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ADVANCED OXIDATION PROCESS FOR SUSTAINABLE WATER MANAGEMENT

Effects of short- and long-term exposures of humic acid on the Anammox activity and microbial community

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Abstract
Humic acid has a controversial effect on the biological treatment processes. Here, we have investigated humic acid effects on the Anammox activity by studying the nitrogen removal efficiencies in batch and continuous conditions and analyzing the microbial community using Fluorescence in situ hybridization (FISH) technique. The results showed that the Anammox activity was affected by the presence of humic acid at a concentration higher than 70 mg/L. In fact, in the presence of humic acid concentration of 200 mg/L, the Anammox activity decreased to 57% in batch and under continuous condition, the ammonium removal efficiencies of the reactor decreased from 78 to 41%. This reduction of Anammox activity after humic acid addition was highlighted by FISH analysis which revealed a considerable reduction of the abundance of Anammox bacteria and the bacteria living in symbiosis with them. Furthermore, a total inhibition of *Candidatus Brocadia fulgida* was observed. However, humic acid has promoted heterotrophic denitrifying bacteria which became dominant in the reactor. In fact, the evolution of the organic matter in the reactor showed that the added humic acid was used as carbon source by heterotrophic bacteria which explained the shift of metabolism to the favor of heterotrophic denitrifying bacteria. Accordingly, humic acid should be controlled in the influent to avoid Anammox activity inhibition.

Keywords Anammox bacteria · Heterotrophic bacteria · Humic acid · Inhibition · Nitrogen

Introduction

Nitrogen is an essential nutriment for all living organisms since it is an important constituent of proteins and nucleic acids. However, eutrophication due to nitrogen pollution is one of the most serious environmental issues all over the world. In fact, ammonium nitrogen, often present in different types of wastewater, can end up in different water bodies causing an important reduction of dissolved oxygen which lead to the toxicity of aquatic organisms (Wahab et al. 2010a).

In order to control nitrogen pollution, different physicochemical and biological methods including adsorption, chemical precipitation, ion exchange, air stripping, and biological nitrification and denitrification were proposed for the removal of ammonium from wastewater (Wahab et al. 2010b, 2012). Nevertheless, conventional wastewater treatment technologies are associated with many operational problems, energy demand, and high costs. In fact, in nitrification and denitrification process, there is often demand for energy for aeration, during the aerobic stage, and requires the addition of an external source of organic matter during the anaerobic stage.

Anaerobic ammonium oxidation (Anammox) could be considered as an innovative and promising process for nitrogen removal under anoxic conditions, during which ammonium is directly oxidized to nitrogen gas.

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Accordingly, Anammox demands less oxygen and no external carbon source is required which makes the process cost-effective alternative to conventional processes of nitrogen removal (Jetten et al. 1997). From a technical point of view, several studies reported a high nitrogen removal in Anammox reactors and some full-scale processes have been established successively (Lackner et al. 2014). For instance, Tang et al. (2010) reported a high nitrogen removal rate of 74.3–76.7 kg N/m^3/day in a lab-scale Anammox UASB reactor. However, this reactor required long start time due to the very slow growth rate and low biomass yield of Anammox bacteria (Strous et al. 1998). In fact, the anaerobic processes are characterized by their long start-up period and their high sensitivity to the presence of inhibitors (Wahab et al. 2014). Therefore, it is necessary to identify the toxic substances affecting Anammox process and to understand their effect on the whole process and especially on the microbial community distribution. A variety of inhibitory substances, such as substrates (ammonia and nitrite) (Fernández et al. 2012), organic matter, salts, heavy metals, phosphate, and sulfide, have been described to affect the Anammox process (Jin et al. 2012). However, the impact of humic acid on the Anammox process is still unknown. Humic acid are charged polyelectrolyte complexes due to the presence of carboxylic, phenolic, ketonic, aromatic, and aliphatic groups and interact with both living and non-living matter (Steinberg et al. 2008).

The majority of full-scale installations of Anammox process counting about 100 worldwide (Lackner et al. 2014) are mainly applied to treat the sludge digestion reject water in which humic acid can reach up to mass fractions of 1.5% of total solids (Fernandes et al. 2014). Many studies have demonstrated that humic acid can function as electron shuttles in anaerobic environments affecting the hydrolysis step of the anaerobic digestion and decreases the methanogenic activity (Zhou et al. 2014; Khadem et al. 2017). Since humic acid can affect the anaerobic treatment processes and the majority of the Anammox installations are intended to treat effluents containing humic acid in different concentrations, thus, it is important to determine its effect on anaerobic ammonium oxidation.

Accordingly, in this study, the inhibitory effect of humic acid on the Anammox process was investigated. Firstly, batch experiments were performed to study the effect of different humic acid concentrations on Anammox activity and to determine the threshold concentration. Secondly, long-term experiments were carried out in a sequencing batch reactor (SBR) to investigate the effect of the gradual addition of humic acid on the stability and the performance of the Anammox process under continuous operation. In addition, we have also investigated the response of the Anammox bacteria to humic acid addition in the SBR by comparing the microbial community composition before and after humic acid addition.

Materials and methods

Short-term effects of humic acid on the Anammox activity

In order to determine the short-term effect of humic acid on the Anammox activity, batch experiments were conducted using 38-mL vials with 25 mL working volume, each closed with gas-tight covers following the procedure described by Dapena-Mora et al. (2007). The vials were inoculated with Anammox bacteria previously washed in phosphate buffer (0.14 g/L KH_2PO_4 and 0.75 g/L K_2HPO_4). The liquid phase and headspace of each vial were flushed with argon gas to remove the oxygen, ensuring the anoxic conditions. The vials were incubated for 30 min in an incubator shaker at 30 °C at a constant shaking rate of 150 rpm until stabilization. Then, a concentration of 70 mg N/L of each nitrite and ammonium was added to the vials. The humic acid concentration ranges tested were 50, 75, 100, and 200 mg/L, and it was added with the substrates. After initial equalization to the atmospheric pressure, each vial was placed again in the incubator shaker. The headspace pressure inside the vial was measured periodically by using a differential pressure transducer (Centerpoint Electronics). The analysis of the produced biogas composition indicated that more than 99% of the produced gas was N_2 (Dapena-Mora et al. 2007). Control experiments, without humic acid addition, were also carried out under the same conditions. All experiments were performed in duplicate.

Long-term effects of humic acid on the Anammox process performance

The study of the long-term effects of humic acid on the Anammox process was carried out in a sequencing batch reactor of 3.75 L of working volume operated during 150 days. The reactor was operated in anoxic conditions in cycles of 6 h distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min), and draw (15 min), according to Dapena-Mora et al. (2004). The exchange volume was fixed at 25%, so the hydraulic retention time was fixed at 1 day. Two peristaltic pumps were used to feed and draw the unit. The reactor was provided with a thermostatic system to keep the temperature at 30 °C. The pH value was not controlled and ranged between 7.5 and 8.0. The complete mixture inside the reactor was achieved using a mechanical stirrer (150 rpm). From day 107 onwards, humic acid was added into the feeding media and its concentration was gradually increased from 50 to 200 mg/L. Anoxic conditions were ensured in the reactor by flushing argon gas. Thus, in this case, the effect over the Anammox activity was discussed in terms of nitrogen balance in the reactor compared to the widely reported Anammox stoichiometry.

Anammox biomass and feeding media

The Anammox biomass was retrieved from a laboratory scale reactor. The total suspended solid (TSS) was estimated at 1.30 g/L. The reactor was fed with a synthetic autotrophic medium with a composition similar to that described by Dapena-Mora et al. (2004) (Table 1). The ammonium to nitrite molar ratio in the feeding media was fixed at 1.0 (100 mg N/L of each).

Substrates and humic acid preparation

Analytical grade ammonium chloride salt (NH_4Cl) and sodium nitrite ($NaNO_2$) were used for the preparation of the stock NH_4^+ and NO_2^- solutions of 2300 mg N/L, respectively. A stock humic acid solution of 1000 mg/L was also prepared using humic acid with chemical formula $C_{10}H_{12}O_5N$ (Sigma-Aldrich). Ammonium, nitrite, and humic acid solutions of different concentrations were prepared by diluting the stock solutions with distilled water. The initial pH was adjusted to the desired value using diluted solutions of HCl or NaOH (0.1 N).

Analytical methods

Physico-chemical parameters like nitrate, nitrite, ammonium concentrations, concentrations of TSS, and volatile suspended solids (VSS) were determined according to Standard Methods (APHA 1998): TSS (dried at 105 °C method and VSS: dried at 550 °C) with a detection limit of 1 mg/L. Total ammonia-nitrogen (NH_4^+-N) was determined spectrophotometrically by phenate method with applicable concentrations ranged between 0.02 and 2 mg/L. Nitrate concentration was determined following the method 4500-NO_3^--N-B (ultraviolet spectrophotometric screening method) described in the Standard Methods for the Examination of Water and Wastewater with detection limit of 0.05 mg NO_3-N/L. Nitrite concentration was determined following the method 4500-NO_2-B. The colorimetric method (B) is suitable for concentrations of 5 to 1000 μg NO_2-N/L. Total organic carbon (TOC) was measured with a Shimadzu analyzer (TOC-5000) as the difference between the concentrations of the total carbon (TC) and the inorganic carbon (IC). The total carbon is obtained from CO_2 produced during the combustion of the sample at 680 °C, and the inorganic carbon is determined from CO_2 produced during the chemical decomposition of the sample with H_3PO_4 (25%) at room temperature.

Table 1 Feeding media composition

Compound	Concentration (g/L)
$(NH_4)Cl$	0.382
$NaNO_2$	0.493
$KHCO_3$	1.250
KH_2PO_4	0.057
$CaCl_2$	0.227
$MgSO_4{\cdot}7H_2O$	0.2
$FeSO_4{\cdot}7H_2O$	0.011
KNO_3	0.0728
EDTA	0.006
Trace solution	0.2 mL/L

Calculations

SAA

The N_2 production rate was determined from the maximum slope (α) of the curve describing the pressure increase in the vial throughout the time of the experiment Eq. (1).

$$\frac{dN_2}{dt} = \frac{\alpha \cdot V_G}{R \cdot T} \qquad \left(\frac{\text{mol } N_2}{\text{min}}\right) \tag{1}$$

being V_G: volume of the headspace; T: temperature; and R: ideal gas coefficient.

The SAA was calculated from the nitrogen gas production rate divided by the biomass concentration in the vial X (g VSS/L).

$$\text{SAA} = \frac{\frac{dN_2}{dt}}{X \cdot V_L} \cdot \frac{28 \text{ g N}}{\text{mol } N_2} \cdot \frac{1440 \text{ min}}{\text{day}} \qquad \left(\frac{\text{g } N_2\text{–N}}{\text{g VSS} \cdot \text{day}}\right) \tag{2}$$

V_L: volume of liquid phase in the vial (L).

Activity percentage

The percentage of Anammox activity maintained when inhibitory compounds were added was calculated as Eq. (3).

$$\text{SAA } (\%) = \text{SAA}/\text{SAA}_0 \times 100 \tag{3}$$

SAA maximum specific activity of the test with humic acid.

SAA_0 maximum specific activity of the control (without humic acid).

Fluorescence In Situ Hybridization (FISH)

Biomass samples were collected from the reactor before and after humic acid addition and were analyzed by the Fluorescence in situ hybridization (FISH) technique. FISH is a molecular

technique used to identify the presence of certain microorganisms using rRNA-targeted oligonucleotide probes. This protocol has been adapted from Amann et al. (1995) in order to identify and evaluate the abundance of nitrifying microbes and Anammox bacteria by selective hybridization with FISH probes.

The 16S rRNA sequences of targeted oligonucleotides used in this study and their target bacteria, formamide concentration, and applied fluorochromes are shown in Table 2. The total biomass was stained by DAPI (4′,6-diamidine-2-phenylindole) right before the imaging process by epifluorescence microscope. It displays a blue color under the microscope.

Generally, there are three steps to apply the FISH technique. After taken from the reactor, all samples were fixed with paraformaldehyde and then stored in the freezer at − 20 °C. Phosphate-buffered saline (1 × PBS) was used to wash the samples. Then, the slides were dehydrated by ethanol series 50, 80, and 98%. Samples were hybridized at 48 °C for 1.5–2.0 h with 8 μL of hybridization buffer and 1 μL of each gene probe and the complementary competition probe. After hybridization, the slides were washed at 48 °C for 20 min in washing buffer and then in distilled water and left at room conditions until they dried. The positive results for *Bacteria* domain were identified with an equimolar mixture of EUB338I, EUB338II, and EUB338III.

Fluorescence detection was carried out with an Olympus BX51-RFAA microscope. Images were captured with an Olympus MX10 CCD Camera using the fluorescence imaging software CELL. An amount of 20 random photos of each sample were taken for quantifying the bio-volume of targeted microorganisms. The software "Digital Image Analysis in Microbial Ecology (DAIME)" was used to analyze the image. This program has been designed for analyzing the images obtained by FISH and other fluorescence techniques (Nielsen et al. 2009).

Results and discussion

Short-term effects of humic acid on Anammox activity

In order to apply the Anammox process to treat wastewater rich in ammonium and containing humic acid, it is necessary to know the effect of different concentrations of humic acid on the Specific Anammox Activity (SAA).

The results indicated that in the absence of humic acid (control assay), the SAA was about 0.3 g N_2-N/(g VSS day), which is regarded as 100% Anammox activity. This relatively high activity was maintained in the presence of humic acid at concentrations ranging between 50 and 75 mg/L. However, humic acid concentrations of 100 mg/L provoked a 36% decrease of the Anammox activity and reached a 43% of activity reduction when increasing humic acid concentration to 200 mg/L (Fig. 1). Accordingly, the Anammox bacteria can tolerate the presence of humic acid at concentrations lower than 100 mg/L. Over this threshold concentration, the Anammox activity will be seriously reduced, and at high humic acid concentration, the effects on the Anammox activity would be even higher.

Some studies have demonstrated the positive effect of humic acid on some processes such as the photolytic degradation of organic pollutant (Li and Hu 2016) and denitrification

Table 2 Targeted organisms and corresponding formamide (FA) percentages for the used oligonucleotide probes employed in this study

Probe	Probe sequence (5′ → 3′)	% FA	Targeted organisms
EUB338I	GCTGCCTCCCGTAGGAGT	0–50	Most *Bacteria*
EUB338II	GCAGCCACCCGTAGGTGT	0–50	Planctomycetales
EUB338III	GCTGCCACCCGTACGTGT	0–50	Verrucomicrobiales
PLA886	GCCTTGCGACCATACTACC	35	Planctomycetes
PS56a	GCTGGCCTAGCCTTC	0–5	Pseudomonadales
LGC354A	TGG AAG ATT CCC TAC	35	Firmicutes
Xan 940	GCGCGTTTCGCTCCCGAT	30%	Xanthomonadales
PLA46	GACTTGCATGCCTAATCC	30	Planctomycetales
Nso1225	CGCCATTGTATTACGTGTGA	35	*Betaproteobacterial* AOB
Nso190	CGA TCC CCT GCT TTT CTC C	35	*Betaproteobacterial* AOB
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	35	Most members of the Phylum *Nitrospirae*
NIT3	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter* spp.
Amx368	CCTTTCGGGCATTGCGAA	15	*All ANAMMOX* bacteria
Kst157	GTT CCG ATT GCT CGA AAC	25	*Candidatus* Kuenenia stuttgartiensis
Apr 820	AAACCCCTCTACCGAGTGCCC	40	*Candidatus Jettenia*
BAN162	CGG TAG CCC CAA TTG CTT	40	*Candidatus Brocadia* anammoxidans
GNSB941	AAACCACACGCTCCGCT	35	Phylum *Chloroflexi*

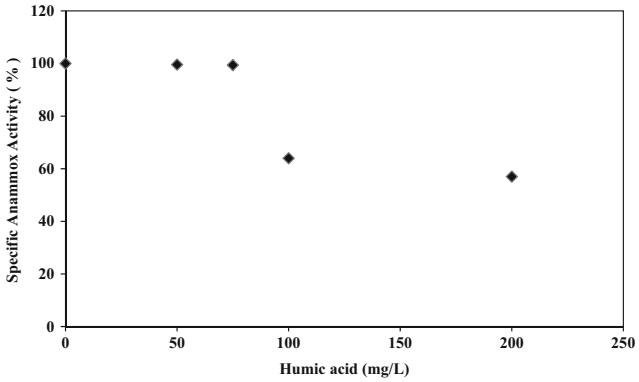

Fig. 1 Inhibitory effect of different concentrations of humic acids on Specific Anammox Activity (SAA)

process for landfill leachate (Dong et al. 2017). However, a negative effect was recorded by Khadem et al. (2017) in anaerobic digester, indicating that in presence of humic acid, methane production was reduced by 89%. According to Khadem et al. (2017) results and the results presented in this study, humic acid seemed to affect the anaerobic microorganisms in different anaerobic processes like Anammox bacteria in Anammox process and methanogenic in anaerobic digestion.

In order to compare the inhibitory effect of humic acid with other reported inhibitors, the inhibition percentages of different inorganic and organic substances under batch condition are presented in Table 3. According to these information, it is clear that for the same range of concentration, the inhibition of humic acid and phenol is similar. In fact, phenol is one of the main by-products of the humic acid biodegradation. Thus, it can be suggested that the inhibition of the Anammox activity in the present study may be due to the effect of humic acid itself or to the effect of phenol resulting from humic acid degradation. Further studies could be made to determine the main mechanism of inhibition.

Nitrogen compound evolution

Results of Anammox process performances of the reactor before and after the addition of humic acid are shown in Fig. 2. In order to understand the effect of humic acid on the Anammox process under continuous condition, the reactor was operated over 107 days without humic acid addition (phase 1) to reach a stable and effective Anammox activity. During the first 3 weeks of this phase, the consumption of ammonium was around 77% but high nitrite accumulation was observed (up to 80 mg NO_2^--N/L) with a conversion of only 30% (Fig. 2b). Moreover, concentration of approximately 60 mg NO_3^--N/L has been measured exceeding the 26% of the converted ammonium, as it was expected according to the Anammox stoichiometric reaction (Strous et al. 1998). Therefore, in this period, the predominant process taking place was the aerobic nitrogen oxidation whereas only 28% of the incoming nitrogen was removed. This could be explained by the fact that ammonium was oxidized by the ammonium oxidizing bacteria (AOB), and nitrate was produced not only by Anammox bacteria but also by nitrite oxidizing bacteria (NOB) present in the reactor and profiting from small concentrations of dissolved oxygen entering the non-hermetically closed reactor.

From week 5 of phase 1 onwards, nitrite and ammonium were consumed approximately at the same percentage of 80% (Fig. 2b). These percentages corresponded to an average nitrite consumption to ammonia consumption ratio of 1.1:1.0 (Fig. 2c), which was a bit lower than reported in the literature for the Anammox reaction (1.32) and 1.37:1.00 (Helmer et al. 2001). Toh and Ashbolt (2002) also obtained lower Anammox, stoichiometric coefficient of NO_2^--N consumption/NH_4^+-N consumption, equal to 1.1. Campos et al. (2009) reported that the coefficient obtained in their experiment of enrichment of Anammox

Table 3 Research on the substances inhibition of Anammox

Inhibitor	Experimental conditions (only batch assays)	Inhibition (%)	Reference
Nitrite	350 mg N/L	50	Dapena-Mora et al. (2007)
	224 mg N/L	50	Oshiki et al. (2011)
Ammonium	770 mg N/L	50	Dapena-Mora et al. (2007)
	100 mg NH_3-N/L	80	Fernández et al. (2012)
Zinc	2 mg Zn^{+2}/L	37	Daverey et al. (2014)
Phosphorus	20 mmol P/L	50	Dapena-Mora et al. (2007)
NaCl	230 mmol/L	50	Dapena-Mora et al. (2007)
Sucrose	700 mg/L	98	Tang et al. (2010)
Propionate	1 mmol/L	1	Oshiki et al. (2011)
Glucose	1 mmol/L	5	Oshiki et al. (2011)
Phenol	25–300 mg/L	0–50	Pereira et al. (2014)
Humic acid	20–200 mg/L	43	This study

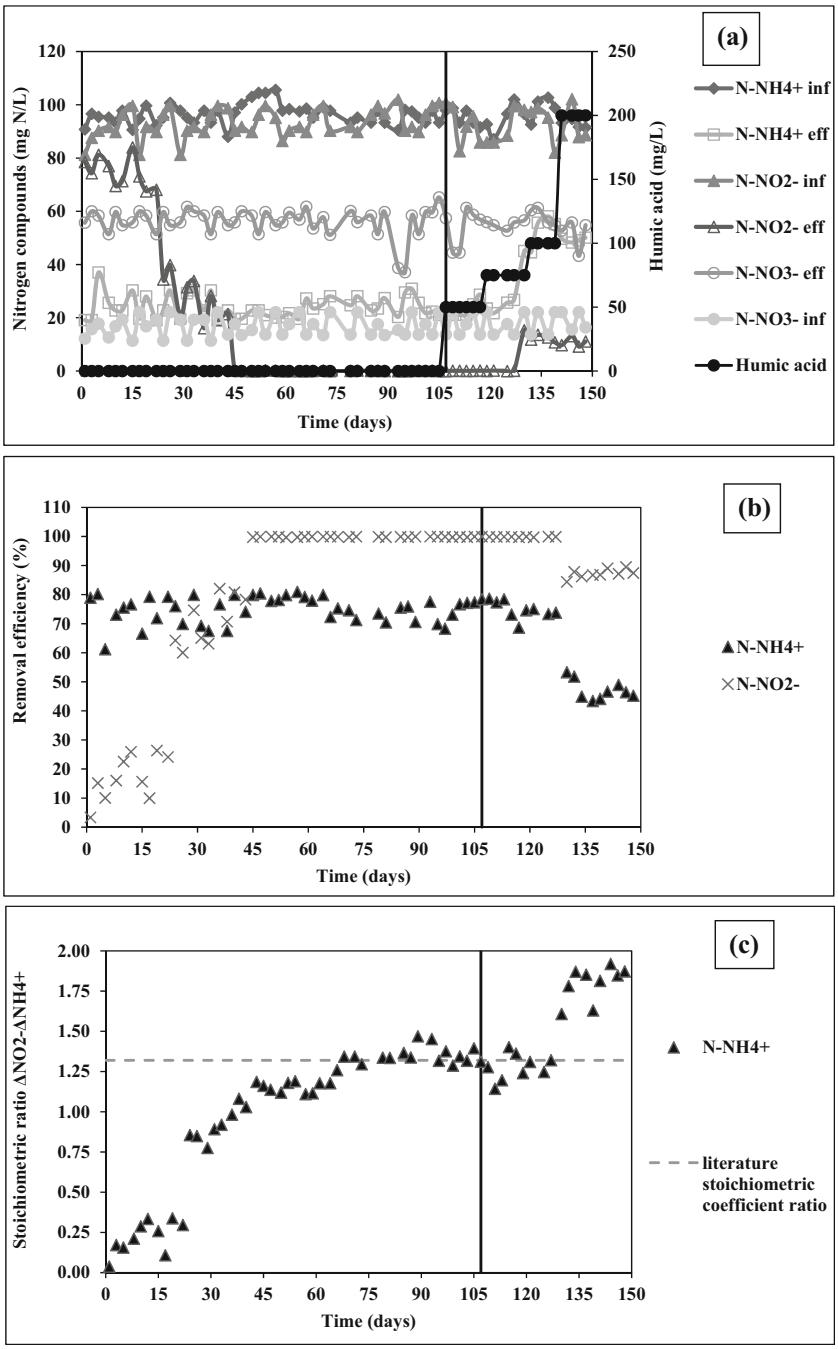

Fig. 2 Performance of the Anammox SBR before and after humic acid addition in the feeding. **a** Nitrogen concentrations in the influent and effluent. **b** Nitrogen removal efficiencies. **c** Evolution of the stoichiometric ratio $\Delta NO_2^-/\Delta NH_4^+$

bacteria remained close to 1.0. The ammonium consumed in excess may be attributed to the existence of other bacteria, such as ammonia oxidizing bacteria which were already detected in previous studies with Anammox reactors (Wang et al. 2010). Furthermore, the presence of these bacteria could be favorable to Anammox bacteria since they were able to consume oxygen that might leak into the reactor (Dapena-Mora et al. 2004). In contrast, higher coefficient was obtained in SRB Anammox by Chamchoi and Suwanchai (2007), equal to 1.5, on average, and also by Quan et al. (2008), which obtained a coefficient equal to 1.46, in an anaerobic upflow reactor operated under Anammox condition.

From the tenth week of operation onwards and until the end of phase 1, the consumption of NH_4^+ and NO_2^- increased to 70.63 and 96.51 mg/L, respectively (Fig. 2a), resulting in high nitrogen removal efficiency values of 60.0 ± 6.7% for ammonium and 91.9 ± 9.9% for nitrite (Fig. 2b). These values corresponded to an average NO_2^-/NH_4^+ ratio equal to 1.33 values close to those reported in the literature for the Anammox reaction (1.32), which is an indicator of a stable Anammox activity (Fig. 2c). Similar values were reported by

Strous et al. (1998). In this period, nitrate production was still higher than the expected value, and the ratio of nitrate production to ammonia removed ratio was 0.43 still higher than the previous reported values for the Anammox process of 0.256 (Strous et al. 1998). This phenomenon was also reported in previous studies, for instance, the ratio 0.44 was observed by Xiong et al. (2013), indicating the co-existence of Anammox bacteria and nitrifying bacteria activities.

In contrast to the results obtained in the present study, the mean stoichiometric coefficient of NO_3^--N/NH_4^+-N consumption obtained by Chamchoi and Suwanchai (2007) was lower, equal to 0.04. The authors suggested that this may be related to the presence of other groups of microorganisms co-existing with Anammox bacteria, probably responsible for the reduction of nitrate, as denitrifying bacteria, for example.

After reaching a stable Anammox activity at the end of phase 1, humic acid was progressively added at concentrations from 50 to 200 mg/L from day 108 onwards and for approximately 40 days (phase 2). According to Fig. 2, the supply of concentration of humic acid in the reactor up to 75 mg/L did not affect the Anammox process performance which remained stable. These findings were in accordance with the batch experiment results (Fig. 1). In fact, when the concentration of humic acid varied from 50 to 75 mg/L, the removal efficiency of nitrite was close to 100% and approximately of 78% for ammonium, reaching a total nitrogen removal efficiency of 58%. In contrast, when the concentration of humic acid was increased up to 100 mg/L, the Anammox process performance was suddenly deteriorated, and the effluent concentrations of ammonium and nitrite increased up to 56 and 12 mg N/L, respectively (Fig. 2a), confirming again the results previously obtained in batch experiments, which indicated the negative effect of humic acid on Anammox metabolism under this concentration range (Fig. 1). In fact, when the concentration of humic acid was increased from 100 to 200 mg/L, the removal efficiency of nitrite decreased to 87.6%, while that of ammonium removal goes down to 41% and total nitrogen removal efficiency 39.45%. Thus, the addition of humic acid over 100 mg/L seems to affect the stability of the Anammox process by reducing the ammonium removal efficiency. With respect to the Anammox stoichiometry, at these high humic acid concentrations, the NO_2^-/NH_4^+ ratio remained stable for concentrations of humic acid under 100 mg/L when it increased to 1.89, which indicated an instability state of the Anammox process (Fig. 2c). According to these results, it can be suggested that the presence of dissolved organic matter like humic acid enhances the consumption of nitrite more than that of ammonium. The phenomenon was also reported in previous studies by Toh and Ashbolt (2002) and Pereira et al. (2014) who indicated that the addition of phenol increased nitrite consumption and changed the stoichiometric characteristics of the Anammox metabolism. These results suggested that the presence of organic matter at high concentration in Anammox reactor allowed a metabolic shift to the favor of the heterotrophic denitrifying bacteria. Therefore, the nitrite was probably used as an electron acceptor by heterotrophic bacteria for the oxidation of the organic matter of the humic acid present in the reactor.

Evolution of the organic matter consumption

In order to understand the effect of organic matter increase on the perturbation of the Anammox process, the evolution of the total organic carbon (TOC) concentration in the reactor was measured (Fig. 3). According to this figure, the addition of humic acid increased the total organic carbon concentration from 13.2 ± 7.6 mg/L at the end of phase 1 to 60.0 ± 10.3 mg/L in phase 2. Despite this increase in the influent, the reactor presented almost the same concentration of total organic carbon in the effluent. This indicated that the microbial community in the reactor consumed the additional organic matter provided by humic acid supply. The nitrite was probably used as an electron acceptor by the heterotrophic denitrifying bacteria for the oxidation of the additional organic matter added to

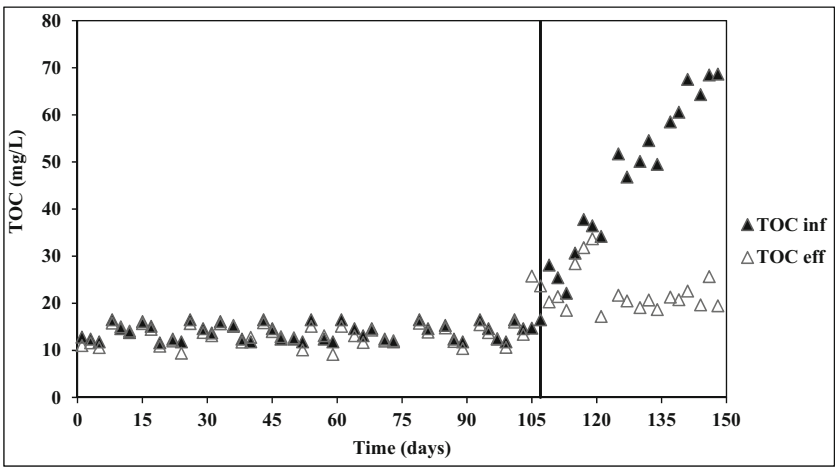

Fig. 3 Evolution of the total organic carbon (TOC) concentration in the Anammox SBR before and after humic acid addition

the reactor as humic acid. Some studies reported that the Anammox bacteria can co-exist with the heterotrophic denitrifying bacteria (Wang et al. 2010). Hou et al. (2013) reported that Anammox process may be coupled closely to denitrification reaction. The denitrification process was likely a primary source of nitrite for Anammox process (Meyer et al. 2005). This co-existence can play an important role in treating nitrogen, since these bacteria can use the nitrate produced by Anammox bacteria as electron acceptor or the nitrite (Lan et al. 2011). This behavior can explain the high removal of nitrite (87%) in the presence of high humic acid concentration, which enhances the total nitrogen removal efficiency of the Anammox sequencing batch reactor. According to these results, it can be suggested that the presence of significant humic acid concentrations, apart from inhibiting the Anammox activity, might result in an excessive growth of heterotrophic denitrifying bacteria which would contribute to further destabilization of the Anammox activity and modification of the diversity and composition of the microbial community.

Microbial community composition determined by FISH technique

In order to determine the effect of humic acid on the microbial community composition, the FISH technique was used to analyze the microbial community before (at the end of phase 1 (days 105)) and after humic acid addition (at the end of phase 2 (days 143)) in the reactor. The results of the FISH technique indicated that the Anammox bacteria remained in the reactor throughout the operation time, even after feeding the humic acid. Thus, the presence of adverse conditions (i.e., the presence of humic acid, which is a toxic organic compound) in the reactor did not contribute to the elimination of these microorganisms, although it affected their activity, since the removal of nitrogenous compounds was compromised after feeding the reactor with high concentrations of humic acid (200 mg/L), reducing by 41% the removal efficiency of ammonia, compared to the period in which the humic acid was not added in the reactor. Similar results were observed by Pereira et al. (2014) who indicated that after the addition of 300 mg/L phenol, the Anammox bacteria remained in the reactor but the ammonium removal efficiency was reduced to 47%.

The results revealed also that *Planctomycetes* was the most abundant phylum, before humic acid addition, with a relative abundance of 48% (Fig. 4a). Indeed, it is well known that the Anammox process is mediated by the *Planctomycetes* phylum: a monophyletic group of obligatory anaerobic chemoautotrophic bacteria (Strous et al. 1999). According to this observation, the stability of Anammox activity in the reactor, before humic acid addition, was confirmed by the high abundance of *Planctomycetes* as dominant group. However, after humic acid addition, the abundance of this group significantly decreased, reaching a relative abundance less than 24% (Fig. 4b), indicating a negative effect of humic acid on this phylum

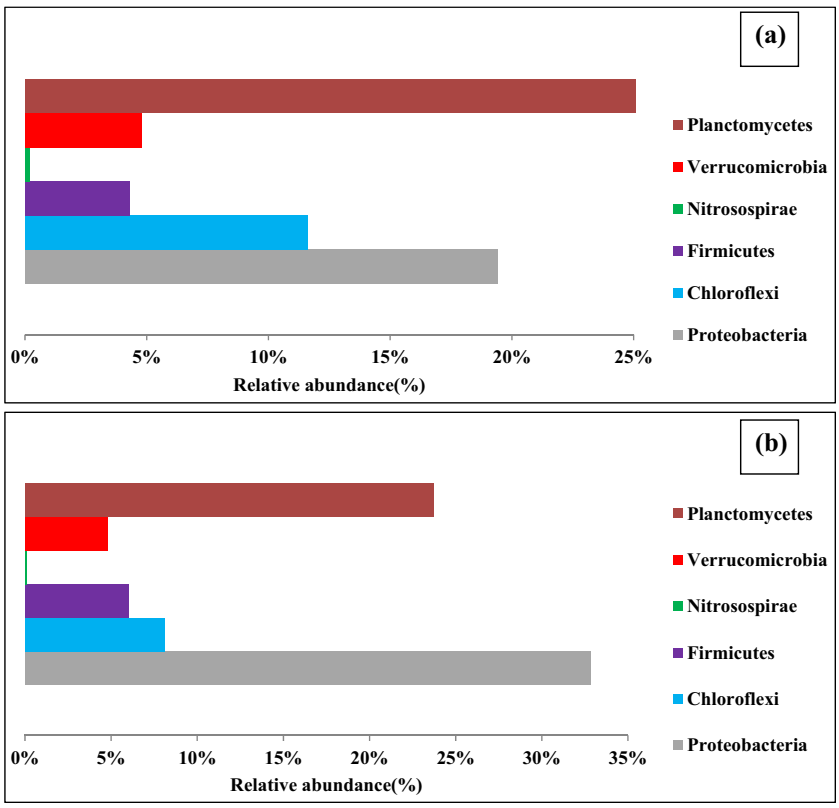

Fig. 4 Relative abundance of bacterial populations at the phylum level **a** before and **b** after humic acid addition

and the whole Anammox process. The chemoautotrophic bacteria abundance reduction confirmed as well the changes of the stoichiometric ratio nitrite consumption and ammonium depletion obtained during continuous assays after humic acid addition.

In order to determine which group of Anammox bacteria was more vulnerable to the presence of humic acid, different populations were analyzed (*Candidatus* Kuenenia stuttgartiensis, *Candidatus Brocadia* anammoxidans, and *Candidatus Brocadia fulgida*) (Figs. 5 and 6). Results indicated that *Candidatus* Kuenenia was the dominant Anammox bacteria in the reactor before and after humic acid addition with relative abundance of 23 and 15%, respectively (Fig. 5), followed by *Candidatus Brocadia* with a relative abundance of 18% before humic acid addition and 10% after humic acid addition (Fig. 5a and b. In contrary, Van der Star et al. (2008) reported that *Candidatus Brocadia* was the dominant Anammox bacteria.

Despite the important reduction of abundances, no significant population shifts of Anammox bacteria were observed after humic acid addition, in terms of dominant populations. *Candidatus Jettenia*, which presented a low abundance of 5% before humic acid addition, was also affected by the presence of high concentration of humic acid and its richness decreased to reach a value lower than 1%. Similar results were reported by Pereira et al. (2014) who indicated that by DGGE, the dominant order of the total *Planctomycetes* was *Brocadiales*, of which *Candidatus Brocadia* dominated. Nevertheless, *Candidatus Jettenia* was detected at very low abundance (0.5% of the total *Brocadiales*) which also indicated that the addition of a high concentration of phenol (300 mg/L) negatively affected this group. The low abundance of *Candidatus Jettenia* among all Anammox bacteria was also reported by Sonthiphand et al. (2014).

According to these results, it is clear that the abundance of Anammox bacteria was strongly affected by the presence of high humic acid concentration without recording any population shift inside the *Candidatus Brocadia* genus. Indeed, in lab-scale bioreactors, population shifts of Anammox bacteria have been frequently reported under various conditions. The growth rate and affinity to a limiting substrate were demonstrated to be among the causes that led to population shifts. However, in the present study, the presence of humic acid at high concentrations did not lead to any Anammox population shift but decrease the abundance of all bacteria of *Candidatus Brocadia* genus.

In fact, there were two dominant species of *Candidatus Brocadia* in the reactor during the period without humic acid, *Candidatus Brocadia* sp. 40 and *Candidatus Brocadia fulgida* with relative abundances of 9 and 4%, respectively. However, in the presence of 200 mg/L of humic acid, *Candidatus Brocadia fulgida* were not detected anymore while the relative abundance of *Candidatus Brocadia* sp. 40 decreased from 9 to 4%. Thus, it can be suggested that the humic acid affects the abundance of most of the Anammox bacteria which decrease in abundance. However, some bacteria like *Candidatus Brocadia fulgida* do not tolerate the presence of humic acid and are totally inhibited. These results suggest that

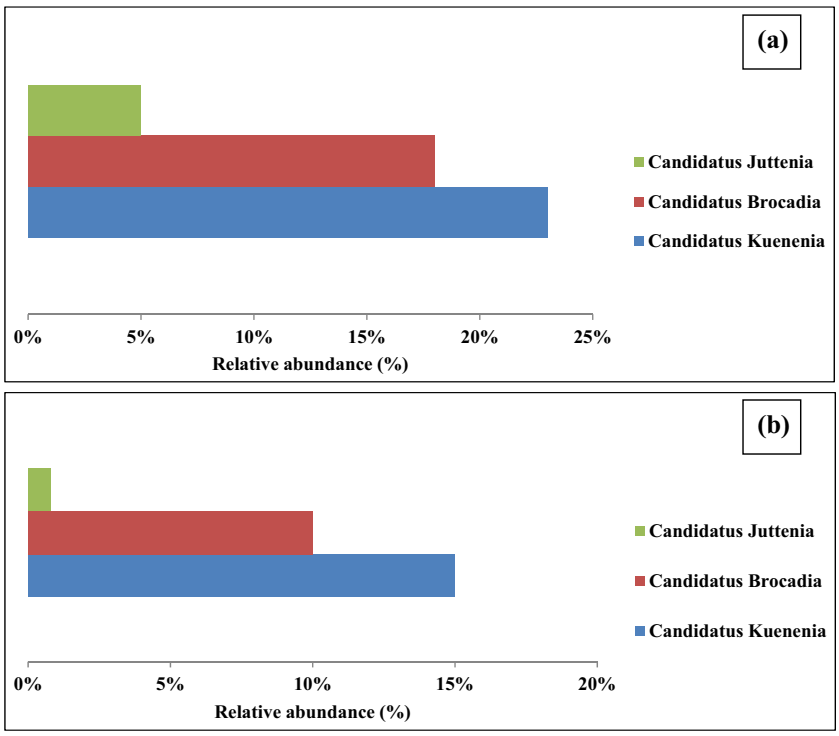

Fig. 5 Relative abundance of the Anammox groups **a** before and **b** after humic acid addition

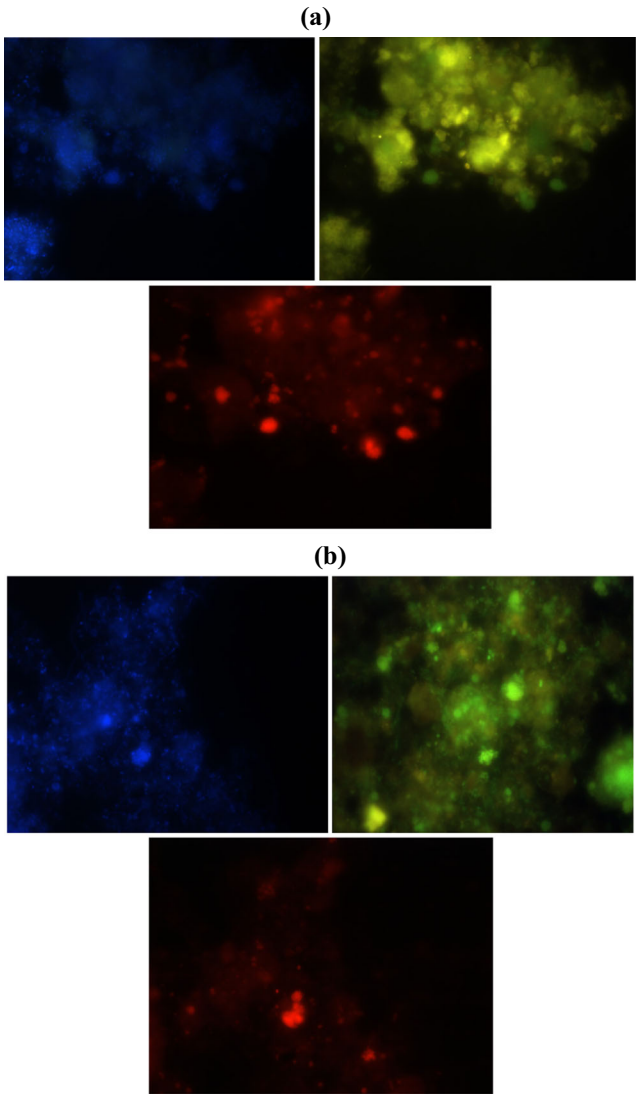

Fig. 6 Detection of Anammox by FISH technique. Top left, DAPI (all DNA (blue)); top right, EUB338mix probe (all bacteria (fluos, green)); bottom, probe Kst157 (*Candidatus* Kuenenia (Cy3, red)) **a** for days 105 and **b** for days 143

Candidatus Brocadia sp. 40 has a higher tolerance for humic acid than *Candidatus Brocadia fulgida*. Similar results were obtained by Pereira et al. (2014), who indicated that Candidatus *Brocadia* sp. 40 has a higher tolerance for phenol than *Candidatus Brocadia fulgida*. These results agree with previous studies, indicating that *Candidatus Brocadia* were the most abundant Anammox species in wastewater treatment plants where organic compounds were present together with ammonium, nitrite, and nitrate (Hu et al. 2010).

In contrast, a population shift was observed with *Proteobacteria* phylum, which became the dominant phylum at the end of the unstable phase (after humic acid addition) instead of *Planctomycetes* which became the second abundant phylum. In fact, the relative abundance of the phylum *Proteobacteria* increased after humic acid addition from 19 to 33% and became the dominant phylum suggesting that heterotrophic denitrification was the favored metabolism in reactor after humic acid addition. The most dominant orders of *Proteobacteria* was *Pseudomonadales* (Fig. 7). Their relative abundance increased from 14 to 21%. The increase of these microorganisms in the presence of high concentration of organic matter has been previously reported by Pereira et al. (2014), who demonstrated that *Rhodocylales* and *Pseudomonales* in the presence of phenol stress increased from 14 to 37%, suggesting that heterotrophic bacteria used light as an energy source and phenol as a carbon source. Moreover, Ullhyan and Ghosh (2012) indicated that *Pseudomonales* was associated with the degradation of aromatic compounds. Accordingly, the increase of *Pseudomonales* abundance in the reactor after humic acid addition could be due to the release of phenolic compounds from humic acid degradation. In fact, the released phenol seemed to be used as carbon by *Pseudomonales* which increased in abundance explaining the fact that the total organic carbon (TOC) in the effluent did not increase even after the addition of high humic acid concentration which were probably consumed by *Pseudomonales*. The degradation of phenol by *Pseudomonales* in anaerobic condition was reported also by Sueoka et al. (2009).

Unlike the groups mentioned above, the proportion of *Xanthomonadales* decreased from 7.6 to 0.3% in the presence of high levels of humic acid, indicating that the presence of humic acid was not favorable to all *Proteobacteria* phylum due to the presence of competition or toxic effect of humic or the compounds resulting from its degradation like phenol. Pereira et al. (2014) reported that the *Xanthomonadales* decreased after phenol stress.

According to these results, we can deduce that the addition of humic acid seemed to affect the abundance of the bacterial community by decreasing the abundance of Anammox bacteria which led to the reduction of ammonium removal by approximately 50% (from 78 to 41%) and increasing heterotrophic denitrification bacteria abundance which use humic acid and compounds resulting from its degradation as carbon source. Because denitrifiers can grow faster using organic matter, as carbon source, a competition phenomenon with Anammox bacteria might be developed for nitrite (Gonzalez-Gil et al. 2014).

The FISH analysis indicated as well that the relative abundance of phylum *Chloroflexi* was affected by humic acid addition which decreased from 13 to 6%. The presence of this group of organisms in Anammox reactor has been indicated by several studies (Cho et al. 2010; Costa et al. 2014). In fact, several studies suggested that this phylum lived in symbiosis with Anammox bacteria (Zhang et al. 2012) by using cellular compounds, polysaccharides, and proteins from dead cells and metabolites produced by Anammox bacteria (Cao et al. 2016; Kindaichi et al. 2012). Thus, the reduction of

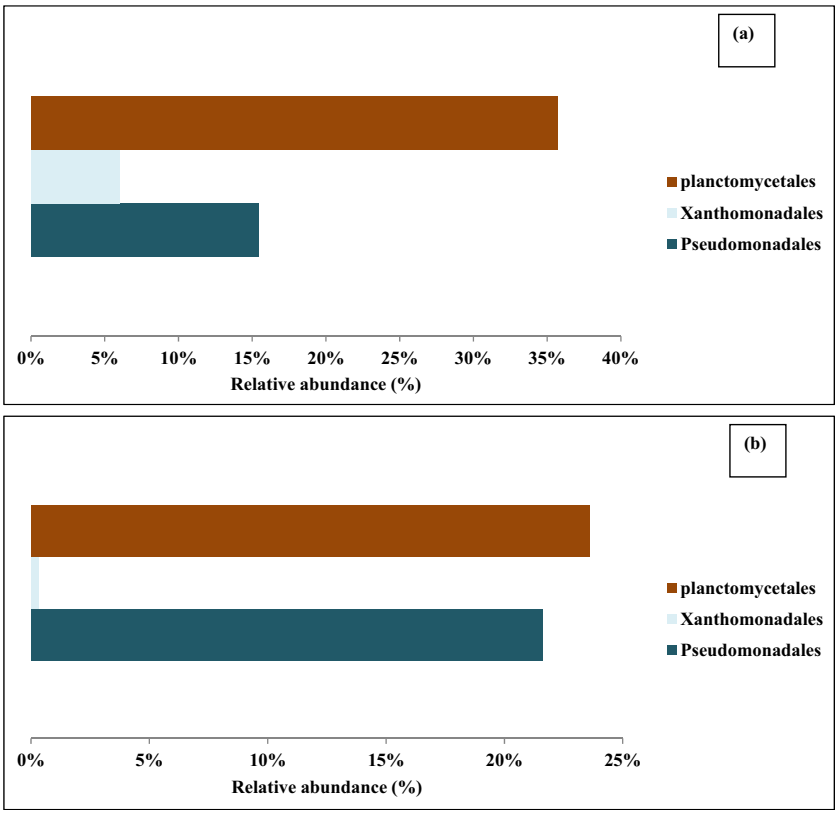

Fig. 7 Relative abundance at the order **a** before and **b** after humic acid addition

Chloroflexi abundance could be explained by the drop of Anammox bacteria abundance. As a consequence, we can conclude that the presence of high humic acid concentration did affect not only the abundance of Anammox bacteria but also the bacteria living in symbiosis with them.

For the other phylum, there were no relevant changes in their abundances. In fact, *Firmicutes* and *Verrucomicrobia* were detected in the two samples but at low abundance (4–5%), as well as *Nitrospirae* but with very low abundance (lower than 1%). This type of bacteria is characterized by being strict aerobic (Buchanan and Gibbons 1974). The presence of these bacteria could explain the higher stoichiometric coefficient for the production of NO_3^-/NH_4^+ consumption found in the present study compared with the literature stoichiometric coefficient. Zekker et al. (2014) reported that *Nitrospira* may survive in anaerobic environment and were responsible of the accumulation of nitrate in the effluent. This could explain as well the high nitrate concentration recorded in the present study in comparison to the theoretical concentration expected. Several studies reported the presence of *Nitrospirae* in Anammox reactors suggesting their benefic effect on Anammox bacteria since they are able to eliminate dissolved oxygen and consume the excess nitrite which avoided the inhibition of the Anammox process (Egli et al. 2003; Costa et al. 2014).

According to these microbial results, it is very crucial to control the concentration of humic acid in the Anammox reactor to avoid a shift of metabolism and an inhibition of the Anammox bacteria which may lead to the deterioration and even the failure of the Anammox process.

The results of previous studies and the results of this study demonstrate that humic acid negatively affects the nitrogen removal performance of Anammox reactors, as proved by the reduction in the nitrogen removal efficiency and by the change in the stoichiometric ratio of nitrite consumption to ammonium depletion. However, as demonstrated in the present study, the presence of humic acid did not eliminate the Anammox bacteria. Instead, the proportion of Anammox bacteria decreased and the abundance of denitrifying bacteria increased.

Conclusion

To sum up, this study attracts attention for the first time to the humic acid effects on the Anammox process which could be inhibitory over a threshold concentration of 100 mg L^{-1} and reduced dramatically the nitrogen removal by 50%, as evidenced by the change of the stoichiometric ratio of nitrite consumption to ammonium depletion. This performance deterioration is mainly due to the decrease of the Anammox bacteria abundance and the microbial order living in symbiosis with them, due to a phenomenon of "out-competition" with the heterotrophic bacteria which grew faster in the presence of

humic acid and became the dominant bacteria in the reactor. Since the humic acid biodegradation could lead to the release of phenolics and heavy metals, it is interesting to investigate the concentration of these compounds during the Anammox process to decipher the inhibition mechanism occurred in the presence of humic acid.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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