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pH ROLE IN PARTIAL NITRITATION OF ANAEROBIC DIGESTER REJECT WATER. REACTION RATES, INHIBITION, AND BIOMASS PROPERTIES

The nitritation process (ammonia oxidation to nitrite) is successfully applied in different processes used for reject water treatment. The impact of pH was investigated in experiments performed at pH 6.0, 6.5, and 7.0 using real dewatering liquor. The overall process performance in terms of the effluent quality and stability was compared. Efficient nitratation suppression was achieved at pH 6.5 and below due to the strong free nitrous acid (FNA) inhibition of nitrite oxidizers. The nxr gene activity showed the highest NOB suppression at pH 6.5. The process rate depended on the pH and nitrogen loading, and was in the range of 13.2–16.5, 8.7–23.3, and 34.0–37.9 g N/(kg·VSS·h), at pH 6.0, 6.5, and 7.0, respectively. The highest inhibition constant was estimated to be 0.905 g HNO_2-N/m^3 at pH 6.0. Decrease in pH also improved the AOB tolerance to free ammonia up to K_{i,NH_3} = 97.2 mgNH_3-N/dm^3. The affinity constants were 0.40, 0.34, and 0.12 mg NH_3-N/dm^3 at pH 6.0, 6.5, and 7.0, respectively. Microbial analysis revealed that the flocculation could be attributed to the *Accumulibacter* and *Competibacter* extracellular polymeric substance (EPS) production, which can also serve as a protector agent for nitrifiers.

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1. INTRODUCTION

Different process configurations (i.e., Anammox, shortcut nitrification-denitrification) used for ammonia-rich wastewater treatment require a stable nitritation process [1]. As nitrate formation is unwanted there, appropriate selective factors for nitrite-oxidizing bacteria (NOB) suppression must be applied. Temperature, solid retention time (SRT), dissolved oxygen level, aeration approach, free ammonia (FA), and free nitrous acid (FNA) inhibition are all often presented as the most effective ways to achieve this goal [2, 3]. As pH plays a significant role in the nitrification process, this parameter was extensively investigated, suggesting the optimal pH range for ammonia oxidation in the pH range 6.5–8.5. Recently, robust acidic nitrification was reported even at pH <5.0 [4, 5].

pH and ammonium/nitrite concentrations are key factors influencing FA/FNA formation [6]. Activated sludge treating nitrogen-rich wastewater is particularly sensitive to pH fluctuations. Elevated pH enhances ammonia formation, reducing substrate limitation but potentially causing toxicity, while FNA toxicity increases under acidic conditions. Since nitrifying consortia can adapt to varying conditions, biomass properties are often examined regarding FA/FNA affinity or inhibition [7]. These factors directly affect process rates; thus, understanding their interrelations under different pH conditions is essential for process optimization.

In sidestream processes, biomass flocculation can often be a problem [8]. Many factors can affect the morphology of sludge flocs, i.e., dissolved oxygen concentration, pH, SRT, or extracellular polymeric substance (EPS) production [9]. Process conditions should therefore always be accounted for when designing or optimizing the sidestream process, since an insufficient level of biomass flocculation could pose a threat to process stability.

The discussed aspects emphasize the strong influence of pH on nitritation stability and efficiency. Understanding the combined effects of pH on FA/FNA formation, NOB inhibition, AOB substrate availability, and biomass dynamics is crucial for optimizing sidestream nitritation. This study examined pH values between 6.0–7.0 in a sequencing batch reactor (SBR) treating ammonium-rich reject water. The experiments compared the impact of pH on nitritation rate, NOB suppression stability, and biomass response to increased loading. AOB characteristics were evaluated through respirometric tests, while microscopic and next-generation sequencing (NGS) analyses assessed floc structure and microbial taxonomy. AOB and NOB activities were further quantified using quantitative polymerase chain reaction (qPCR) to determine the pH influence on these populations.

2. MATERIALS AND METHODS

Inoculum and medium characteristics. Conventional activated sludge (CAS) samples were collected from a large municipal wastewater treatment plant (WWTP). CAS

samples were separately examined in terms of the AOB kinetic parameters, microbial community structure, and floc characteristics. Reactors were fed with real reject water from the same WWTP. The composition of the reject water was (average±SD): ammonium 840.6±100.2 g NH_4^+-N/m^3, total COD 502.4±142.5 g O_2/m^3, soluble COD 375.8±56.3 g O_2/m^3, alkalinity 3740±405 g $CaCO_3$/m^3 and total suspended solids 92.3±42.6 g/m^3.

Experimental setup and operation. A 150 dm^3 SBR fed with reject water was used for all experiments. The reactor equipment allowed control of the operating conditions in terms of pH, temperature, dissolved oxygen (DO) level, and mixing intensity. All experiments were conducted at 25 °C. DO in all experiments was set at 1 g O_2/m^3. Each SBR operation cycle (240 min) consisted of: aeration (180 min, including five feeding phases, 5 min each), sedimentation (45 min), decantation, and an idle phase (15 min in total).

Experiment description. The nitritation of reject water was carried out in three SBRs operated at pH 7.0 (Start-up 1), (Start-up 2), and 6.0 (Start-up 3). Operation was divided into three phases: sStart-up, stable operation, and increased load. Additional analyses, including microscopy and kinetic tests, were conducted at selected stages. The start-up phase goal was to achieve a high nitrite accumulation rate (NAR > 90%) under a moderate nitrogen loading rate (NLR) ~0.2 kg NH_4^+–N/(m^3·d). Low SRT was maintained through excess sludge removal to promote NOB washout. In the next phase, operational conditions were stabilized for biomass adaptation, maintaining the same NLR. Batch tests were used to estimate kinetic parameters, and microbial and microscopic analyses were performed. In the final stage, increased load, NLR was raised by at least 30% by reducing the hydraulic retention time (HRT) while keeping other parameters constant to assess process resilience.

Analytical methods. The compositions of influent and effluent were regularly measured using Hach's (Germany) with a DR3900 spectrophotometer using photometric LCK cuvette tests. Alkalinity was monitored according to PN-EN ISO 9963-1:2001. The concentrations of the total and volatile suspended solids (TSS and VSS) were measured in accordance with PN-EN ISO 872:2007.

Batch tests. Nitritation performance was measured by a series of ammonium uptake rate (AUR) measurements at all the stages of the experiments. During the regular operation of the SBRs, the measurements were performed by collecting mixed liquor samples at set time intervals. All AUR results were standardized regarding VSS concentration and temperature ($\Theta = 1.072$).

Respirometric tests were performed using a BM-T Advance respirometer (Surcis, Spain) to evaluate the inhibitory effect of FNA ($K_{i,FNA}$, according to Monod's kinetics) and ammonia affinity/inhibition ($K_{s,NH3}$, $K_{i,NH3}$ according to Haldane's kinetics) of the

cultivated AOBs. The model K_i/K_s parameters were estimated using the least square estimation method [10].

Microscopic analysis. Samples were collected directly from the reactors and immediately processed using an optical microscope (Olympus CX33) and a cellSens Imaging Software for the presence of filamentous microorganisms [11]. Image analysis was carried out using a Digital Image Analysis in Microbial Ecology (DAIME). Approximately 50 images of the raw sludge sample were analysed [11].

The activated sludge samples were collected and placed into sterile polypropylene probes and frozen at –20 °C until genetic material isolation. DNA isolation was performed with GeneMATRIX Environmental DNA and RNA Purification Kit (Eurx, Poland) according to the manufacturer's protocol. The obtained DNA underwent high-throughput sequencing based on the 16S rRNA coding gene with an Illumina system [12].

The activity of ammonia and nitrite oxidizers in the activated sludge of the experimental SBRs was analyzed with qPCR on functional gene ammonia monooxygenase (amoA) and nitrite oxidoreductase (nxr) for AOB and NOB (including comammox), respectively [13]. The quantity and purity of the isolated RNA were measured with a Qubit 4® Fluorometer (Invitrogen, USA). The qPCR of the functional genes (amoA and nxr) was performed with QuantStudio™5, Real-Time PCR System, 96-well, 0.2 cm^3 (TermoFisher Scientific, USA) with a PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA), according to the manufacturer's instructions as previously described [14]. The results were presented as a ratio to a reference gene (16S rRNA coding gene) as described by Livak and Schmittgen [15].

3. RESULTS AND DISCUSSION

The concentrations of nitrogen forms obtained in effluent are presented in Fig. 1, and the operating parameters for each start-up are presented in Table 1.

3.1. START-UP

During the initial period, NO_2-N accumulation was observed, indicating constant NOB washout (Fig. 1). The end of this phase was considered when NAR exceeded 90% (at NLR > 0.2 kg NH_4^+-N/(m^3·d)). Factors inducing the NOB inhibition were short SRT (11–16 days), elevated temperature (25 °C), and low DO (1.0–1.4 g O_2/m^3). The levels of FA (0.03–1.18 g NH_3-N/m^3) and FNA (0.05–0.30 g HNO_2-N/m^3), both known as important NOB inhibitors [16], varied depending on pH, as well as ammonium and nitrite concentrations.

The duration of this phase varied depending on the maintained pH and reached 31 (pH 7.0), 34 (pH 6.5), and 26 days (pH 6.0). The start-up time was relatively short,

especially at the lowest tested pH. Other reports [17] showed a rapid nitritation start-up of mature landfill leachate in a pilot-scale reactor within 35 days at pH 7.3–7.8.

Table 1

Operating parameters

pH	DO [g/m^3]	T [°C]	SRT [day]	Concentration		
				NLR [kg N/(m^3·d)]	FNA [g N/m^3]	FA [g N/m^3]
Start-up						
7.0	0.92±0.04	25.2±0.4	16.0±10.8	0.28±0.07	0.05±0.05	0.03±0.04
6.5	1.26±0.93	25.0±0.1	12.4±4.0	0.21±0.09	0.13±0.18	1.18±2.41
6.0	1.41±0.56	25.0±0.2	10.8±3.2	0.23±0.02	0.29±0.38	0.21±0.08
Stable operation						
7.0	1.03±1.06	26.3±1.1	34.6±6.7	0.33±0.04	0.12±0.02	0.004±0.005
6.5	0.98±0.26	25.0±0.0	62.4±24.9	0.19±0.03	0.50±0.06	0.19±0.07
6.0	1.24±0.53	25.0±0.0	20.2±4.6	0.23±0.03	0.91±0.17	0.35±0.12
Increased load						
7.0	1.08±2.64	27.4±2.1	28.4±9.9	0.43±0.05	0.03±0.03	0.036±0.07
6.5	0.93±0.02	25.0±0.0	17.3±4.8	0.33±0.06	0.23±0.12	1.35±1.31
6.0	0.98±0.01	25.0±0.0	13.5±2.2	0.31±0.08	0.89±0.06	0.36±0.07

3.2. STABLE OPERATION

The stable phase lasted 29–43 days, showing clear differences in nitrogen species among reactors (Fig. 1). Efficient NOB inhibition was maintained at pH 6.0–6.5, while at pH 7.0 nitrates accumulated, indicating NOB recovery. At pH 6.5, nitrate declined, and nitrite accumulation was high, with stable ammonium concentration (~100 g NH_4^+-N/m^3). Although operational parameters such as DO, temperature, and SRT were comparable, the FNA and FA levels differed substantially – 2.8 and 46 times higher at pH 6.5 than at pH 7.0, respectively – suggesting FNA-driven NOB inhibition. The highest FNA was at pH 6.0, where nitrates remained below 10 g NO_3^-–N/m^3 and ammonium accumulation reached 500 g NH_4^+–N/m^3.

3.3. INCREASED NLR

During increased NLR, the process stability was tested. The NLR was increased by ~0.1 kg NH_4^+–N/m^3 d (Table 1). The complete inhibition of NOB was maintained (at pH 6.0), while ammonia concentration in the effluent slightly increased during this phase (see Fig. 1c). Despite the increased load, the process was stable and had the potential for recovery to efficiency comparable to that observed during the previous phase. At pH 6.5, nitrite accumulation was still over 90%; however, ammonia oxidation was less ef-

ficient (Fig. 1b). At pH 7.0, despite increased loading (resulting in higher FA concentration), NOB suppression was not maintained – in the final weeks of this phase complete nitrification occurred (see Fig. 1a).

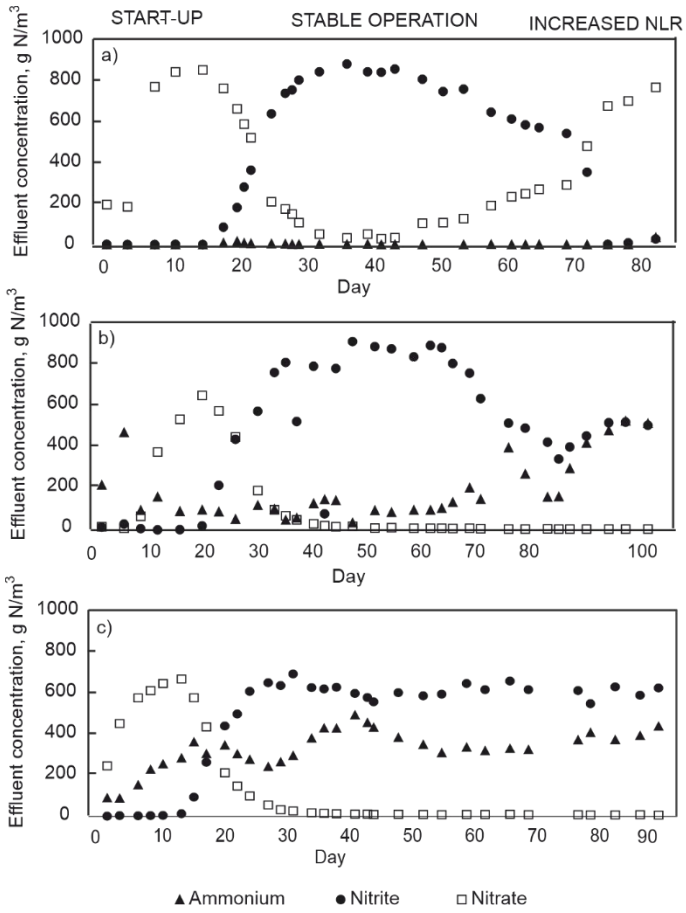

Fig. 1. Nitrogen forms in the effluent during:
a) start-up 1 (pH 7.0), b) start-up 2 (pH 6.5), c) start-up 3 (pH 6.0)

Nitrite accumulation rate (NAR). The accumulation of nitrite occurred in all experiments pH lower than neutral was required to maintain stable NOB suppression. This effect seems to be related to the concentration of FNA. A higher pH value in the reactor resulted in a lower FNA concentration, and consequently, the decreased selection pressure on NOB, enabling their restoration and reinduced nitrate production. Based on these observations, the pH range suitable for stable nitritation of dewatering liquor was not higher than 6.5. Reducing pH to 6.0 and the consequent rise of FNA concentration

resulted in a noticeably faster accumulation of nitrite. Further reduction of pH is unwarranted, as the activity of AOB in wastewater treatment usually decreases and completely disappears at pH below 6.0 [5]. However, there are reports of effective nitrification at a much lower pH [4]. Examples of strains successively conducting a nitritation process at pH below 6.0 are *Ca. Nitrosogobus* [19] and *Nitrosococcus*. *Nitrosoglobus* appeared in the activated sludge of the start-up at pH 6.0 at the end of the experiment, and these results suggest that it plays a role in effective nitrification (Fig. 2).

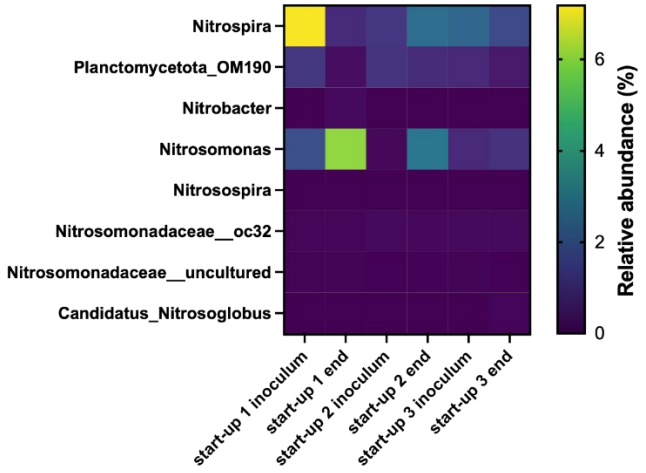

Fig. 2. Relative abundance of the dominant genera of bacteria typically recognized as ammonia (including annamox) and nitrite oxidizers in activated sludge, present in the inoculum and the final sample for: start-up at pH = 7.0 (start-up 1), pH = 6.5 (start-up 2)

Ammonium oxidation rate (AOR). The lowest AOR observed at pH 6.0 resulted from limited alkalinity addition. Although raw reject water initially had a high pH (7.5–7.8), pH decreased during nitritation and was maintained at 6.0 with minimal bicarbonate dosing. Since alkalinity buffers H^+ ions, its deficiency restricted complete ammonium oxidation, allowing only 64% of the influent load to be theoretically oxidized; however, most of the available ammonium was removed without external alkalinity (59%). At pH 6.5, higher alkalinity dosing improved AOR to 80–90%, confirming its key role in AOB activity, while at pH 7.0, it enabled complete ammonium removal. Despite low alkalinity, AOR stability at pH 6.0 indicated robust process performance. It suggests that the optimal selection of pH for the nitritation process should be considered in terms of two contrary aspects: 1) more acidic to maintain high FNA concentration and stable suppression of NOB, and 2) relatively high to provide a sufficient process rate.

Ammonium uptake rate (AUR). The process dynamics during the stable operation period varied significantly. The highest AUR values were achieved at pH 7.0

(37.9±10.7 g N/(kg VSS·h)), while AUR rates were 8.7±2.2 and 13.2±2.3 g N/(kg VSS·h) at pH 6.5 and 6.0, respectively.

Compared to the results from other studies, these values were relatively high, suggesting a high enrichment of cultivated sludge with nitrifiers. In other reports, during synthetic wastewater treatment in the acidic environment, this value could reach 7.1 at pH 3.8 and 15.9 at pH 4.4 for suspended and attached biomasses, respectively [18]. Municipal wastewater treatment achieved a nitritation rate of 6.7 g N/(kg VSS·h) at pH as low as 4.5 in a membrane bioreactor [19]. In SBR treating reject water, the AUR values can be found from 7.6 to 33.2 g N/(kgVSS·h) at an optimal pH range 6.5–8.5 [20–22]. Also, the effect of using real wastewater instead of a synthetic medium can affect the AUR values. Torà et al. [2] observed a very high AUR level of 75.9–76.5 g N/(kg VSS·h) treating synthetic or mixed influents, while a maximal rate of only 58.9 g N/(kg VSS·h) was reached when the reactor switched to real reject water in the same operating conditions (pH = 7.5). The AUR values achieved in this study were presented against the background of literature reports in Fig. 3.

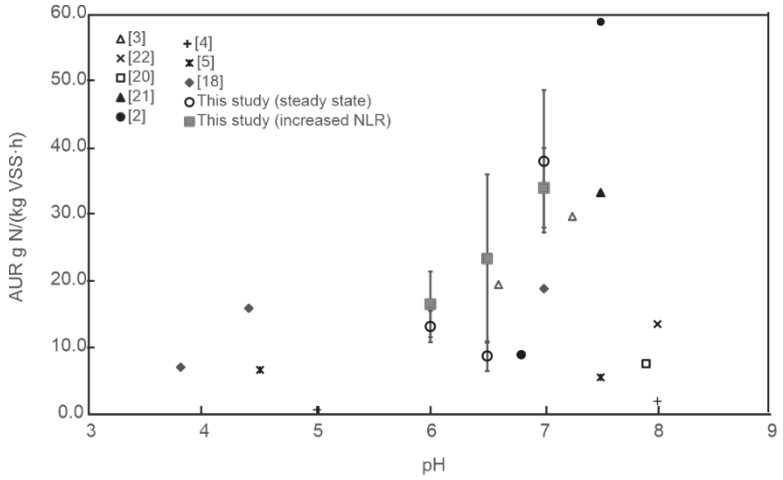

Fig. 3. Nitritation process rates at different pH taken from this study and other literature reports; all values standardized to 25 °C (θ = 1.072)

3.4. AOB ADAPTATION

Ammonia: affinity and inhibition. At the lowest pH tested (start-up 3), the additional alkalinity was negligible, resulting in continuous NH_4^+-N accumulation during normal operation in a steady state (av. 62±39 g NH_4^+-N/m^3). Despite that, the FA level was, on average, 0.35±0.12 g NH_3-N/m^3. At pH 6.5, the NH_4-N accumulation was much lower (91±22 g NH_4^+-N/m^3 on average), which resulted in low FA: 0.07±0.05 g NH_3-N/m^3. As the operation at pH 7.0 required the alkalinity addition, the substrate level was almost

completely depleted, resulting in very low FA (0.004±0.003 g NH_3-N/m^3). Affinity measurements revealed that the estimated $K_{s,NH3}$ values were 0.40, 0.34 and 0.12 g NH_3-N/m^3 at pH 6.0, 6.5, and 7.0, respectively (Fig. 4a). The CAS used as an inoculum was characterised by high substrate affinity ($K_{s,NH3}$ = 0.016±0.005 g NH_3-N/m^3). Compared to other studies, the observed values were typical of sidestream AOB (0.24–0.88 g NH_3-N/m^3) rather than for CAS (~0.0075 g NH_3-N/m^3) [23]. The obtained $K_{s,NH3}$ values had a clear dependence on the FA level in the SBR. These results suggested the domination of nitrifier species classified as r-strategists in terms of substrate affinity [24].

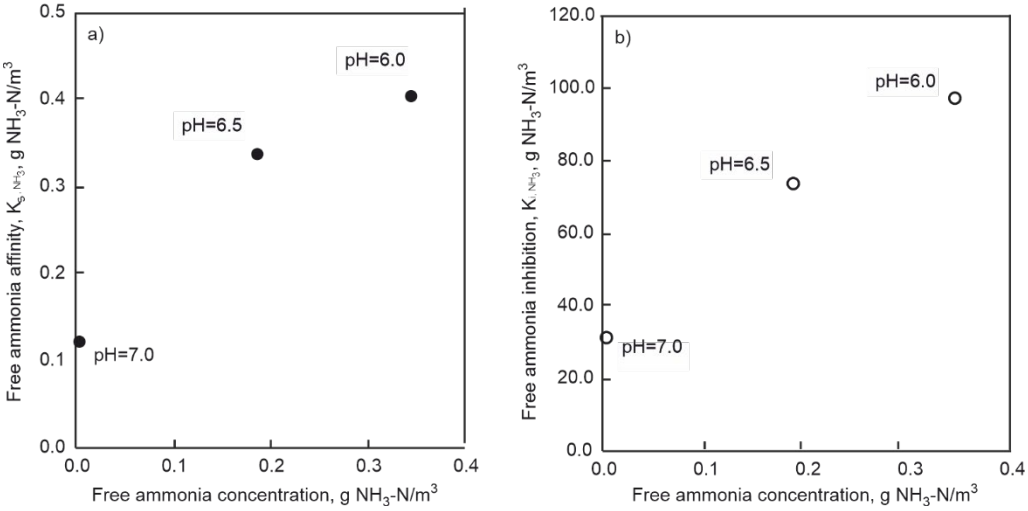

Fig. 4. Ammonia affinity ($K_{s,NH3}$) (a), and inhibition ($K_{i,NH3}$) (b) of cultivated AOB biomass and the level of NH_3-N in the reactor

In terms of substrate inhibition, the observed biomass characteristics were different than those in the case of the affinity constant. The $K_{i,NH3}$ values estimated for biomass cultivated at pH 6.0 and 6.5 revealed similar vulnerability to FA toxicity: 97.2 and 73.7 g NH_3-N/m^3, respectively (Fig. 4b). The lowest $K_{i,NH3}$ was at pH 7.0 (31.3 g NH_3-N/m^3). Higher FA concentration in the reactor was the trigger to develop more ammonia-tolerant AOBs, which was confirmed in other studies [25], also visible in the community structure in Fig. 2.

FNA tolerance. Cultivated biomass was exposed to a wide range of FNA concentrations, from 0.124 to 0.905 g HNO_2-N/m^3, at pH 7.0 and 6.0, respectively. Despite a higher nitrite concentration in the reactor at a higher AOR level (start-up 1), the pH level was the dominant factor in HNO_2 formation. Respirometric test results showed that as pH increases, fewer FNA-tolerant nitrifiers will be present in the system (lower $K_{i,FNA}$, Fig. 5). The AOBs cultivated in this experiment can be considered as typical for well-known nitrifiers with $K_{i,FNA}$ within the range of 0.1–0.5 g HNO_2-N/m^3 [23, 26] and

significantly lower than recently described *Candidatus Nitrosoglobus* grown in a more acidic environment (pH < 5.0) [5].

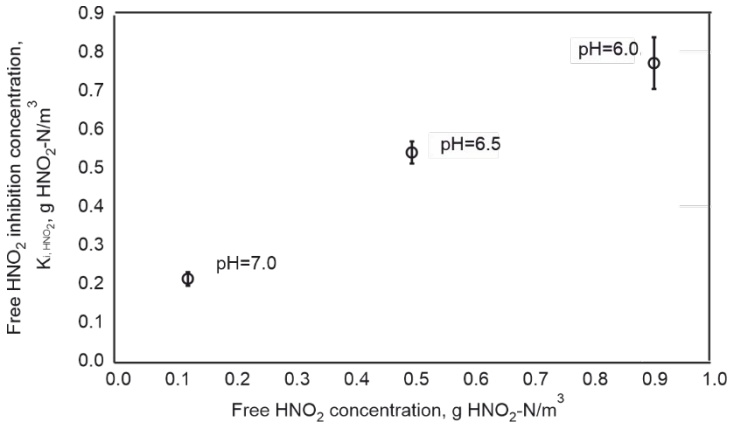

Fig. 5. FNA inhibition coefficient ($K_{i, FNA}$)
of cultivated AOB biomass and the level of HNO_2-N in the reactor

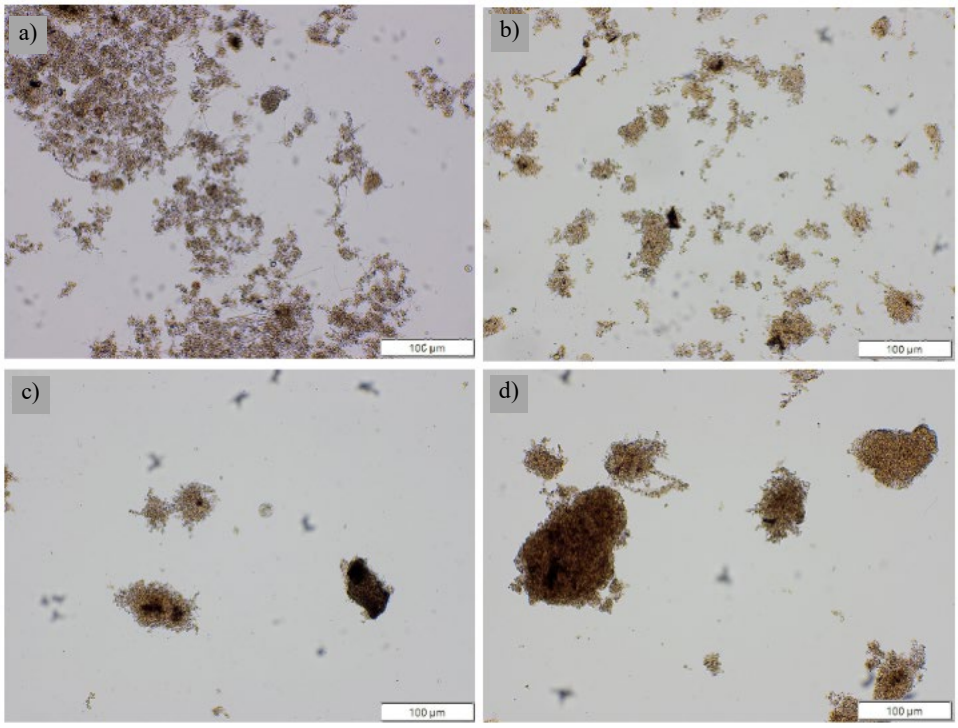

Fig. 6. CAS from WWTP used as inoculum (a) , start-up 3 (Ph 6.0) (b),
start-up 2 (pH 6.5) (c), start-up 1 (pH = 7.0)

This result is also supported by metataxonomic data in which *Nitrosoglobus* representatives were absent in all but one (the end of the experiment with pH 6.0) sample of the activated sludge in this experiment (Fig. 6).

3.5. BIOMASS AND MICROBIAL COMMUNITY CHARACTERISTICS

The analysis revealed significant differences between the sludge samples. A high number of filamentous bacteria (filament index *FI* = 3.5) was found in the CAS (inoculum), while their amount gradually decreased and ultimately disappeared altogether reaching 0 in all experiments. These results are supported with data obtained with next generation sequencing at the genus level (Fig. 7a).

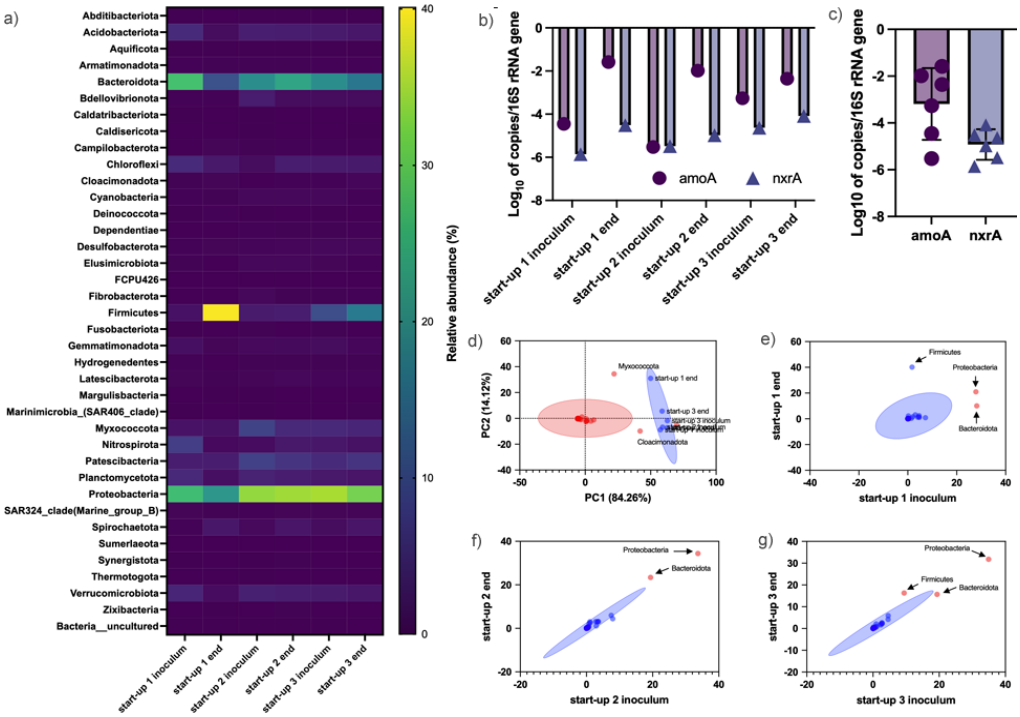

Fig. 7. Relative abundance of the dominant bacterial phyla in activated sludge in the inoculum and the final sample for: start-up at pH = 7.0 (start-up 1), pH = 6.5 (start-up 2), and pH = 6.0 (start-up 3) (a); log amoA and nxrA gene copies relative to 16S rRNA (b); grouped log amoA and nxrA gene copies relative to 16S rRNA across the experiments (c); PCA analysis showing clustering between bacterial phyla and experimental time points (d); K-means clustering between inoculum and end points in experiments conducted at pH 7.0 (e), pH 6.5 (f), and pH 6.0 (g)

The inoculum contained a relatively high abundance of Zooglea (1.224%), while in the experiments, this abundance was lower: 0.043 and 0.151% for start-up 2 and 3, respectively. Regardless of the initial presence of these bacteria, their relative abundance

decreased and ultimately reached 0.17, 0.038, and 0.091%, respectively, for samples from start-ups 1, 2, and 3.

In addition, significant changes in the sludge morphology have been observed. While the inoculum contained a loose structure and irregularly shaped flocs (mostly >100 μm in size), samples from the end of the respective experiments (Fig. 6) differed significantly. pH 6.0 resulted in the morphology resembling that of the inoculum, showing a predominance of the small flocs (97% fell within the 10–100 μm range) with a compact structure and an irregular shape. The start-up at pH 6.5 showed an increase in floc size (80% of flocs within 10–100 μm range) and a more compact structure and regular shape. During the start-up at pH 7.0, flocs were the biggest (only 72% flocs within the 10–100 μm range) and their structure was much more compact and regularly shaped.

While temperature and DO concentration can affect activated sludge morphology [9], a lack of differences between start-ups regarding those parameters implies that these differences could be pH-related, especially in terms of FNA and FA formation. Higher concentrations of these compounds can distort the structure of sludge flocs by interfering with EPS synthesis, leading to the formation of smaller and more dispersed flocs [27, 28].

On the other hand, very low concentrations of FNA can be beneficial, as nitrifying bacteria can produce more EPS as a defensive response [27], while high concentrations at the pH of 6.0 led to weaker and more dispersed flocs. The presence of Protozoa and Metazoa, found in the samples from all start-ups, may have also affected the structure of the activated sludge, as those organisms remove free-swimming bacteria and prevent excessive growth of activated sludge flocs [11].

The 16S rRNA coding gene revealed that, at the phylum level in all 3 start-ups, Proteobacteria was the most abundant group. However, its relative abundance slightly differed: for start-up 1, it dropped from 27.83 to 20.94%. For start-up 2 at pH 6.5, the abundance of Proteobacteria increased slightly from 33.68 to 34.37%, while for start-up 3 at pH 6.0, it decreased from 34.91 to 31.78%. The second most abundant phylum in all activated sludge samples was Bacterioidota, even though its relative abundance decreased in all cases. For start-up 1, it went from 28.18% to 10.2%, while for start-up 2 and 3, it decreased from a comparable level of 19.36% to 23.36 and 15.66%, respectively.

The relative abundance of Nitrospirota, the third most abundant phylum in activated sludge inoculum for start-up at pH 7.0 (7.181%), decreased in this experiment to 0.83%. For the start-ups at pH of 6.5 and 6.0, the inoculum was less rich, at a level of 1.128 and 2.349%, respectively. The abundance of these bacteria also decreased to 1.606% for pH 6.0, but interestingly, in the case of the experiment at pH 6.5, it increased to 2.562%.

The other groups with a relatively high abundance in inoculating sludge were: Acidobacteriota, Chloroflexi, Patescibacteria, Planctomycetota and Firmicutes. The last group's relative abundance was low in the inoculum for the start-up at pH 7.0 – only

1.726% – but it increased to 40.1%. In other start-ups, this increase was not as pronounced: from 2.792 to 3.096% and 9.394 to 16.28%, respectively, for start-ups at pH 6.5 and 6.0. These results show that pH 7.0 seems to be the most suitable for the development of Firmicutes. It should also be noted that representatives of this group can sporulate under unfavourable conditions, which means that these bacteria could still have been present in large quantities in the other two experiments. However, due to the unfavourable environmental conditions, the less vegetative forms were present in the activated sludge samples.

The relationship between the dominant bacterial types was analyzed using *K*-means clustering across different pH conditions at the beginning and end of the experiment. In start-up 1 (Fig. 7e), Proteobacteria and Bacterioidota form a distinct cluster separate from the other bacterial types. However, despite the considerable distance of Firmicutes from the blue-marked cluster, it is still included within it. This may suggest a dependency of Firmicutes on the rest of the bacterial community at pH 7. During start-up 2, Firmicutes is grouped with the other bacterial types (Fig. 7f), and only at pH 6.0 (Fig. 7g) it forms a separate cluster along with Proteobacteria and Bacterioidota. The Firmicutes phylum demonstrates increased activity under acidic conditions (pH 5.0) [29], which may indicate its potential to dominate at the lowest pH tested. Moreover, selected species within the Firmicutes phylum are known to exhibit high activity across a wide pH range (5.0–7.0) [30], which could explain the significant distance from the blue cluster observed at pH 7.0 (Fig. 7e). On the other hand, the PCA analysis (Fig. 7d) did not reveal a significant relationship between the three dominant types (Proteobacteria, Bacterioidota, and Firmicutes) and the pH conditions or the time points of the experiments.

There was a relatively high number of unculturable nitrifiers in the activated sludge for all start-ups. However, all the analysed samples were rich in several genera known to be AOB and NOB representatives. Figure 2 shows the changeability of the genera typically recognised as ammonia and nitrite oxidizers in the activated sludge. For the start-up at pH 7.0, the dominant genus was *Nitrosomonas*, which was present in the inoculum with a relative abundance of 1.723%, which increased to 7.99%. For the other two experiments, the tendency was similar. At pH 6.5, the relative abundance of this bacterium increased from 0.089 to 2.816%, while for start-up at pH 6.0, from 0.8 to 1.19%. *Nitrosospira* was absent in all experimental samples analysed in this experiment. *Nitrosoglobus* was only observed at the end of the experiment at pH 6.0 with its relative abundance of 0.075%. The inoculating activated sludge in the start-up at pH 7.0 was rich in *Nitrospira*, representing NOB. It was 7.181%, and it decreased to 0.83%. In the start-up at pH 6.0, the presence of *Nitrospira* also decreased from 2.349 to 1.606%. Only in the case of the start-up at pH 6.5 did its abundance increase from 1.128 to 2.56%. The presence of *Nitrobacter* was noted only at the end of the experiment at pH 7.0, with a relative abundance of 0.16%. In all three experiments, the relative abundance of Planctomycetota_OM190 decreased, which was most likely caused by the inhibiting influence of oxygen, as the experiment was performed at similar DO. Nonetheless, it cannot

be excluded that this genus could have played some role in ammonia oxidation during these experiments, as it was present during all 3. It has been previously described that anammox bacteria are located deep within the activated sludge flocs and therefore are protected from the inhibitive influence of oxygen in aerobic systems by AOB located on the outside layer of the flocs. Such a location can support the thesis of the AnOB role in ammonia removal also in aerobic conditions [14].

To present the influence of pH change on the AOB and NOB community, the gene activity was analysed with RNA-based qPCR. The analyses were performed on the functional genes amoA and nxr, presenting the activity of the AOB and NOB groups during the experiment, while the results were presented as Log10 of copies of genes related to 16S rRNA (Figs. 7b, c). This means that lower values of Log10 reflect the higher gene activity. In all 3 experiments, the relative activity of ammonia oxidizers was far higher in inoculate than in nitrite oxidizers, seen in Fig. 7c. Also, the activity of the amoA gene increased heavily at the end of all 3 experiments, with the highest increase in pH 7.0 (Fig. 7b). The activity of the nxr gene during start-ups for all 3 tested pHs also increased, with the lowest change at pH 6.0. It could be stated that the tested pH levels for all 3 start-ups have not changed the process environment to a degree sufficient for the permanent inhibition of NOB. However, the lowest tested pH, 6.0, seems to be the most efficient for such inhibition, as the increase of the nxr gene is the lowest.

Nonetheless, both AOB and NOB present in the experimental activated sludge will adapt to the pH changes, influencing FA/FNA concentrations and their inhibiting influence in a longer experiment due to the change in the AOB/NOB representatives, dominant in the community. That building tolerance would also be facilitated by the presence of other microorganisms whose role is to protect other community members against the influence of unfavourable changes in environmental conditions. In the communities in all 3 experiments, microorganisms such as *Accumulibacter* and *Competibacter* were present in relatively high numbers. These bacteria, known as phosphate- and glycogen--accumulating microorganisms, respectively, produce large amounts of EPS. At the lowest tested pH 6.0, the relative abundance of *Competibacter* increased (from 1.02 to 2.12%), while *Accumulibacter* was at a comparable level to that of the inoculum (from 0.88 to 0.72%, Fig. 7a). It could be suspected that such a community shift linked with the environmental change caused the increase of EPS production by these bacteria, which served as protection for the AOB/NOB and the rest of the activated sludge community.

4. CONCLUSIONS

- The nitritation rate varied from 13.2–16.5 to 34.0–37.9 g N/(kgVSS·h), at pH 6.0 and 7.0, respectively.

- At pH 7.0, nitratation was re-established after temporary inhibition, as NOB suppression was insufficient.
- DECREASE IN PH IMPROVED AOB TOLERANCE TO FA UP TO KI,NH3 = 97.2 MG NH3-N/DM3, and affected substrate affinity. K_{s,NH_3} values of 0.40, 0.34, and 0.12 mg NH_3-N/dm^3 were observed at pH 6.0, 6.5 and 7.0, respectively.
- AOB tolerance for FNA was up to 0.905 mg HNO_2-N/dm^3 at pH 6.0.
- The highest FNA concentration caused the formation of the smallest and most dispersed flocs, while neutral pH resulted in the most regular and compact flocs.
- The relative abundance of *Nitrosomonas* increased in all start-ups. *Nitrospira*, the most abundant NOB representative, was highly abundant at the pH 7.0 inoculum. No *Nitrosospira* representatives were observed in all 3 experiments.
- The relative activity of the AOB and NOB functional genes increases at pH 7.0, 6.5, and 7.0, with the highest NOB inhibition at pH 6.5. The relative abundance of *amoA* was far higher than *nxr* in all samples.
- Bacterial genera such as *Accumulibacter* and *Competibacter* may play an important role in EPS production at low pHs, which serves as a protective agent for AOB/NOB community.

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