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RESEARCH PAPER

Short-Term Effects of Operating Parameters and Wastewater Constituents on the Performance of Free-cell Candidatus Brocadia and Candidatus Scalindua Anammox Enrichment

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Abstract

Anammox, is a state-of-art nitrogen removal technology for domestic and industrial wastewater treatment. Anaerobic chemolithoautotrophic Anammox species are quite sensitive to operating parameters and various inorganic and organic compounds. Previous Anammox research was mainly on mixed cultures including Ca.Brocadia and/or Ca.Kuenenia species that are abundant in domestic wastewater. Little information exists about the mixed Anammox cultures including Ca.Scalindua species known as marine Anammox species that also present in WWTPs receiving sea-water infiltrated sewage, saline-rich wastewater in coastal cities and saline-rich industrial wastewater. In this study, the influence of operating parameters and organic and inorganic wastewater constituents on the performance of mixed suspended Ca.Brocadia and Ca.Scalindua Anammox enrichment was evaluated based on Specific Anammox Activity (SAA). Response surface methodology was used to model the relationship between pH, DO and temperature changes with SAA. Optimum pH and temperature were identified as 7.36 and 32.7^{0}C, respectively. Short-term inhibitory (IC_{50}) values of acetate, propionate and glucose were identified as 1000–1500, 3300 and 3600–5700 mg COD/L, respectively. NO_2^--N caused Anammox inhibition above 50 mg/l. IC_{50} values for SO_4^{2-}, PO_4^{3-}-P and K^+ were determined as 3500 mg SO_4^{2-}/L, 1384 mg PO_4^{3-}-P/L and 2400 mg K^+/L. The study provides a comprehensive insight into the tolerance of Ca.Brocadia and Ca.Scalindua enrichment against changing operational conditions and potential inhibiting compounds and facilitates optimization of operational strategies for the efficient performance of engineered Anammox systems. The findings will also contribute to future research activities that will focus on composite inhibitors and metabolic inhibition pathways.

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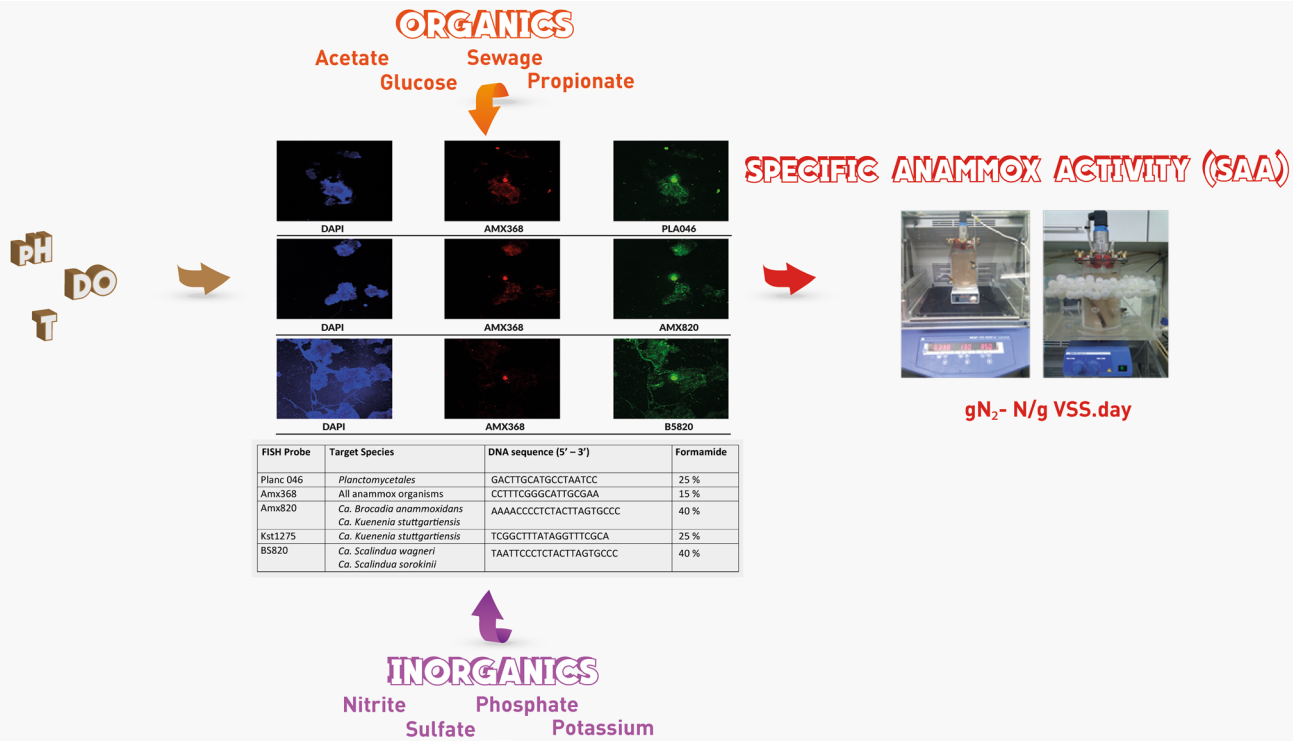
Graphical abstract



FISH Probe	Target Species	DNA sequence (5′ – 3′)	Formamide
Planc 046	*Planctomycetales*	GACTTGCATGCCTAATCC	25 %
Amx368	All anammox organisms	CCTTTCGGGCATTGCGAA	15 %
Amx820	*Ca. Brocadia anammoxidans* *Ca. Kuenenia stuttgartiensis*	AAAACCCCTCTACTTAGTGCCC	40 %
Kst1275	*Ca. Kuenenia stuttgartiensis*	TCGGCTTTATAGGTTTCGCA	25 %
BS820	*Ca. Scalindua wagneri* *Ca. Scalindua sorokinii*	TAATTCCCTCTACTTAGTGCCC	40 %

Highlights

- Optimum pH and temperature were found lower than the other Anammox species.
- Oxygen tolerance was more than the other Anammox species.
- Acetate was identified more inhibitory than propionate and glucose.
- NO_2^--N, which is the e- acceptor of Anammox, caused inhibition above 50 mg l^{-1}.
- IC_{50} values of SO_4^{2-}, PO_4^{3-}-P and K^+ were identified.

Keywords Anammox · Inhibition · Inorganics · Organics · Response Surface Methodology (RSM) · Specific Anammox Activity (SAA)

Abbreviations

Anammox	Anaerobic Ammonium Oxidation
Anova	Analysis of Variance
Ar	Argon
Ca.	Candidatus
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FA	Free ammonia
FNA	Free nitrous acid
FISH	Fluorescence In-Situ Hybridization
IC_{50}	Inhibitory Concentration (50%)
K^+	Potassium
MLVSS	Mixed Liquor Volatile Suspended Solids
NH_4^+-N	Ammonium nitrogen
NO_2^--N	Nitrite nitrogen
N_2	Nitrogen gas
O_2	Oxygen
PO_4^{3-}-P	Phosphate phosphorus
RSM	Response Surface Methodology
S_o/X_o	Initial substrate initial biomass ratio
SAA	Specific Anammox Activity
SBR	Sequencing Batch Reactor
SO_4^{2-}	Sulfate
WWTP	Wastewater treatment plant

Introduction

Anammox (anaerobic ammonium oxidation) process was discovered about 25 years ago by Mulder et al. (1995) and since then has been widely applied for the sidestream treatment. Anammox process is now receiving more attention, especially for mainstream applications due to the paradigm shift from energy-negative to energy-neutral or

positive operation of wastewater treatment plants. As being an anaerobic chemolithoautotrophic biological conversion process, Anammox process allows the saving of oxygen and organic matter, minimizes sludge production and prevents greenhouse gases emissions and hence is very attractive for energy-neutral or positive plants. However, the high sensitivity of Anammox bacteria to changing environmental conditions and various compounds is one of the major limitations of these applications.

As discovered not long ago, the first Anammox enrichments were from wastewater environments from where it was discovered in 1995. Hence, the initial focus on the behavior of Anammox biomass at different pH, temperature, DO conditions and in the presence of various organic and inorganic compounds was mainly studied with Ca. Brocadia and/or Ca. Kuenenia species common in freshwater environments, e.g., wastewater. However, besides Ca. Brocadia and Ca. Kuenenia species, there are also four other Anammox species belonging to the genus of Jettenia, Anammoxoglobus, Anammoximicrobium and Scalindua (Wei et al. 2020). Among them, Ca. Scalindua species are known as marine (saline) Anammox species since they have been majorly detected in marine water columns and sediments worldwide. However, in the last 10-year period, the presence of Ca. Scalindua species in wastewater have been reported in various studies (Alpaslan Kocamemi and Dityapak, 2014; Azari et al. 2017; Lanzetta et al. 2021; Schmid et al. 2003). Remarkable abundance of Ca. Scalindua species (15.2–17% in activated sludges, and 15.9–26.8% in granular biofilms) was reported in full-scale Anammox plants (Azari et al. 2017). In WWTPs receiving seawater infiltrated sewage in coastal regions especially due to the use of seawater in flushing toilets and pre-treated saline-rich industrial wastewaters (e.g., fish canning, textile dyeing, and leather processing), harboring of marine and freshwater environments is unavoidable. In such WWTPs, saline Anammox species (i.e., Ca. Scalindua) are always present together with freshwater Anammox species (i.e., Ca. Brocadia and/or Ca. Kuenenia). However, to the best of our knowledge, there is no information about the behavior of an enriched Anammox culture harboring both saline and freshwater species at different pH, temperature, DO conditions and in the presence of various organic and inorganic compounds in wastewater. Influences of operating parameters and wastewater constituents on the performance of the Anammox process were mainly studied with microbial populations with an abundance of freshwater Anammox species Ca. Brocadia and/or Ca. Kuenenia, mostly in biofilm or granular systems under mass transfer limitation. In these studies, the physiological pH and temperature ranges for the Anammox process were reported to be between 6 and 10 (Chamchoi and Nitisoravut 2007; Dapena-Mora et al. 2007; Egli et al. 2001; Jetten et al. 1998; Khin and Annachhatre, 2004; Tao et al. 2012; Strous et al. 1997a, 1997b) and 6–43 °C, (Chamchoi and Nitisoravut, 2007; Arrojo et al. 2006; Dosta et al. 2008; Isaka et al. 2007; Strous et al. 1999) respectively. The optimum values varied between 7 and 8 for pH and 30–45 °C for temperature depending on the sludge type, reactor configurations and experimental conditions. Jetten et al. (1999) reported that dissolved oxygen completely inhibited the Anammox activity when it was deliberately introduced into the enrichment cultures. However, there are also studies indicating the tolerance of Anammox bacteria to low levels of dissolved oxygen (Strous et al. 1997a; Jin et al. 2013; Ma et al. 2016). Various studies identified nitrite (NO_2^-), the electron acceptor of the process, as a potential inhibitory compound for Anammox. There are numerous studies evaluating the inhibitory level of nitrite on Anammox with reported threshold concentrations varying between 5 to 280 mg N/l (Jin et al., 2013). Since the Anammox bacteria are autotrophic, organic matter was usually thought to be a potential inhibiting compound for Anammox. Various forms of nontoxic organic matter (e.g., acetate, propionate, glucose) were evaluated to give an idea about the possible application of Anammox process for the treatment of both nitrogen and organic matter containing wastewater (Jin et al., 2013). The number of studies evaluating the effects of common inorganic wastewater constituents (e.g., sulfate, potassium, phosphate) on Anammox process is very limited with respect to the other studies (Dapena-Mora et al. 2007; Jetten et al. 1998; Carvajal-Arroyo et al. 2013; Van de Graaf et al. 1996). These earlier studies in the literature were performed under various experimental conditions (e.g., different pH, temperature, DO conditions) and evaluated the effect of target operating parameters or wastewater constituents using different methodologies, e.g., monitoring substrate (NH_4^+-N) and electron acceptor (NO_2^--N) depletion rates in the long term or monitoring main product nitrogen (N_2) gas formation rates in the short term. Therefore, the reported values in the literature are usually not consistent and comparable with each other.

Influence of operating parameters and wastewater constituents on the performance of Anammox culture including mixed saline and freshwater Anammox species is still a matter of debate. Further studies evaluating the one response of saline and freshwater Anammox species in the same culture to major operating parameters and wastewater constituents are needed to develop a state-of-the-art operational strategy for Anammox processes in wastewater treatment plants receiving seawater infiltrated sewage, saline wastewater from life activities in coastal cities and pre-treated saline rich industrial wastewaters.

In this study, a series of batch experiments were performed in a sequencing batch reactor (SBR) with highly enriched suspended Anammox culture harboring freshwater Ca. Brocadia and saline Ca. Scalindua species. The

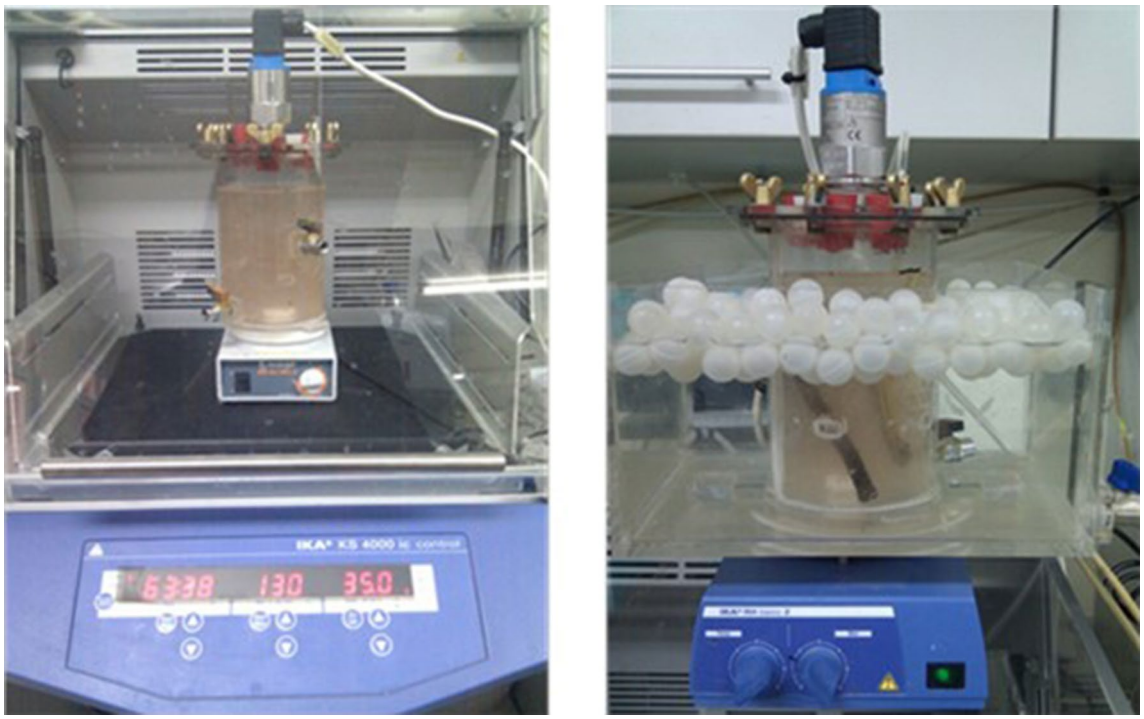


Fig. 1 Batch experimental set-up

experiments were based on monitoring of nitrogen (N_2) gas production and calculation of Specific Anammox Activity (SAA) values. The effect of operating temperature, pH and DO conditions on the Anammox process was evaluated to determine optimal operating values. Short-term inhibitory levels of various organic (acetate, glucose, propionate) and inorganic compounds (nitrite, sulfate, potassium, phosphate) that are commonly present in wastewater were determined.

Materials and Methods

Seed Culture

The enriched Anammox culture was obtained from a lab-scale SBR started up with seed from the first worldwide Anammox unit in Sluisjesdijk sludge treatment plant in Rotterdam, NL and was enriched for suspended Anammox species via gradually increasing ammonia and nitrite loadings for 410 days (Alpaslan Kocamemi and Dityapak, 2013, 2014). Fluorescence in situ hybridization (FISH) analyses were used to identify Ca. Brocadia and Ca. Scalindua species in the enrichment seed. Biomass samples were collected with 50% ethanol (v/v) and stored at -20°C. They were fixed with 4% (v/v) paraformaldehyde. Hybridization with fluorescently labelled Eub 338 I, II, III, Planc 046, Amx368, Amx820, BS820, Kst1275 oligonucleotides was performed as described by Daims et al. (1999). Images were taken with a Leica TCS SP2 confocal laser scanning microscope.

Experimental Set-up and Test Procedure

Experiments were conducted in duplicate in a 1 L plexiglass test reactor equipped with a pressure transducer (Endress Hauser PMC 131) to monitor pressure changes in the headspace due to N_2 gas production (Fig. 1). The test reactor was placed in an incubating shaker (IKA KS 4000 IC Control) to keep the temperature constant during the test period. Complete mixing was ensured by means of a magnetic stirrer (Heidolph MR Hei-Mix L). Initial S_o/X_o values were kept around 0.2–0.4 g NH_4^+-N/ g MLVSS. Unless otherwise stated, pH, DO and water temperature were kept around 7.5–8, 0 mg/L, 32-35^{0}C, respectively.

Before each test, the test reactor was inoculated with enriched Anammox bacteria washed three times with mineral solution. The headspace and liquid phase of the reactor were washed with 95% Ar + % 5 CO_2 gas mixture. The reactor was fed with synthetic wastewater composed of the followings: 5–1,166 mg/l NH_4^+-N, 5–1,400 mg/l NO_2^--N, 25–30 mg/L NO_3^--N and 525–1,250 mg/L $KHCO_3$, 27.2 mg/L K_2HPO_4, 300 mg/L $MgSO_4{\cdot}7H_2O$ and 180 mg/L $CaCl_2{\cdot}2H_2O$. Trace element solutions of which 1 mL was added per each liter of the rector including the followings: To each liter of the reactor, 1 mL of trace element solution I including 5 g/L $Na_2EDTA{\cdot}2H_2O$ and 5 g/L $FeSO_4{\cdot}7H_2O$

and 1 mL of trace element solution II including 15 g/l $Na_2EDTA{\cdot}2H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.011 g/L H_3BO_3, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.12 g/L $CoCl_2$, 0.19 g/L $NiCl_2{\cdot}6H_2O$, and 0.10 g/L Na_2SeO_3. NH_4^+-N and NO_2^--N concentrations ranged between 100–150 mg NH_4^+-N/L and 175–212 mg NO_2^--N/L. Nitrogen gas production was continuously monitored in the headspace against time.

Through the batch experiments of DO parameter, a series of blank experiments were initially performed in the absence of biomass to observe pressure change in headspace due to stripped Ar/CO_2 with the introduction of various concentrations of O_2 gas to the water. In the experiments with biomass, the measured pressure values were corrected with blank results to report pressure change only due to N_2 gas production of Anammox bacteria. Similarly, prior to batch experiments with organic compounds, another series of blank experiments were carried out to exclude possible N_2 gas production by denitrifying organisms in the enriched Anammox seed. In these blank experiments, seed culture was fed with synthetic wastewater, not including ammonia but nitrite, nitrate and target organic compound. The measured SAA values in blank experiments were subtracted from SAA values monitored during experiments with ammonia.

Data Analysis

In each test, pressure changes monitored in the headspace of the test reactor via pressure transducer were plotted against time and linearized to find slope (dP/dt) values. The dP/dt values were placed to the ideal gas law equation to calculate dN_2/dt and then divided by biomass concentration and liquid volume in the reactor to determine corresponding Specific Anammox Activity (SAA) values in accordance with Dapena-Mora et al. (2007) (Eq. 1–3). The results of pH and temperature experiments were further analyzed with one-factor design of response surface methodology (RSM) to find out optimum values of process parameters along with their effects on the SAA. Design Expert 8.0.7.1. free trial package program was used for these analyses.

$$\frac{dP}{dt}V = \frac{dN_2}{dT}RT \tag{1}$$

$$\frac{dN_2}{dt} = \alpha\frac{V_G}{RT} \tag{2}$$

$$SAA = \frac{dN_2/dt}{XV_L}\frac{28gN}{molN_2}\frac{1440\,min}{day} \tag{3}$$

where α is the pressure change in headspace with respect to time (dP/dt) (bar/min), V_G volume of the headspace (L), R ideal gas coefficient (0.00831447 L bar/ K.mol), T liquid temperature (K), X biomass concentration (g VSS/L), V_L liquid volume (L), SAA = g N_2-N g/ VSS.day.

Analytical Methods

NH_4^+-N, NO_2^--N, PO_4^{3-}-P, K^+, SO_4^{2-} were analyzed with Shimadzu 20A-Dual Injection Ion Chromatography instrument operating under 40^0C oven temperature. Cation measurements were performed using Shimpack IC-SC1 (150 × 4.6 mm) using 3.5 mM H_2SO_4 mobile phase under 0.8 mL/min flow. Anion measurements were performed using Shimpack IC-SA2 (250 × 4 mm) using 12 mM $NaHCO_3$ and 0.6 mM Na_2CO_3 mobile phase under 1 mL/min flow. pH, DO and temperature were measured with Hach-Lange HQ40 on-line probes. COD measurements and MLVSS measurements were performed in accordance with Method 5220C-Closed reflux Method and Method 2540G-Total, Fixed, and Volatile Solids in Solid and Semisolid Samples described in Standard Methods (1998).

Statistical Analyses

Statistical analyses of the models obtained for pH, temperature and DO by the one-factor design of RSM were performed through Analysis of variance (ANOVA) in Design Expert 8.0.7.1. free trial package. Central composite design model developed for pH and temperature interaction was tested with ANOVA analysis in Minitab software. IC 50 determination duplicate experiments of organics and inorganics were depicted with 95% confidence interval error bars using Microsoft Excel software.

Results and Discussions

pH

Batch assays with an initial S_o/X_o ratio of 0.4 ± 0.1 were performed in the pH range of 6 to 9 at 32.6 ± 1^0C. SAA values calculated from gauge pressure changes observed in the test reactor against time are shown in Fig. 2a. SAA exhibited a gradual increase with increasing pH from 6 to 7.5–7.8. Above pH 7.5–7.8, SAA decreased gradually with increasing pH. pH values of 6.4 and 8 resulted in an approximately 50% decrease in maximum SAA observed at pH 7.5–7.8. The activity of Anammox bacteria completely ceased at pH 9. These findings demonstrated that similar to all microorganisms, energy yields of Ca. Brocadia and Ca. Scalindua species respond strongly to pH variation which may be due to changing chemical activity of protons, proton motive force, structure/function of macromolecules, especially proteins, mineral dissolution and precipitation, surface complexation, and other redox reactions (Jin and Kirk, 2018). Additionally,

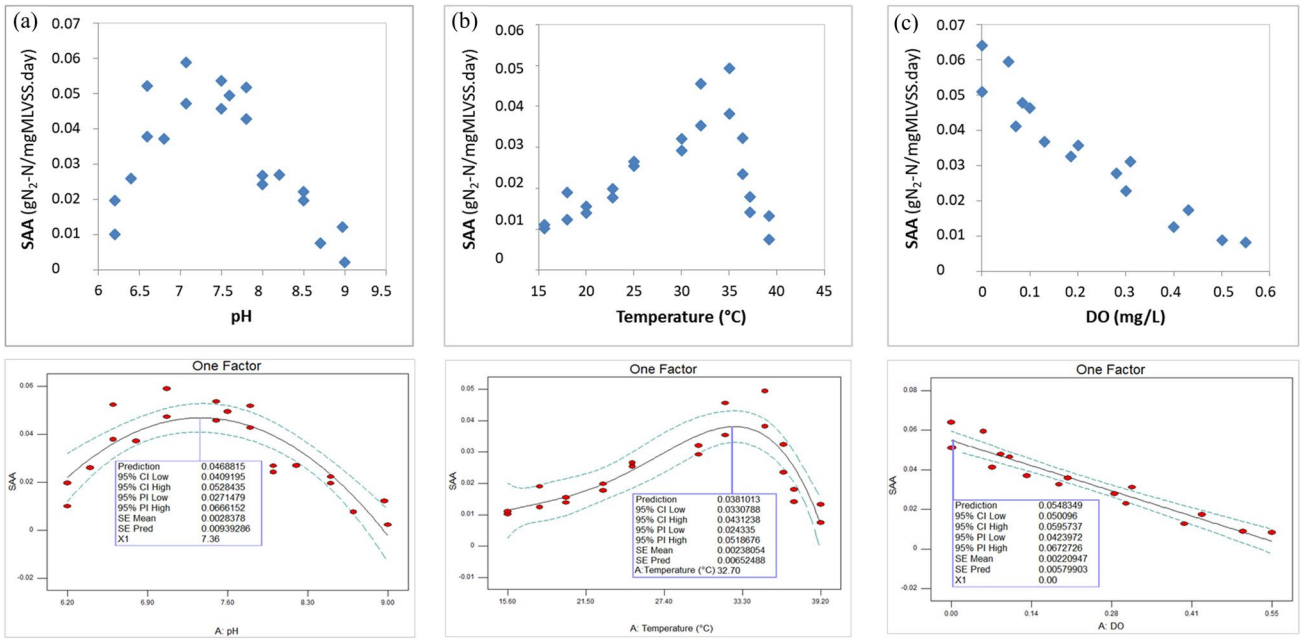


Fig. 2 SAA values observed at various **a** pH, **b** temperature, **c** DO ranges

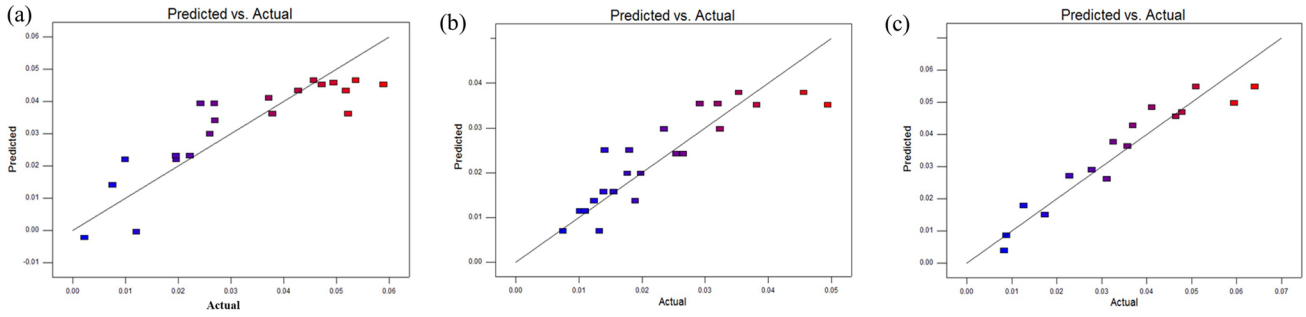
inhibition levels can be linked to the increasing or decreasing free ammonium (FA) and free nitrous acid (FNA) concentrations with varying pH values as reported by other researchers (Talan et al 2021). In the present study, the FA concentrations reaching up to 54 mg/L at pH 9 and FNA concentrations reaching 400 µg/L at pH 6 may be the reason for significant SAA decreases observed at very low and high pH values. Optimum pH for the culture enriched for Ca. Brocadia and Ca. Scalindua Anammox species was identified as 7.36 by one-factor design of RSM (Fig. 2a). The relationship between variable pH and response SAA was modelled by a quadratic polynomial equation (Eq. 4). The ANOVA results of quadratic model (Fig. 3a) showed that the quadratic model developed is significant with a factor of 27.26 ($p < 0.05$).

$$\text{SAA}, \frac{gN_2 - N}{\text{mgMLVSS}.d} = -9.4E-1 + 2.7E-1 \times \text{pH} - 1.8E-1 \times \text{pH}^2 \quad (4)$$

In line with the findings of this study, Awata et al. (2013) reported the optimal pH range of Ca. Scalindua species to be 6.5–8 and mentioned that this range is lower than that of reported for other Anammox species. In a study performed with Ca. Brocadia suspended Anammox species, Carvajal-Arroyo et al. (2013) revealed important losses in activity exceeding 20% as the pH shifted more than 0.3 units from the optimal pH value of 7.5. However, there are numerous studies in literature (Egli et al. 2001; Strous et al. 1999) reporting wider optimum pH ranges. These conflicting findings may be due to initial substrate concentration (i.e., FA concentration), temperature and genus type and morphology (i.e., free or granular cells). The optimum pH for cultures enriched for Brocadia and Kuenenia species was reported as 7.8–8 while reported as 6.8–7 for Brocadia and Anammoxoglobus enrichment (Cho et al. 2020). In view of the present and past studies, it is evident that slight changes in pH may affect the Anammox process efficiency significantly in a positive or negative way. Hence, in Anammox tanks, strict real-time pH monitoring is quite necessary to prevent the inhibition of Anammox culture against sudden pH changes under treatment plant operation conditions. Additionally, for the treatment of a specific type of wastewater (e.g., saline wastewater), the information gained would provide an insight to increase Anammox process efficiency along with minor pH adjustments in the operation. Further research activities are suggested for the evaluation of the specific effect of pH on other Anammox species Ca. Jettenia and Ca. Anammoximicrobium, which has been rarely studied.

Temperature

The short-term effects of wastewater temperature on Anammox activity were evaluated in the temperature range of 15–40^{0}C. The SAA values observed through batch assays that were performed with an initial S_o/X_o ratio of 0.32 ± 0.06 at pH 7.5 are shown in Fig. 2b. Similar to other mesophilic groups of bacteria, temperature increase exhibited a positive effect on Anammox activity. SAA increased gradually up to 32–35 °C range and then decreased gradually for increasing temperature values. Similar to all other microorganisms, the



Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.00437	2	0.002185	27.25602	< 0.0001	significant
A-pH	0.001199	1	0.001199	14.95133	0.0011	
A^2	0.003492	1	0.003492	43.55353	< 0.0001	
Residual	0.001443	18	8.02E-05			
Lack of Fit	0.001145	11	0.000104	2.441327	0.1228	not significant
Pure Error	0.000298	7	4.26E-05			
Cor Total	0.005813	20				
R^2	0.751765					
Adj R^2	0.724184					
Pred R^2	0.647568					
Adeq Precision	14.42596					
F-value	27.26					

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.002238576	4	0.0005596	15.16361	< 0.0001	significant
A-Temperature	0.001043877	1	0.0010439	28.283947	< 0.0001	
A^2	2.67222E-06	1	2.672E-06	0.0724042	0.7911	
A^3	0.000702683	1	0.0007027	19.039276	0.0004	
A^4	5.72383E-05	1	5.724E-05	1.5508776	0.2299	
Residual	0.00062742	17	3.691E05			
Lack of Fit	0.00041821	6	6.97E-05	3.6648442	0.0300	significant
Pure Error	0.000209209	11	1.902E-05			
Cor Total	0.002865995	21				
R^2	0.7811					
Adj R^2	0.7296					
Pred R^2	0.6609					
Adeq Precision	10.702					
F-value	15.16					

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.004039908	1	0.00404	140.5331	< 0.0001	significant
A-DO	0.004039908	1	0.00404	140.5331	< 0.0001	
Residual	0.000402458	14	2.87E-05			
Lack of Fit	0.000317583	13	2.44E-05	0.287826	0.9149	not significant
Pure Error	8.49E-05	1	8.49E-05			
Cor Total	0.004442367	15				
R^2	0.9094					
Adj R^2	0.9029					
Pred R^2	0.8791					
Adeq Precision	26.888					
F-value	140.53					

Fig. 3 ANOVA Analyses for **a** pH, **b** Temperature, **c** DO models in one-factor design of Response Surface Methodology

increasing Ca. Brocadia and Ca. Scalindua growth rate with increasing temperature was mainly due to the increasing affinity of microorganisms for substrate (Nedwell 1999). The decrease in growth rate beyond optimum temperature was mainly due to decreasing membrane fluidity and denaturation of key cellular components especially enzymes (Nedwell 1999). The maximum observed SAA at 35 °C decreased about 20% for every 5 °C decrease from the optimum temperature. When temperature drops from 30 °C to 15 °C, SAA decreases three-fold. One-factor design of RSM (Fig. 2b) estimated the optimum temperature as 32.7^0C for the culture enriched for Ca. Brocadia and Ca. Scalindua Anammox species (Fig. 2b). The relationship between temperature and SAA was modelled by a quartic polynomial equation (Eq. 5). The ANOVA results of the quadratic model (Fig. 3b) showed that the quadratic model developed is significant with F factor of 15.16 ($p < 0.05$).

$$\begin{aligned}\text{SAA}, \frac{\text{g}N_2 - N}{\text{mgMLVSS}.d} &- 1.6E-1 + 3.4E \\ &- 2 \times \text{Temperature}(^\circ\text{C}) \\ &- 2.4E3 \times \text{Temperature}^2(^\circ\text{C}) \\ &+ 7.6E-5 \times \textit{Temperature}^3(^\circ\text{C}) \\ &- 8.6E-7 \times \text{Temperature}^4(^\circ\text{C}) \end{aligned} \quad (5)$$

Similarly, in the study of Awata et al. (2013), the optimum temperature of Ca. Scalandiua species were identified as 30 °C which is lower than that of other Anammox species. In the research with Ca. Brocadia and Ca. Kuenenia species (Dosta et al. 2008; Lotti et al. 2015) an approximately tenfold decrease was observed from 35 °C to 10 °C. In the study of Pedrouso et al. (2021) with Brocadia fulgida Annamox species, temperature decrease from 30^0 to 15^0C resulted five-fold decrease in SAA which is quite higher than the threefold decline in SAA observed in the present study under the same degree of temperature drop. This may indicate that Ca. Scalindua species can be a good candidate for cold-Anammox applications. In consistent with this hypothesis, Kouba et al. (2022) indicated the Ca. Scalindiua species displayed outstanding potential for nitrogen removal from cold streams (10-20^0C) due to their unique ladderane structures and bacteriohopanoids. In the present study, above 35 °C, a sharp decrease in SAA was observed. This decrease reached a value of 76% at 39 °C. The activity completely halted at a temperature around 45 °C. In contrast, in the study of Dosta et al. (2008) with Ca. Kuenenia enrichment, SAA exhibited an exponential increase till the temperature of 40 °C although, lysis was observed beyond 45^0C. Lotti et al. (2015) reported the highest SAA at 30^0C for biomass with Ca. Brocadia enrichment. In the study of Sobotka et al. (2021) the highest SAA value was observed at 40 °C with no information on the abundant type of Anammox species. A

5 °C rise in temperature above 40 °C reduced SAA by almost 50%. Tomaszewski et al. (2017) reported the highest SAA value at 40 °C for Ca. Jettenia cells.

Although Anammox process is identified in a wide variety of environments, the cold temperature tolerance of Anammox species is still one of the major obstacles to the widespread application of the Anammox process in wastewater treatment. The results of the present study give deep insight into the response of the Anammox process including Ca. Brocadia and Ca. Scalindua enrichment under seasonal temperature variations in wastewater treatment plant conditions. Additionally, information gained from these short-term experiments is quite valuable for future work that will evaluate the cultivation conditions of the Anammox biomass in cold-temperature conditions. Future work evaluating the temperature response of less studied Anammox species, e.g., Ca. Anammoxoglobus and Ca. Anammoximicrobium is also suggested to identify Anammox species with the highest ability to adapt to low temperatures.

Dissolved Oxygen

The effect of the presence of dissolved oxygen on the Anammox process was evaluated through batch assays with an initial S_o/X_o ratio of 0.41 ± 0.04 at pH 7.5 and temperature of 32 ± 0.5 °C (Fig. 2c). As being strictly anaerobic, the presence of DO negatively affected the activity of Ca. Brocadia and Ca. Scalindua enrichment (Fig. 2c). A decreasing trend of SAA with increasing DO values was observed to be strongly linear. DO concentration causing 50% decrease in maximum SAA (IC_{50}) was observed as 0.3 mg/L. Obligate anaerobic bacteria, unlike aerobic and facultative bacteria, mostly do not possess appropriate protective mechanisms against the toxic oxygen radical by producing superoxide dismutase (SOD) enzyme. However, some obligate anaerobes do contain SOD which may provide oxygen intolerance of strictly anaerobic species to the low levels of dissolved oxygen (Kato et al., 1997). The slight tolerance (25–30% decrease in SAA) of Ca. Brocadia and Ca. Scalindua enrichment up to DO level of 0.1 mg/L may provide a possibility of coupling the Anammox process with the partial nitritation process in single-stage combined systems. Consistently, in the study of Awata et al. (2021), Ca. Scalindua performance was found better than Ca. Brocadia seeded the MBR system under the presence of low DO. The relationship between dissolved oxygen and SAA was modelled by a linear equation (Eq. 6) with one-factor design of RSM. The ANOVA results of the model (Fig. 3c) showed that the model developed is significant with an F factor of 140.53 ($p < 0.05$).

$$\text{SAA}, \frac{\text{g}N_2 - N}{\text{mgMLVSS}.d} = -2.9E-2 - 2.5E-2 \times \text{DO(mg/L)} \quad (6)$$

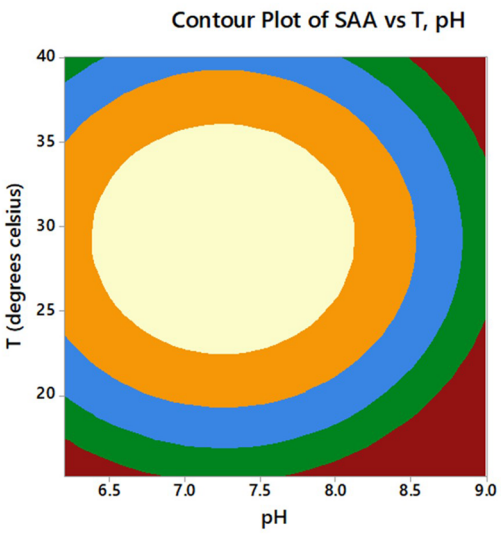
DO tolerance of Anammox bacteria is heavily dependent on species type, i.e., free cells or aggregated. In the study of Strous et al. (1997a) oxygen tension to 0.5% of air saturation (ca. 0.04 mg/L of DO) was reported as completely inhibiting the Anammox activity without reporting the abundant Anammox species. Seuntjens et al. (2018) reported complete inhibition of Ca. Brocadia and Ca. Kuenenia free cell species at micro-aerobic levels of less than 0.04–0.12 mg O_2/L Based on these reported values, Ca. Brocadia and Ca.Scalandiua enrichment is found more tolerant to DO than other Anammox species. The findings of the present study demonstrated that in the Anammox process, DO control is of crucial importance and real-time, sensitive and reliable DO control methods are required for the successful operation of the process under treatment plant conditions. The presence of oxygen-consuming bacteria in the niche of Anammox plants will be a promising strategy to avoid the inhibitory effect of DO on process efficiency. Future researches on the DO inhibition characteristics of less studied Anammox species, e.g., Ca. Anammoxoglobus and Ca. Anammoximicrobium and assessment of DO adaptation of Anammox consortium with corresponding nitrogen removal performance are strongly suggested.

Interactive effect of pH and Temperature

The effect of pH and temperature variables on the SAA under the absence of dissolved oxygen was further analyzed with Central Composite Design of Response Surface Methodology under Minitab 17 software (Fig. 4). The interaction between pH and temperature was modelled by a two-factor design of RSM (Eq. 7). The ANOVA results showed that the model is fitting moderately with an R square of 40.3% but significant with F factor of 4.79 ($p < 0.05$). The middle light-yellow region of the contour plot in Fig. 4 represents that the maximum Anammox activity (i.e. maximum SAA) can be achieved under a temperature range of $24 - 35^0$C with pH values varying between 6.5 and 8. The contour lines can also be used to find how pH can be adjusted to achieve the maximum possible SAA under a constant temperature or vice versa.

$$\begin{aligned}\text{SAA}, \frac{\text{g}N_2 - N}{\text{mgMLVSS}.d} = &-0.718 + 0.1645 \times \text{pH} \\ &+ 0.01094 \times T \\ &- 0.01134 \times \text{pH} \times \text{pH} \\ &- 0.000187 \times T \times T \end{aligned} \quad (7)$$

In literature, the studies evaluating the interactive effect of temperature and pH on Anammox activity is relatively rare. In the study of Daverey et al. (2015), the simultaneous effects of temperature and pH were studied using a seed from a landfill leachate treatment plant and reported that maximum SAA would be above 33^0C and between pH of 7.2



Source	DF	Adj SS	Adj MS	F Value	P-Value
Model	4	0.005821	0.001455	9.11	0.000
Linear	2	0.001529	0.000765	4.79	0.012
pH	1	0.000976	0.000976	6.11	0.017
T	1	0.000565	0.000565	3.54	0.065
Square	2	0.004043	0.002021	12.65	0.000
pH*pH	1	0.002197	0.002197	13.75	0.000
T*T	1	0.002653	0.002653	16.61	0.000
Error	54	0.008626	0.000160		
Lack of Fit	23	0.003360	0.000146	0.86	0.642
Pure Error	31	0.005266	0.000170		
Total	58	0.014448			

S	R-sq	R-sq (adj.)	R-sq (pred.)
0.0126390	40.29%	35.87%	31.70%

Fig. 4 Evaluation of pH and temperature interactions with Central Composite Design of Response Surface Methodology

and 9. Further research activities are necessary especially with less studied Anammox enrichments of Ca. Jettenia, Ca. Anammoxoglobus and Ca. Anammoximicrobium to develop the most optimum operation strategy in Anammox plants under various seasonal conditions.

Organic Compounds

Organics are present in a broad type and concentration range in both domestic and industrial wastewaters. Hence, the effect of organic matter on the activity of Anammox bacteria are crucial for the application for Anammox process applications. In the present study, short-term effect of non-toxic organic compounds acetate, propionate, and glucose on Anammox culture enriched for Ca. Brocadia and Ca. Scalindua species. The effect of carbonaceous content of domestic sewage on Anammox bacteria was also evaluated. The observed SAA values observed through batch assays performed at S_o/X_o ratio of 0.36, pH of 7.5 and temperature of 34 ± 0.72 °C are shown in Fig. 5.

All studied non-toxic organic compounds (Fig. 5) exhibited a negative effect on SAA. Inhibitory effect of acetate on Ca. Brocadia and Ca. Scalindua Anammox species was more severe than the inhibitory effect of propionate and glucose. 50% decrease in SAA (IC_{50}) was observed for 1000–1500 mg COD/L acetate, 3300 mg COD/L propionate and 3600–5700 mg COD/L glucose levels. In batch assays with acetate in the range of 200 – 6200 mg COD/L (Fig. 5a), the gradual decreases observed in SAA up to 1000–1500 mg COD/L remained almost constant till 4000 mg COD/l. However, above 4000 mg COD/L, SAA values sharply decreased and 90% decrease in SAA (IC_{90}) was observed at 6000 mg/L acetate concentration. In the studied propionate range of 255–6300 mg COD/L (Fig. 5b), the gradual increase of propionate inhibition reached 90% at 5000 mg COD/L. The batch assays with glucose in the range of 100–10,000 mg/L (Fig. 5c) showed that no decrease in SAA was observed till 2000 mg COD/L glucose concentration. Above 2000 mg COD/L, SAA decreased gradually with increasing glucose concentration. 35% and 70% inhibitory concentrations (IC_{35} and IC_{70}) were determined as 3685 mg COD/L and 5720 mg COD/L glucose concentrations, respectively. The inhibition of the Anammox process by all studied non-toxic organic compounds cannot be related with the "out-competition" between organic and nitrogen utilizing bacteria since the experiments were performed with Anammox enrichment. Hence, enzyme inactivation is the major responsible mechanism for the observed inhibition of which type identification

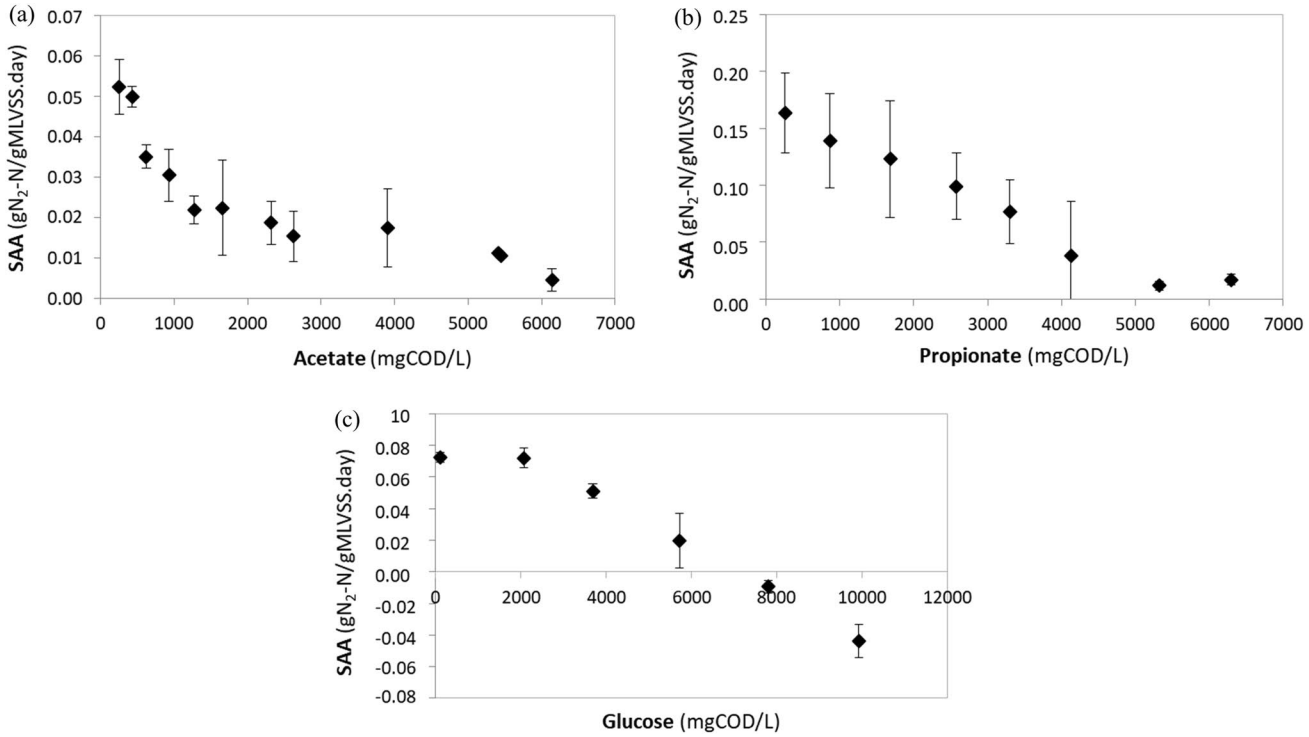


Fig. 5 SAA values observed at various **a** acetate, **b** propionate and **c** glucose concentrations

requires further studies. As opposed to studied non-toxic organic compounds, complex organic composition of sewage had no negative effect on Anammox activity. An aerated grit chamber effluent from a local sewage treatment plant with 215 mg/L soluble COD did not result in any decrease in SAA of Ca. Brocadia and Ca. Scalindua enrichment.

Similarly, in the previous studies, 20% and 70% decreases in maximum SAA were observed at 1280 mg COD/L and 4800 mg COD/L acetate concentrations, respectively (Dapena-Mora et al. 2007; Alpaslan Kocamemi and Dityapak, 2014). Viet et al. (2008) reported that IC_{40} and IC_{70} values for glucose as 3840 mg COD/L and 7680 mg COD/L, respectively. Identification of potential inhibitory behavior of non-toxic organic compounds acetate, propionate and glucose is beneficial to develop methods for relieving Anammox inhibition (e.g. wastewater pre-treatment) prior to treatment of specific types of wastewater including these compounds. Presently, partial nitritation and partial denitrification are two available technologies for nitrite-generation which is a prerequisite for Anammox applications. Since partial nitritation is very difficult to achieve under low ammonium containing cold mainstream conditions, Anammox and partial denitrification coupling technologies are receiving great attention. The identified inhibitory levels of various organic compounds are quite beneficial to determine precise optimal conditions for an Anammox and partial denitrification coupling process without significant inhibition of Anammox process. Further research activities that will evaluate the interactive effect of major operating parameters such as pH and temperature on the inhibitory behavior of these compounds may provide identification of an optimum operation strategy. Additionally, future research activities evaluating the inhibition of the Anammox process with other non-toxic organics and also with toxic organics are strongly recommended to extend the Anammox applications for industrial wastewater treatment. Future studies may focus on the metabolic pathways of the Anammox process in response to inhibitors through the use of metagenomics and metatranscriptomics.

Inorganic Compounds

In this part of the study, effects of inorganic constituents commonly found in both domestic and industrial wastewaters were evaluated through batch assays performed at S_o/X_o ratio of 0.2–0.3, pH of 7.5 and temperature of 32–34 °C. The nitrite batch assays, however, were performed at S_o/X_o ratio of 0.02–0.006, pH of 8.2 and temperature of 35 °C.

Among the inorganic compounds studied, nitrite (NO_2^--N), which is the electron acceptor of Anammox process, caused no significant change in SAA till 50 mg/L (Fig. 6a). Above 50 mg/L, SAA started to decrease gradually. IC_{50} value was determined as 71.5 mg NO_2^--N/L which is significantly lower than the IC_{50} values identified for

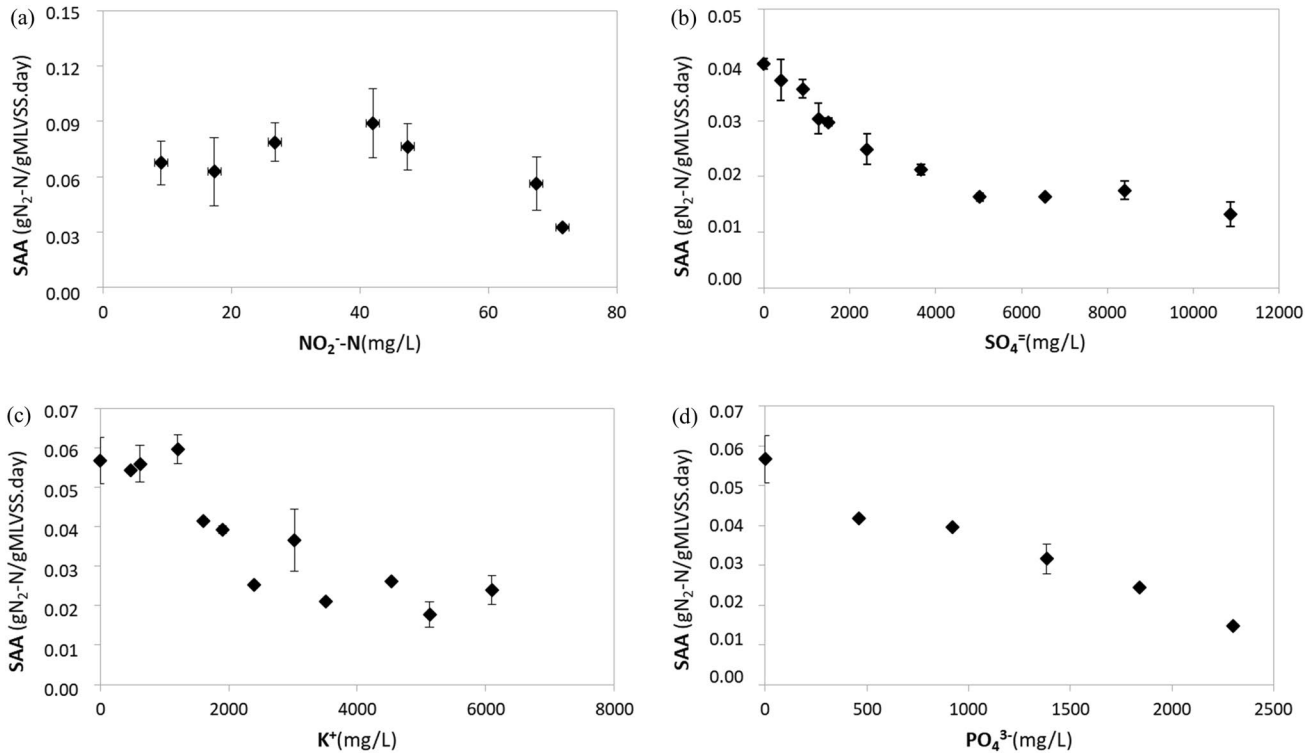


Fig. 6 SAA values observed at various **a** NO_2^--N, **b** SO_4^{2-}, **c** K^+ and **d** PO_4^{3-}-P concentrations

Brocadia (400 mg/l) and Kuenenia (350 mg/L) species in the previous studies (Cho et al. 2020). Similarly, Awata et al. (2013) reported nitrite tolerance of Scalindua enrichment as 7.5 mM (105 mg/L) which is lower than that reported for Ca. Brocadia and Ca. Kuenenia species. The short-term effects of sulfate (SO_4^{2-}) on Anammox (Fig. 6b) were studied up to sulfate concentration of 11,000 mg/l. SAA gradually decreased with increasing sulfate concentration until 5000 mg SO_4^{2-}/L. IC_{50} was identified as 3500 mg SO_4^{2-}/L. At 5000 mg/l, SAA inhibited 60% and no further decrease was observed up to 11,000 mg/L. In the study of Dapena-Mora (2007), SAA activity of Ca. Kuenenia species inhibited 50% at 2400 mg/L SO_4^{2-} and completely halted at 9600 mg/L. Phosphate (PO_4^{3-}-P) was found inhibitory only at very high concentrations, which is mostly not typical in real wastewater. IC_{50} was determined as 1384 mg PO_4^{3-}-P/L. Maximum decrease in SAA was observed around 73% at 2300 mg/L PO_4^{3-}-P. In the studies of Carvajal-Arroyo et al. (2013) and Zhang et al. (2017), IC_{50} value were reported as 784 mg PO_4^{3-}-P/L and 1205 mg PO_4^{3-}-/L for Ca. Brocadia and Kuenenia species, respectively. This indicates that Kuenenia and Scalindua species are more resistant to high concentrations of phosphate. Potassium exhibited no negative effect on Anammox activity up to 1200 mg/L (Fig. 6c). In the concentration range of 1200–6000 mg/L, SAA gradually decreased with increasing K^+ concentration. IC_{50} was found as 2400 mg K^+/L. In the concentration range of 3500–6000 mg/l, SAA inhibition reached the levels of 50–70%. To the best of our knowledge, no previous study has investigated the inhibitory level of K^+ on Anammox species.

The overall results of inhibition batch assays with inorganic compounds indicated that except NO_2^--N, Ca. Brocadia and Ca. Scalindua enrichments have slightly higher IC_{50} values in comparison to the Anammox cultures in which Ca. Brocadia and /or Ca. Kuenenia species are abundant. This finding can be quite beneficial when choosing the most appropriate inoculum to treat wastewaters including these inorganics with different concentrations. Slightly higher IC_{50} value observed for NO_2^--N may be related to the higher NO_2^--N affinity of Ca. Scalindua species with respect to the other Anammox species (Zhang and Okabe, 2020) or inhibition by increasing FNA at high NO_2^--N concentrations. This finding indicates that Anammox systems will be started up with enrichments including Ca. Scalindua species may face inhibition problems during start-up periods during which nitrite accumulation is quite common. Future research activities should focus on composite inhibition studies performed with multiple inhibitory inorganic compounds commonly co-existing in wastewaters. Additionally, the effect of major operating parameters, pH and temperature on the inhibitory levels of inorganic compounds merits future research activities.

Conclusion

The overall findings provided comprehensive insight into the tolerance of Anammox systems enriched for Ca. Brocadia and Ca. Scalindua species against key (pH, DO and temperature) and common organic and inorganic wastewater constituents. The short-term responses identified by monitoring N_2 gas production and calculation of SAA values reveal the potential of using saline Ca. Scalindua species together with common freshwater Ca. Brocadia species and allows to develop an optimum operation strategy for engineered Anammox systems to be designed for the treatment of wastewater with various organic and inorganic constituents. In this study, one-factor design of response surface methodology allowed modeling of pH-SAA, temperature-SAA and DO-SAA relationships. Optimum pH (7.36) and temperature (32.7^0C) values for Ca. Brocadia and Ca. Scalindua Anammox enrichment were found lower than the optimum values identified for Ca. Kuenenia, Ca. Jettenia and Ca. Brocadia- Ca. Kuenenia enrichments. Similar to the other biological processes, an increase in pH and temperature values to optimum values positively affected the Anammox activity of species. As expected from the strict anaerobic nature of Anammox species, the presence of dissolved oxygen inhibited the process. However, IC_{50} value of DO (0.3 mg/L) showed that Ca. Brocadia and Ca. Scalindua enrichment is more tolerant to the presence of DO in comparison to other Anammox species. All studied organic compounds except sewage, exhibited a negative effect on Ca. Brocadia and Ca. Scalindua enrichment. Inhibitory effect of acetate on Anammox species was identified more severe than the inhibitory effect of propionate and glucose. IC_{50} values/ranges of acetate, propionate and glucose were identified as 1000–1500 mg COD/L, 3300 mg COD/L and 3600–5700 mg COD/L, respectively. Among the inorganics studied, NO_2^--N, which is the electron acceptor of the Anammox process, caused inhibition of Anammox above 50 mg/L. IC_{50} value of NO_2^--N is slightly higher than the values observed for other Anammox species. The other studied inorganics (SO_4^{2-}, PO_4^{3-}P and K^+) exhibited an inhibitory effect on Anammox at very high concentrations with very different patterns. IC_{50} values of all studied inorganics (3500 mg SO_4^{2-}/L, 1384 mg PO_4^{3-}-P/L and 2400 mg K^+/L) were found slightly lower than those observed for other Anammox species. In view of the findings, future research activities that will evaluate the composite inhibition of inorganic and organic (non-toxic and toxic) compounds under the interactive effect of major operating parameters are strongly recommended by focusing on metabolic pathways through the use of metagenomics and metatranscriptomics.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Consent to Publish All the authors have read and approved the manuscript and accorded the consent for publication.

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