzed according to *Standard Methods for the Examination of Water & Wastewater* (2012). The DIONEX ICS-1000 ion chromatography system (Thermo Scientific, USA) was utilized for the analysis of NO_3^--N and pH was measured with a Metrohm 691 pH-meter (Metrohm, Switzerland).

RESULTS AND DISCUSSION

Nitrogen removal

Bearing in mind the potential for implementation of the Anammox to the mainstream, in this research the effect of temperature on Anammox activity was studied at 30, 22 and 14 °C. The results of the batch tests show that Anammox bacteria were strongly influenced by temperature regardless of the COD source and COD/N ratios applied. High values of total nitrogen removal (Table 2) were observed at 30 °C (73–79%) and 22 °C (77–84%), but there was no nitrogen removal at 14 °C.

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Table 2 | Removal efficiency of NH_4^+-N, NO_2^--N and $(NH_4^+ + NO_2^-)$-N observed in batch experiments at different incubation temperatures COD/N ratios and COD sources

		NH_4^+-N				NO_2^--N				$(NO_2^-$-N + NH_4^+-N)-N			
		30 °C		22 °C		30 °C		22 °C		30 °C		22 °C	
COD/N ratio	Sample	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
2	Control ($n = 4$)	57	3	71	8	100	0	100	0	76	2	84	4
	Acetate	62	4	60	7	100	0	100	0	79	4	77	4
	Acetate + Starch	61	4	64	3	100	0	100	0	77	4	80	2
	Starch	58	4	65	3	100	0	100	0	77	3	81	2
6	Acetate	59	2	64	1	100	0	100	0	76	1	80	0
	Acetate + Starch	58	4	66	1	100	0	100	0	76	3	82	1
	Starch	55	2	66	2	100	0	100	0	73	2	82	1

SD: standard deviation.

Overall, and despite Anammox bacteria not being active at 14 °C, it was interesting to note the lack of denitrification activity over nitrite by ordinary heterotrophs at 14 °C. It could be argued that at 14 °C, heterotrophic denitrification would occur in the control using the COD available in the sludge matrix after washing the sludge, or based on the acetate and/or starch added as the source of organic matter in the other samples. However, at 14 °C the denitrification kinetics rate (K_1) will be reduced to 5% of the activity reported at 30 °C (Ekama & Wentzel 2008), which could explain the lack of denitrifying activity.

Total nitrogen removal efficiencies in the presence of the different COD sources at 30 and 22 °C were similar to the average control values at both COD/N ratios (2 and 6 gCOD/gN), suggesting that the Anammox bacteria were not affected by the presence of COD sources during the batch experiments. These results imply that temperatures in the range of 22–30 °C do not affect the activity of Anammox bacteria or, at least, do not favor the activity of other microorganisms (e.g. ordinary heterotroph organisms) in detriment to Anammox, regardless of whether a carbon source is present. Long-term studies are needed to validate these observations and to assess the effects on the microbial populations and their interactions. With regard to the effect of the organic carbon source, the NO_2^--N was fully removed at 30 and 22 °C, with or without an organic source added. Overall, and although other factors may also influence the carbon source effects on the performance of the Anammox process, the observations suggest that the presence of starch (SBCOD) and acetate (RBCOD) do not appear to affect the metabolism of Anammox consortia.

Stoichiometric NO_2^--N/NH_4^+-N conversion ratios and NO_3^--$N_{produced}$/NH_4^+-$N_{consumed}$

The values of the NO_2^--N/NH_4^+-N conversion ratios observed in the batch experiments at the different incubation temperatures, COD/N ratios and COD sources are summarized in Table 3.

At 30 °C with COD/N ratios of 2 and 6 gCOD/gN, the measured conversion ratios in the control were 1.36 gNO_2^--N/gNH_4^+-N. This ratio is very close to the ratio reported by Strous *et al.* (1998) for Anammox metabolism, i.e. 1.32 gNO_2^--N/gNH_4^+-N. The stoichiometric values for NO_2^--N/NH_4^+-N conversion ratios under the influence of organic matter at 30 °C showed a range of 1.17–1.36 gNO_2^--N/gNH_4^+-N.

In this regard, the conversion ratios at the initial COD/N ratio of 6 gCOD/gN show values lower than those observed in the control bottles, with a minimum of 1.17 gNO_2^--N/

Table 3 | NO_2^--N/NH_4^+-N conversion ratios observed in batch experiments at different incubation temperatures COD/N ratios and COD sources

		Temperature 30 °C		22 °C	
COD/N ratio	Sample	gNO_2^--N/ gNH_4^+-N	SD	gNO_2^--N/ gNH_4^+-N	SD
2	Control ($n = 4$)	1.36	0.09	1.19	0.09
	Acetate	1.36	0.16	1.26	0.16
	Acetate + Starch	1.23	0.20	1.25	0.04
	Starch	1.43	0.04	1.25	0.01
6	Acetate	1.21	0.01	1.32	0.01
	Acetate + Starch	1.30	0.04	1.32	0.01
	Starch	1.17	0.33	1.36	0.08

SD: standard deviation.

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gNH_4^+-N, when starch was present. On the other hand, at a COD/N ratio of 2 gCOD/gN, the lowest value for the conversion ratio was 1.23 gNO_2^--N/gNH_4^+-N when acetate and starch were present. However, when starch was added as a single organic compound, the highest stoichiometric ratio was obtained, 1.43 gNO_2^--N/gNH_4^+-N. The batch tests, carried out at 22 °C with a COD/N of 2 gCOD/gN, did not have significant variations in the results for almost all samples; the conversion ratios were 1.25–1.26 gNO_2^--N/gNH_4^+-N. Higher stoichiometric values were obtained when the COD/N ratio was increased to 6 gCOD/gN, at the same temperature, when all samples with organic matter added had conversion ratios between 1.32–1.36 gNO_2^--N/gNH_4^+-N. Overall, the stoichiometric values obtained can be used as a baseline to assess the potential occurrence of denitrification by ordinary heterotrophic organisms during batch tests. In this regard, if the COD was consumed by ordinary heterotrophic organisms denitrifying over nitrite then, for instance, in accordance with Ekama & Wentzel (2008), 360 mg COD/L would lead to about 162 mg VSS/L (using $Y_H = 0.45$ gVSS/gCOD), requiring around 16.2 mg NH_4^+-N/L (assuming an N-requirement of 0.10 gN/gVSS) and consuming about 68.4 mg NO_2^--N/L (based on the electron equivalents of NO_2^--N to oxygen) that would result in an NO_2^--N to NH_4^+-N ratio of about 4.22 gNO_2^--N/gNH_4^+-N. This ratio is considerably much higher than those obtained during the tests that on average lay around 1.17–1.43 gNO_2^--N/gNH_4^+-N, suggesting that if certain COD was consumed by ordinary heterotrophic organisms for denitrification over NO_2^--N it was negligible, implying that the N-removed via Anammox was the dominant N-removal pathway. In general, the stoichiometric NO_2^--N/NH_4^+-N conversion ratios found in the batch tests of this research are in accordance with the values reported by other authors. At 20 °C, Hendrickx *et al.* (2012) have found values between 1.11 and 1.54 gNO_2^--N/gNH_4^+-N during the treatment of an influent consisting of synthetic wastewater and 20% of an anaerobic effluent (on a volume basis). The anaerobic effluent was supplied from a laboratory-scale UASB system used for the treatment of sewage. The concentrations of NH_4^+-N and NO_2^--N in the influent were similar to those applied in this study: 30 mg/L for each nitrogen species. In the same way, in a kinetic study with Anammox cultures dominated by the species *Candidatus Brocadia caroliniensis* (flocculent sludge) and *Candidatus Brocadia fulgida* (granular sludge), Puyol *et al.* (2013) used the obtained kinetics parameters and growing models for the estimation of the stoichiometry of the Anammox metabolism. The proposed conversion ratio of NO_2^--N/NH_4^+-N is 1.278 gNO_2^--N/gNH_4^+-N; this result is also close to the values of 1.25–1.26 gNO_2^--N/gNH_4^+-N, which were found using *Candidatus Brocadia fulgida* in this study at COD/N of 2 gCOD/gN and 22 °C. With respect to the conversion ratios at 14 °C, there was no bacterial activity at this temperature. (A discussion about this topic was covered in the previous section.)

Irrespective of the COD/N ratio and COD source, the NO_3^--N produced at 30 and 22 °C was below the detection limit (0.50 mg NO_3^--N/L), except for the control, which was assessed at a COD/N ratio of 6 gCOD/gN and at 22 °C. Under these conditions, the conversion ratio of NO_3^--$N_{produced}$/NH_4^+-$N_{consumed}$ was 0.11 gNO_3^--N/gNH_4^+-N, which is lower than the ratio reported by Strous *et al.* (1998) of 0.26 gNO_3^--N/gNH_4^+-N. The absence of nitrate production suggests a process of nitrogen removal over NO_3^--N by heterotrophs and possibly by the *Candidatus Brocadia fulgida* using the existing organic carbon sources. This species of Anammox bacteria has the capacity for oxidizing formate, acetate and propionate using as electron acceptor nitrite and/or nitrate (Kartal *et al.* 2008). The coupled process of the oxidation of acetate and the nitrate reduction by *Candidatus Brocadia fulgida* has a specific activity of 0.95 μmol/min g of protein.

CONCLUSIONS

- The Anammox process shows potential as a post-treatment for anaerobic sewage treatment, particularly for warm and moderate climates (but not for low temperatures).
- Slowly biodegradable COD (starch) and readily biodegradable COD (acetate) did not affect the metabolism of the Anammox consortia.
- Observed NO_2^--N/NH_4^+-N conversion ratios show that Anammox bacteria were not impacted by denitrifying organisms in any of the batch tests executed. However, batch tests do not predict long-term reactor performance. Therefore, the next step in this research is to carry out long-term experiments under similar conditions. In this way, the population dynamics of Anammox bacteria and other relevant microbial populations (ammonium oxidizing bacteria, nitrite oxidizing bacteria and ordinary heterotrophic organisms) under the given process conditions can be assessed.

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