Intermittent Aeration Suppresses Nitrite-Oxidizing Bacteria in Membrane-Aerated Biofilms

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Abstract

Autotrophic ammonium oxidation in membrane-aerated biofilm reactors (MABRs) can make treatment of ammonium-rich wastewaters more energy-efficient, especially within the context of short-cut ammonium removal. The challenge is to exclusively enrich ammonium-oxidizing bacteria (AOB). To achieve nitritation, strategies to suppress nitrite-oxidizing bacteria (NOB) are needed, which are ideally grounded on an understanding of underlying mechanisms. In this study, a counter-diffusion nitrifying biofilm reactor was operated under intermittent aeration. During eight months operation, AOB dominated, while NOB were suppressed. Based on dissolved oxygen (DO), ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) profiles within the biofilm and in the bulk, a 1-dimensional nitrifying biofilm model was developed and calibrated. The model was utilized to explore the potential mechanisms of NOB suppression associated with intermittent aeration, considering DO limitation, direct pH effects on enzymatic activities, and indirect pH effects on activity via substrate speciation. The model predicted strong periodic shifts in the spatial gradients of DO, pH, free ammonia and free nitrous acid, associated with aerated and non-aerated phases. NOB suppression during intermittent aeration was mostly explained by periodic inhibition caused by free ammonia due to transient periodic pH upshifts. Dissolved oxygen limitation did not govern NOB suppression. Different intermittent aeration strategies were then evaluated for nitritation success in intermittently aerated MABRs: both aeration intermittency and residual free ammonia turned to be effective control parameters.

Introduction

Short-cut ammonium (NH_4^+) removal via nitrite (NO_2^-) is more energy- and cost- efficient than traditional NH_4^+ removal via nitrate (NO_3^-) due to reduced aeration and external electron donor requirements.[1,2,3] This process requires full nitritation (oxidation of all NH_4^+ to NO_2^-) and zero nitratation (oxidation of none of the NO_2^- to NO_3^-); in other words minimal activity of nitrite-oxidizing bacteria (NOB) and maximal activity of ammonium-oxidizing bacteria (AOB). Similar conditions– with only partial nitritation- can also be exploited to convert NH_4^+ to a 50:50 mixture of NO_2^- and NH_4^+, which can then be coupled to anoxic NH_4^+ oxidation to attain even more resource efficient ammonium removal.[4,5]

Various conditions have been successfully tested to suppress NOB over AOB activity or wash-out NOB over AOB biomass to attain nitritation in suspended growth systems. They include the operation of bioreactors at limited dissolved oxygen (DO) concentrations,[6] at high temperature combined with low solids retention times,[1] and at elevated free ammonia (FA) and/or free nitrous acid (FNA) concentrations.[7] In all cases NOB suppression or outcompetition versus AOB is based on differential growth kinetics. Sometimes, the proper choice of system inoculum also accelerates AOB over NOB selection.[8] By contrast, maintaining long-term nitritation in biofilm-based reactors can be more challenging[9] due to long solids retention times in biofilms that interfere with outcompetition based on kinetic principles. Finding operational conditions and confirming mechanisms that suppress NOB in biofilms remains a challenge. On the one hand, the existence of strong spatial chemical gradients (e.g. of DO, pH and nitrogenous species) in nitrifying biofilms[10] makes it difficult to prescribe environmental conditions that favor AOB over NOB in the system. On the other hand, the existence of multiple simultaneous chemical gradients complicates identification of the underlying mechanism(s) that suppresses NOB. For example, pH and DO gradients occur simultaneously in active nitrifying biofilms:[11] it is difficult to unravel to what extent nitritation failure or success is associated with the differential effect of oxygen (AOB and NOB having different oxygen affinities)[12] or the differential effects of pH (AOB and NOB responding differently to pH– as a consequence of the pH-dependent maximum growth rates[13,14] and the pH-dependent speciation of FA and FNA which act as both substrates and inhibitors).

Mathematical models are one way to describe multiple processes that occur simultaneously in time and space in nitrifying biofilms.[15,16] A multi-species nitrifying biofilm model (MSNBM) was explicitly developed to study the competition between AOB and NOB; effects of DO, pH, FA and FNA on growth kinetics were incorporated in a spatially explicit way to evaluate operational conditions for NOB suppression in co-diffusion biofilms.[3,17] Park et al.[17] showed that FA inhibition of NOB was more efficient in nascent biofilms (when residual NH_4^+ was still high), but that DO limitation was the dominant mechanism of NOB suppression in established biofilms. Besides bulk DO and influent NH_4^+ concentration, the model suggested that bulk buffer capacity was another means to manipulate NOB suppression by affecting pH gradients within biofilms.

While AOB/NOB competition in conventional co-diffusion biofilms has been studied in some detail,[3,17,18] there are less studies on AOB/NOB competition in the context of nitritation in counter-diffusion biofilms. Counter–diffusion biofilms develop in membrane-aerated biofilm reactors (MABRs), where air delivery is via the biofilm base.[19] MABRs have been broadly explored for autotrophic N removal.[11,20,21] In counter-diffusion nitrifying MABRs, active bacteria thrive at the base of the biofilm, where they utilize oxygen supplied from the membrane lumen. Growth of bacteria– including NOB- at the biofilm base would limit the chance for outcompetition, once established, due to spatial protection by the overlying biofilm layers. Efficient operation of MABRs to attain long-term nitritation has, to our knowledge, not been documented, with the exception of one, highly-loaded (33 g-N/m^2/day) fully NH_4^+ penetrated MABR where controlling DO concentrations at the membrane-biofilm interface sufficed to maintain nitritation.[22]

Recently, Pellicer-Nàcher et al.[21] observed that fully nitratation MABRs accumulated NO_2^- immediately after switching from continuous to intermittent aeration, even at elevated oxygen loadings. The causal link between nitritation onset and aeration regime change were not explored. Here we report additional experimental evidence of NOB suppression in intermittently aerated MABRs and we develop and calibrate an improved MSNBM incorporating explicit pH calculation. Using the calibrated model, we systematically evaluate potential causes for NOB suppression associated with intermittent aeration. From this analysis, we identify the periodic FA inhibition- caused by transient pH upshifts and decreases at the biofilm base- as the

likely key cause for NOB suppression. A suitable operational window for an effective nitritation control in counter-diffusion systems is finally proposed.

Materials and Methods

2.1 Reactor Operation and Measurement Methods

Reactor Configuration and Operation

The counter-diffusion MABRs consisted of two tubular gas filled PDMS membranes (3100506, Labmarket, Germany), both fixed in parallel to its longer dimension (Figure S1). The system had a liquid volume of 0.83 L (reactors: 31.5×5×3.5 cm) and was inoculated with enriched nitrifying biomass.[21] To start up the system, the reactor was first run in a batch mode with an initial NH_4^+ concentration at 300 mg-N/L and continuous aeration. The onset of NH_4^+ consumption without oxygen accumulation in the bulk suggested biomass attachment around the membranes. Subsequently, the MABR was operated in continuous flow mode under intermittent aeration. Synthetic wastewater was fed continuously with an NH_4^+ concentration at 75 mg-N/L and without external organic carbon. Hydraulic retention time was 12 hours. The intermittent aeration strategy consisted of a 6-hour aeration period (100% air) followed by a 6-hour non-aeration period (100% N_2). The aeration cycles were controlled by a set of solenoid valves and the pressure in the lumen was 35 kPa. The bulk phase was completely mixed by recirculating at 1.5 L/min. DO and pH were measured with electrodes in the recirculation line (CellOX 325 and Sentix 41, WTW, Germany). Bulk pH was not controlled and remained at 7.2±0.2 due to adequate buffer capacity (molar ratio in the influent: HCO_3^-/NH_4^+ = 2.1). Reactor temperature was at 32.5 ±0.7°C, which was above ambient temperature due to the unintentional heat added by the recirculation pump. N concentrations (NH_4^+, NO_2^- and NO_3^-) were measured with colorimetric test kits (Spectroquant 14776, 00683, 09713; Merck, Germany).

Microelectrode Measurements

Commercially available DO microelectrode (OX-10, Unisense, Denmark) and lab-made potentiometric microelectrodes for NH_4^+, NO_2^- and NO_3^-[23] were used for in-situ profiling measurements within the biofilm. Profiling measurements were performed after biofilms reaching steady state. Microelectrodes were

controlled by a motorized micromanipulator to a precision up to 10 μm, and began from the top of the biofilm. During measurements, the influent and recirculation were kept unchanged. For each profile, replicates (n > 3) were made and the average was considered in model fitting. Besides calibration following the protocols, the signal drift of N-species sensors over time was corrected by measuring N concentrations from effluent before and after profiling.

2.2 Model Development

The MSNBM is a one-dimensional model based on Terada et al.,[24] incorporating additional explicit pH calculation (Table S1). It was implemented in AQUASIM V2.1 with two compartments: a completely mixed gas compartment and a biofilm compartment containing biofilm and bulk liquid.[25] In the counter-diffusion regime, a physical diffusion link connects the gas compartment to the base of the biofilm, defined as

$$A \cdot k_{M,i}\left(\frac{1}{H_i} C_{i,air} - C_{i,base}\right) \quad (1)$$

where $C_{i,air}$ and $C_{i,base}$ are concentrations of carbon dioxide (CO_2) or oxygen (O_2) in the gas compartment and at the biofilm base (mg/L), H is the non-dimensional Henry's Law coefficient (1.32 for CO_2, 34.55 for O_2, 33 ℃), $k_{M,i}$ is the silicone membrane gas mass transfer coefficient ($k_{M,O2}$ = 6 m/d, $k_{M,CO2}$ = 0.8 m/d, Table S3). Gas transfer of N_2 and NH_3 are not modeled. Other major modeling assumptions- regarding biofilm structure, diffusion mass transfer and boundary layer thickness are as in Terada et al.[24] Process rate expressions are shown (Table S2). The calibrated nitrification model incorporating pH is available from the corresponding author.

Biological Processes

The MSNBM includes 3 active microbial groups- AOB, NOB, heterotrophs (HB) and inerts accumulated during decay processes. For the two-step nitrification process, FA and FNA are considered as true substrates for growth and inhibition in nitritation and nitratation.[26] The growth rate expressions are described as follows,

$$\text{AOB: } \mu_{AOB} \cdot X_{AOB} \cdot \frac{S_{O2}}{K_{O2}^{AOB}+S_{O2}} \cdot \frac{S_{FA}}{K_{FA}^{AOB}+S_{FA}+S_{FA}\cdot S_{FA}/K_{I,FA}^{AOB}} \cdot \frac{K_{I,FNA}^{AOB}}{K_{I,FNA}^{AOB}+S_{FNA}} \quad (2)$$

$$\text{NOB: } \mu_{NOB} \cdot X_{NOB} \cdot \frac{S_{O2}}{K_{O2}^{NOB}+S_{O2}} \cdot \frac{S_{FNA}}{K_{FNA}^{NOB}+S_{FNA}+S_{FNA}\cdot S_{FNA}/K_{I,FNA}^{NOB}} \cdot \frac{K_{I,FA}^{NOB}}{K_{I,FA}^{NOB}+S_{FA}} \quad (3)$$

where μ is the specific growth rate coefficient (1/day), dependent on local pH and μ_{max}; S_{O2}, S_{FA} and S_{FNA} are O_2, FA and FNA concentrations (mg/L), respectively; K_{O2}, K_{FA} and K_{FNA} are half-saturation coefficients (mg/L);$K_{I,FA}$ and $K_{I,FNA}$ are inhibition coefficients (mg/L). Growth substrate inhibitions (FA for AOB, FNA for NOB) are incorporated with the Andrews equation. Other inhibitions (FA for NOB, FNA for AOB) are described with a noncompetitive inhibition term.

For the denitrification process, NO_2^- and NO_3^- are modeled as separate electron acceptors. To avoid unnecessary complexity and focus on AOB/NOB competition, no intermediates (NO or N_2O) are considered. Bacteria have different decay rates in aeration and non-aeration periods: to simplify the model, AOB/NOB are assumed not to decay under anoxic or anaerobic conditions,[27] meanwhile, HB decay is modified by an anoxic reduction factor during non-aeration periods.

Chemical Process: pH Calculation

The one-dimensional model can keep track of local pH changes perpendicular to the membrane substratum. pH along biofilm depth is calculated based on the proton production via nitrification and consumption via denitrification, the equilibrium reaction with bicarbonate buffer, and CO_2 stripping to the membrane lumen. The consumption of inorganic carbon for autotrophic growth is neglected as it has insignificant influence on pH changes under conditions when inorganic carbon is not limiting.

Protons produced and consumed in bioprocesses are listed in the stoichiometry matrix. The acid-base balance reaction with bicarbonate buffer is assumed to occur much faster than biological processes.[28]

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3\ (CO_2) \qquad \text{rate: } \left(\frac{S_{HCO3}\ \cdot S_H}{K_{a,HCO3}} - S_{CO2}\right) \cdot 10^7 \qquad (4)$$

where S_H, S_{HCO3-} and $S_{H2CO3(CO2)}$ are concentrations of proton, bicarbonate and the sum of carbonic acid and dissolved carbon dioxide, respectively (μmol/L); $K_{a,HCO3}$ is the dissociation equilibrium constant of carbonic acid (0.574 μmol/L, 33 ℃, 1 atm). Protons produced in the nitritation process titrate HCO_3^- to H_2CO_3, and over-saturated CO_2 diffuses from the biofilm base to the membrane lumen (Equation 1). Acid-base reactions with phosphate ions were minor and neglected, as the molar ratio of $H_2PO_4^-/HCO_3^-$ in influent was lower than 3%.

Limitations/Inhibitions of AOB/NOB Activity

The growth rate expressions of AOB and NOB consider DO and pH effects. DO limitation is assessed by oxygen affinity constants. Two pH effects are included. (1) pH-enzyme effect: pH can affect nitrifying activity directly by changing the enzyme reaction mechanism or increasing the demand for maintenance energy.[28,29] A Gaussian bell-shaped curve is chosen to model the pH-enzyme dependency of specific growth rates.[13]

$$\mu = \frac{\mu_{max}}{2}\left\{1 + cos\left[\frac{\pi}{\omega}\cdot(pH - pH_{opt})\right]\right\} \qquad |pH - pH_{opt}| < \omega \qquad (5)$$

where μ_{max} is the maximum specific growth rate at the optimal pH- pH_{opt}, ω is the pH range within which μ is larger than a half of μ_{max}. (2) pH substrate-speciation effect: local pH values determine FA/FNA speciation from total NH_4^+/NO_2^-. The speciation between ionized/unionized species is assumed at instantaneous equilibrium.[30]

$$S_{FA} = \frac{K_{a,NH3}\cdot S_{NH4}}{S_H} \qquad S_{FNA} = \frac{S_{NO2}\cdot S_H}{K_{a,NO2}} \qquad (6)$$

where $K_{a,NH3}$ and $K_{a,NO2}$ are dissociation equilibrium constants of ammonium and nitrous acid, respectively (0.000794 and 628.96 μmol/L (33 ℃, 1 atm)). Substrate-speciation will result in differential degrees of FA/FNA inhibition.

2.3 Sensitivity Analysis and Parameter Estimation

To investigate the most determinant parameters on reactor performance, a sensitivity analysis was performed. Initial values of kinetic parameters were taken from ASMN model.[26] The optimal pH ranges for AOB and NOB growth kinetics (pH_{opt} and ω) were from Park et al..[13] The temperature correction for μ_{max} and b_{max} are from Hao et al..[31] The MSNBM was first run in continuous aeration with default values for 300 days to achieve a stable nitrifying biofilm. Then a local sensitivity analysis was performed after switching to intermittent aeration- giving individual parameter a 100% value change while all others remained constant.[25] Reactor performances were evaluated in terms of ammonium removal efficiency (ARE, $\frac{S_{NH\ ,in}-S_{NH4,eff}}{S_{NH\ ,in}}$%),

178 nitrate production efficiency (NaE, $\frac{S_{NO3,eff}}{S_{NH4,in}-S_{NH4,eff}}$%), nitritation efficiency (NE, $\frac{S_{NO2,eff}}{S_{NH4,in}-S_{NH4,eff}}$%) and

179 NOB fraction (fNOB, $\frac{NOB}{NOB+AOB}$%). The normalized sensitivity function is defined as,

180 $$\delta_j = \sqrt{average\left(Sens_{i,j}^2\right)} \; and \; Sens_{i,j} = p_{i,j}\frac{\Delta y_j}{\Delta p_{i,j}}, \quad (7)$$

181 where δ_j, y_j and $p_{i,j}$ are the sensitivity function, the output reactor performances (ARE, NaE, NE or fNOB),

182 and the input parameters, respectively. $Sens_{i,j}$ was evaluated at different times during the aeration cycles

183 (time interval of 0.01 day) and at 20 equidistant points within the biofilm or 1 point in the bulk phase. The

184 averaged value was considered in the sensitivity analysis and parameter sensitivity was ranked for each

185 targeted performance metric. We focused on biokinetic and stoichiometric parameters related to AOB and

186 NOB, as HB parameters are of secondary importance in nitrifying biofilms.[32]

187 The most sensitive parameters were calibrated with steady state experimental data. The model calibration

188 was carried out by trial and error through adjusting the parameter values one by one to minimize the fitting

189 error. Root mean squared error was used to assess the quality of model-data fit as the objective function,

190 $$\text{RMSE} = \sqrt{average\left(\sum_j \sum_i \left(\frac{y_{model,i,j}-y_{meas,i,j}}{y_{meas,j,average}}\right)^2\right)} \quad (8)$$

191 where j is the targeted variable measured or estimated (NH_4^+, NO_2^-, NO_3^- and DO), i is a sample point along

192 biofilm depth (i =20). The model was validated with additional experimental data from this MABR and

193 experimental data from a separate membrane-aerated biofilm reactor (MABR2) operated under 4 different

194 ammonium surface loadings (Table S5, detailed description of the experimental data used in model

195 calibration and validation).[33] The calibrated parameters were checked by comparing RMSE in the calibration

196 with RMSE in the validation and the Janus coefficient (J) was calculated,[34]

197 $$J^2 = \frac{{RMSE_{val}}^2}{{RMSE_{cal}}^2} \quad (9)$$

198 **2.4 Model Simulations**

199 The calibrated MSNBM was run in 3 scenarios (Table S6, detailed description of each simulation scenario):

200 (1) To validate the model with extra experimental data, the calibrated MSNBM was ran in intermittent
201 aeration (6-hour aeration period and 6-hour non-aeration period) under different NH_4^+ surface loadings or in
202 continuous aeration in a batch test. Then the determinant factor(s) that govern NOB suppression in this
203 MABR was explored with the validated model.

204 (2) To clarify why NOB suppression occurred after switching to intermittent aeration from continuous
205 aeration, the model was run in continuous aeration to achieve a nitrifying biofilm, then aeration was switched
206 to the same intermittent aeration as scenario 1.

207 (3) To optimize the operational window for nitritation in intermittently aerated MABRs, different
208 intermittent aeration strategies and influent concentrations were simulated in MSNBM after achieving a
209 nitrifying biofilm in continuous aeration. The effects of aeration intermittency and residual NH_4^+ (FA)
210 concentrations on NOB suppression were evaluated.

211 # Results and Discussion

212 ### 3.1 Model Calibration and Evaluation

213 A sensitivity function, considering the sum of reactor performances (ARE, NaE, NE and fNOB), was
214 calculated to rank parameters (Figure S2). The most sensitive parameter is μ_{max}^{AOB}, followed by $K_{I,FA}^{AOB}$, μ_{max}^{NOB},
215 $K_{I,FA}^{NOB}$, K_{O2}^{AOB} and K_{O2}^{NOB}. The ranking shows that μ_{max} is the most determinant among all kinetic parameters in
216 nitrogen conversion simulations. It is consistent with the sensitivity analysis of Wang et al.[32] who ranked
217 kinetic parameters in terms of nitritation performance and biofilm development in nitrifying biofilm reactors.
218 The higher sensitivity regarding performance within the biofilm (Figure S2B) versus the bulk (Figure S2A)
219 suggests that in-situ microprofiling data is more informative in model calibration than bulk measurements,
220 which were typically used.[22,35] Therefore, microprofiling measurements (NH_4^+, NO_2^-, NO_3^- and DO) in the
221 first aeration hour at steady state were used to calibrate sensitive parameter(s). Microprofiles in the last
222 aeration hour (NH_4^+, NO_2^-, NO_3^- and DO) and bulk profiles in an intermittent aeration cycle (NH_4^+, NO_2^-,
223 NO_3^-, DO and pH) at steady state were used for validation. Additional validation of the model and its
224 parameter estimates was obtained by fitting the initial reactor performance (NH_4^+, NO_2^- and NO_3^-) when

operated in batch start-up mode, and by fitting the biofilm performance (NH_4^+, NO_2^-, NO_3^- and pH) of a separately operated MABR under different NH_4^+ surface loadings.

By fitting the most sensitive parameter- μ_{max}^{AOB} in the reported range,[12] the RMSE decreased to 0.5 and the deviation in NO_3^- fitting contributed the most to the error. Thus, the next most sensitive parameter- μ_{max}^{NOB}- in NO_3^- sensitivity ranking (Figure S3) was added to the calibration and RMSE decreased to 0.1. Values of μ_{max}^{AOB} and μ_{max}^{NOB} were within a reasonable range: the estimated maximum growth rates at the optimal pH were 2.35 d^{-1} for AOB and 2.15 d^{-1} for NOB (Table 1). Predicted microprofiles agree with measurements in the first aeration hour at steady state (Figure 1A): NH_4^+ is consumed along biofilm depth and NO_2^- is produced; NO_3^- remains at lower concentrations than NO_2^- within the biofilm; DO penetrates 60 μm into the biofilm base. The greatest divergence in the overall fitting corresponds to NO_2^- at the biofilm base (6 mg-N/L) but only overestimates FNA concentrations by 0.002 mg-N/L. Errors in DO fitting at the membrane-biofilm interface (6.6 mg/L predicted versus 1.7 mg/L measured) have a minor influence on the oxygen competition between AOB and NOB (Table S4), consistent with Lackner and Smets[36] who reported that oxygen concentrations at interfaces were not decisive in nitritation performance in MABRs. Additionally, uncertainty in measuring the interface DO could be caused by microbial activities on the membrane and an efficiency factor E (1.3 ~ 4.3) was suggested to correct measured values.[33]

MSNBM predicts consistent profiles in the different model validations. It predicted lower NH_4^+ and higher NO_2^- within the biofilm in the last aeration hour (Figure 1B) and uniform dynamic variations of bulk concentrations in a 12-hour intermittent aeration cycle. For example, it captured the pH decreases in the 6-hour aeration phase and increases in the 6-hour non-aeration phase (Figure 1C). It also predicted simultaneous production of NO_2^- and NO_3^- in the batch mode data validation (Figure S4A) and predicted NH_4^+ consumption and NO_2^- production following the tendencies observed in MABR2 (Figure S4B). Janus coefficients were around 1.9 (± 0.5), showing that the RMSEs were within the same order of magnitude in calibration and validations.

3.2 Model-based Exploration of NOB Suppression in Intermittently Aerated MABRs

NOB suppression is the result of indirect and direct (competitive) interactions between AOB and NOB in the local environment. Net microbial activities are captured in the specific growth rates: biomass types with the higher specific growth rate will win the local competition. In the studied system, oxygen was provided intermittently from membrane lumen. The biomass type with the higher specific growth rate (AOB or NOB) thus dominated the oxygen utilization.

Consistent with experimental reactor operations, simulations were initiated with fully-nitrifying biomass and subject to intermittent aeration. Both simulation and experimental data showed that after 2 weeks in intermittent aeration bulk N concentrations became stable, especially NO_3^- was below 1 mg-N/L indicating efficient suppression of NOB activity (Figure S9). To illustrate the competition in the first nitrifying stage, profiles of specific growth rates of AOB and NOB during an aeration cycle (6 hours) are plotted at day 15 (Figure 2A). The averaged μ at time intervals shows kinetic variations over time: (1) 0-15 minutes, with the onset of aeration microbial activities recover from the previous non-aeration period and increase dramatically; (2) 15-180 minutes, AOB activity becomes stable, while NOB activity still recovers; (3) 180-360 minutes, both AOB and NOB activity reach pseudo steady state. The model shows the ratio of μ_{AOB} to μ_{NOB} increases in the intermittent aeration, compared to the ratio of μ_{max}^{AOB} to μ_{max}^{NOB} in continuous aeration (1.5±0.15 versus 1.1). AOB preferentially utilize oxygen to support growth while NOB are outcompeted or their activity is suppressed.

To assess the relative contribution of DO/pH effects on NOB suppression, individual factors influencing growth rates were calculated spatially (at different biofilm depths) and temporally (at different times in the cycle). Considering the effective DO penetration depth, only results in the first 100-μm at the biofilm base are shown (Figure 2B).

DO Limitation in NOB Suppression

O_2 is a growth substrate for both AOB and NOB. In counter-diffusion biofilms O_2 is provided via the lumen and NH_4^+ via the bulk. In the biofilm, DO penetrates only 60 μm during aeration periods with the highest concentration at the membrane-biofilm interface (biofilm depth= 0 μm), presenting spatial variations (Figure

S5A). Besides, DO varies over time during aeration cycles. DO at the membrane-biofilm interface is 0 mg/L at the onset of aeration and quickly increases to the maximum concentration within 15 minutes. Afterwards, DO concentrations within the biofilm remain stable until the end of aeration.

The DO limitation effect was evaluated based on oxygen concentrations within the biofilm (Figure 2B, 1-DO limitation). In aeration periods, during the first 15 minutes DO strongly limits both AOB and NOB activities. During the following period, the limitation is alleviated as DO increases and stabilizes, but still remains strong above 30 μm. With a lower DO affinity NOB are more oxygen-limited than AOB. However, the relatively stronger limitation to NOB is insignificant in its suppression. Model results show that oxygen transfer and its diffusion mostly affects NH_4^+ oxidation efficiency rather than nitritation efficiency (Table S7).

pH-enzyme Effect on NOB Suppression

Because pH affects AOB/NOB kinetics directly and indirectly, it is necessary to incorporate pH effects in models.[14,37] Here MSNBM predicts local pH values within the biofilm and the response to transient aeration phases (Figure S5B). While measurements showed that bulk pH remained relatively stable (±0.2), pH within the biofilm, especially in the DO-penetrated zone, showed considerable variations (±0.6). At the onset of aeration the model indicates a transient pH upshift at the biofilm base (0-15 minutes). The accumulated alkalinity is attributed to continuous CO_2 diffusion from the biofilm base to the membrane lumen where N_2 gas flows through in the previous non-aeration period and slight denitrification activities. As aeration continues, pH decreases due to proton production associated with NH_4^+ oxidation. Simulations predict that pH within the biofilm becomes lower than in the bulk after 1-hour aeration and decreases slowly afterwards. At the end of aeration pH at the biofilm base is 0.4 units lower than the average bulk pH, which will increase again in the following non-aerated phase. Thus pH varies periodically in the intermittently aerated biofilms, a pattern similar but slower than DO variations.

The pH-enzyme effect was assessed based on local pH values (Figure 2B, 2- pH-enzyme effect). It favors NOB growth over AOB as NOB have a lower pH_{opt} (NOB: 7.7 versus AOB: 8.4) and pH varies in the optimal range for its growth. Moreover, the pH-enzyme effect is also insignificant in the overall AOB/NOB

competition due to their robust growth in broad pH ranges and the relatively small pH variations in the system.

pH Substrate-speciation Effects on NOB Suppression

FA/FNA concentrations rely on pH values as well as total NH_4^+/NO_2^- concentrations. In counter-diffusion biofilms, NH_4^+, provided via the bulk, is oxidized at the biofilm base producing NO_2^- which diffuses backward into the bulk.[10] Based on ionic N concentrations, FA and FNA speciation synchronizes with pH variations (Figure S5C and S5D). For instance, at the onset of aeration FA concentration is high due to NH_4^+ and alkalinity accumulation from the previous non-aeration period. During the following aeration period, FA concentration decreases, as pH drops and NH_4^+ consumption continues. On the other hand, FNA shows reversed variations: increasing as aeration progresses and with biofilm depth as a result of the proton and NO_2^- production.

The pH substrate-speciation effect was assessed based on FA/FNA concentrations within the biofilm (Figure 2B, 3- FA/FNA inhibition). During the first 15 minutes, FA strongly inhibits AOB/NOB microbial activities (FA > $K_{I,FA}$). Afterwards, the inhibition is alleviated as FA decreases. Noticeably, FA inhibits AOB and NOB in different ways: the inhibition effect remains strong for NOB throughout the aeration period (from 0.26 to 0.62), while it obviously weakens for AOB (from 0.54 to 0.89). FA inhibition rapidly becomes the most determinant factor in suppressing NOB over AOB. As FNA concentrations are always an order of magnitude lower than $K_{I,FNA}$, its inhibition effect on microbial activities is always minor thereby contributing little to NOB suppression.

Besides the inhibitor effect ($K_I/(K_I + S)$), FA/FNA exhibit the substrate limitation effect ($S/(K_S + S)$) in biological processes (Equation 2). However, FA and FNA concentrations are far above the substrate affinities (K_{FA}^{AOB} and K_{FNA}^{NOB}) in the system, making the substrate limitation effects negligible.

Overall, FA inhibition caused by pH substrate-speciation is the crucial factor in suppressing NOB in the intermittently aerated biofilm reactors. Nitritation success is insensitive to oxygen affinity constants or DO concentrations at the membrane-biofilm interface- a conclusion different from previous studies.[38,39] Downing and Nerenberg[22] suggested manipulating interface DO as an effective method to control shortcut nitrification

in MABRs: with a lower interface DO, more NO_2^- accumulated. However, their biofilms performed at low nitrification rates with a low influent NH_4^+ concentration- 3 mg-N/L, suggesting little FA inhibition and no NO_2^- accumulation or significant pH gradients. The single DO gradient within the biofilm present the interface DO as a key role in nitritation success. This method might not apply for N-rich wastewater treatment. For example Lackner and Smets[36] concluded that nitritation success based only on interface DO was not possible in a counter-diffusion biofilm with high influent NH_4^+ concentrations (20-800 mg-N/L), and nitritation efficiency was not predicted from oxygen affinity constants.

Counter- and co-diffusion biofilms have different mechanisms of NOB suppression due to different spatial structures and population distributions.[32,35,36] In counter-diffusion biofilms, the theoretically optimal habitat for NOB is the biofilm base, where both S_{O2}/K_{O2} and S_{FNA}/K_{FNA} have the highest values. By contrast, the base is not the optimal for AOB growth, as S_{O2}/K_{O2} and S_{FA}/K_{FA} cannot have the maximum at the same spatial position. Outcompeting NOB can be more difficult in counter-diffusion over co-diffusion biofilms, where microbes (AOB and NOB) share the optimal habitats at the biofilm top near the biofilm/liquid interphase. Others have similarly observed that NOB could survive better in counter- versus co- diffusion biofilms, even when operated under constant oxygen limited (DO < 0.1 mg/L) and high pH (8.0-8.3) conditions in the bulk.[32] The inherent system geometry of membrane-aerated biofilms complicates NOB inhibition/washout. Besides, when applying intermittent aeration, periodic pH variations at the biofilm base exert a significant effect on NOB dynamics in counter-diffusion biofilms because of continuous CO_2 diffusion to the gas lumen. However, such pH variations are not expected in co-diffusion biofilms. Many studies have highlighted the benefits of low DO with high FA to maintain shortcut NH_4^+ removal in co-diffusion biofilms.[17,40] Park et al.[3] explored simultaneous effects of DO and FA/FNA in lab-scale co-diffusion nitrifying biofilms, and found that NO_2^- accumulated due to DO limitation or FA inhibition and long-term NOB suppression could not be maintained without DO limitation involved. The results were consistent with Brockmann and Morgenroth[41] who suggested that oxygen limitation was the main mechanism for NOB suppression and FA inhibition was not necessarily required in co-diffusion biofilms. However, DO limitation in nitritation counter-diffusion biofilms appears not as significant as reported for co-

diffusion biofilms, consistent with the observation that nitritation could not be achieved by solely manipulating air pressure in the membrane lumen in MABRs.[21]

3.3 Potential explanation of NOB Suppression in the study of Pellicer-Nàcher et al. (2010)

To answer why NO_2^- accumulated after switching from continuous to intermittent aeration in MABRs, simulations were carried out with the calibrated MSNBM in continuous aeration for 200 days followed by intermittent aeration (6-hour aeration and 6-hour non-aeration cycles). The simulation shows a nitrifying biofilm during continuous aeration (NE = 0%) indicating no NOB suppression (Table 2- continuous aeration). After switching to intermittent aeration the model predicts NOB suppression- NO_3^- decrease and NE increase (Table 2- strategy A, Figure S6). To find the critical factor for NOB suppression, variations of individual pH/DO effect on AOB/NOB competition were assessed: each effect $\frac{effect_{AOB}}{effect_{NOB}}$ in intermittent aeration (for instance at day 215) was normalized by its value during continuous aeration. A value higher than 1 means the effect favors NOB suppression in intermittent aeration, and lower than 1 that it favors NOB growth.

Only FA inhibition is identified to favor NO_2^- accumulation after switching the aeration strategy, while DO limitation, pH-enzyme effect and FNA inhibition remain unchanged (Figure S7). FA inhibition shows certain varying patterns in intermittent aeration: (1) it is overall enhanced due to an increased residual NH_4^+; (2) it is particularly strong during the first 15 minutes of aeration. The simulated increase of residual NH_4^+ after changing to intermittent aeration was also observed in the study of Pellicer-Nàcher et al.:[21] in reactor B bulk NH_4^+ increased by 100 mg/L at stage 1 and 2 (intermittent aeration) compared to stage 0 (continuous aeration). Compared to continuous aeration, MABRs in intermittent aeration display a tradeoff between NH_4^+ removal efficiency and nitritation efficiency (Table 2). Nitritation is assisted by the evaluated residual NH_4^+, which underlines the importance of a minimum NH_4^+ concentration in the bulk. Pérez et al.[18] also highlighted the need for minimum residual NH_4^+ for NOB suppression in co-diffusion biofilms, but attributed the nitritation success to differential oxygen limitation rather than FA inhibition- as NOB were outcompeted due to the strong oxygen limiting conditions imposed by a high residual NH_4^+. The strong FA inhibition at the onset of aeration is due to pH upshifts at the biofilm base in the previous anoxic phases. It

causes a longer lag phase of NOB activity over AOB, which could be another reason in the nitritation success. Theoretically, NOB locate at the biofilm base, if enriched in MABRs, thus pH upshift at the base is more efficient to prompt FA inhibition than increasing bulk pH. This lag phase has also been observed in other intermittently aerated systems.[42,43] Kornaros et al.[44] and Gilbert et al.[45] attributed the lag phase to a long (enzyme) reactivation time in NOB nitrogen metabolism after anoxic exposure in batch continuous stirred-tank reactors. However, the possibility for pH variations was not considered in those studies, even though CO_2 stripping could slowly increase bulk pH.[46]

3.4 Nitritation in Various Intermittent Aeration Strategies

For an intermittent aeration system with certain NH_4^+/O_2 surface loadings, the aeration duration determines residual NH_4^+ concentrations: a longer aeration lowers residual NH_4^+. The aeration intermittency determines pH upshift times and the variation range of bulk concentrations: a higher frequency causes more pH upshifts and a narrow variation range. This information can be utilized to optimize intermittent aeration strategies for efficient nitritation in MABRs (Table 2). MSNBM simulation shows that a higher aeration intermittency can accelerate NOB suppression (A and C) due to more times of pH upshift in non-aeration phases to retard NOB activity while slightly affecting AOB activity, or decelerate NOB suppression (B and A) due to the relatively high bulk NH_4^+ (pH) at the onset of aeration phases even the averaged bulk concentrations are the same. Longer aeration duration (D) leads to a slower nitritation process but a higher NH_4^+ removal efficiency, while keeping the same aeration intermittency. It is consistent with the observation in Mota et al.[47] that intermittently aerated reactors with longer anoxic phase had the lower NOB abundance and relatively higher NH_4^+ effluent concentrations. Both studies suggest that the maximum aeration duration should be set to ensure nitritation success in intermittent aeration, and a specific to the treated wastewater ratio of aeration to non-aeration phase is needed to balance NOB suppression against NH_4^+ removal.[48] Simulation with high NH_4^+ concentrations predicts fast nitritation in the intermittent aeration (E), and vice versa slow nitritation with low influent NH_4^+ (F). Further simulation with low NH_4^+ concentrations but high bulk pH (G) shows efficient nitritation, confirming a key factor in NOB suppression was bulk FA rather than residual NH_4^+ (more simulations in Table S8). In an intermittent aeration regime, the bulk FA can provide a rapid indicator

405 of the nitritation potential of MABRs (Figure S10). It reveals that aeration duration and aeration
406 intermittency are two key criteria that affect nitritation efficiency in MABRs at a certain influent loading.

407 In conclusion, we provide experimental evidence that intermittent aeration supports efficient nitritation in
408 membrane aerated biofilm reactors (MABRs). A pH-explicit 1-D multispecies nitrifying biofilm model
409 (MSNBM) is developed and calibrated: model analysis reveals that NOB suppression - associated with
410 intermittent aeration - is primarily governed by *periodic* FA inhibition as the consequence of transient pH
411 upshifts during non-aeration. These pH upshifts are mainly caused by alkalinity increases due to CO_2
412 stripping to the membrane lumen (which also occurs during aeration) *plus* the cessation of proton production
413 (which only occurs during aeration). In counter diffusion biofilms pH effect is more important than DO
414 (limitation) effect on NOB suppression. Both aeration intermittency and duration are effective control factors
415 to obtain nitritation success in intermittently membrane-aerated biofilms, and maintaining nitritation and
416 NH_4^+ removal efficiency is more easily ensured if operated with high buffer capacities.

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548 **Table 1.** Kinetic parameter values of AOB and NOB in the calibrated model.

Kinetic parameters	AOB	NOB	References
μ_{max}: the maximum specific growth rate, 1/d	2.35 2.72[1]	2.15 1.75[1]	this study
K_{O2}: half-saturation coefficient for O_2, mg/L	0.6	1.2	Hiatt and Grady[26]
Y: autotrophic yield, mgCOD/mgN	0.18	0.06	Hiatt and Grady[26]
K_{FA}^{AOB}, K_{FNA}^{NOB}: half-saturation coefficient, mg/L	0.0075	0.0001	Hiatt and Grady[26]
$K_{I,FA}$: free ammonia inhibition coefficient, mg/L	1	0.2	Hiatt and Grady[26]
$K_{I,FNA}$: free nitrous acid inhibition coefficient, mg/L	0.1	0.04	Hiatt and Grady[26]
b_{max}: decay coefficient, 1/d	0.17	0.073	Hao et al.[31]
pH_{opt} (ω): optimal pH	8.4(3.2)	7.7(2.4)	Park et al.[13]

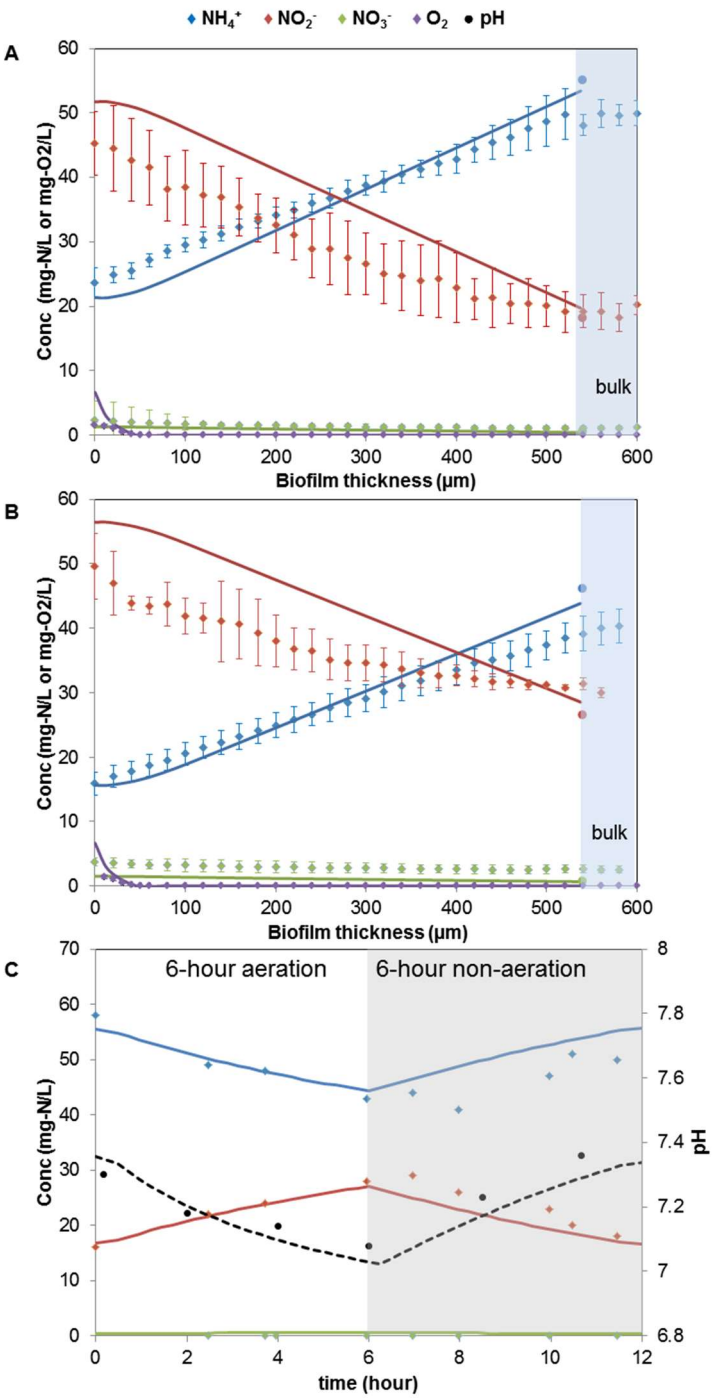
549 [1]default growth rates in ASMN with temperature correction (33°C)

550

Table 2. Predicted nitritation efficiencies (NE, %) in various intermittent aeration strategies

Simulation Case	Influent[2]		Effluent (Bulk)			
	$NH_4^+{}_{in}$ (mg-N/L)	Buffer capacity[2]	NH_4^+ (mg-N/L)	pH	FA[3] (mg-N/L)	$NE_{normalized}$[4]
continuous	75	2.1	39	6.96	0.27	0.01
A: 6+6[1]	75	2.1	53.0 ± 5	7.23 ± 0.15	0.71	1.00[4]
B: 1+1	75	2.1	52.5 ± 1	7.22 ± 0.02	0.69	0.73
C: 12+12	75	2.1	53.1 ± 10	7.25 ± 0.25	0.78	0.79
D: 8+4	75	2.1	47.8 ± 4	7.14 ± 0.15	0.52	0.41
E: 6+6	100	2.1	72.0 ± 7	7.25 ± 0.15	1.02	1.74
F: 6+6	50	2.1	35.0 ± 3	7.20 ± 0.15	0.45	0.21
G: 6+6	50	5	31.2 ± 5	7.41 ± 0.10	0.64	0.83

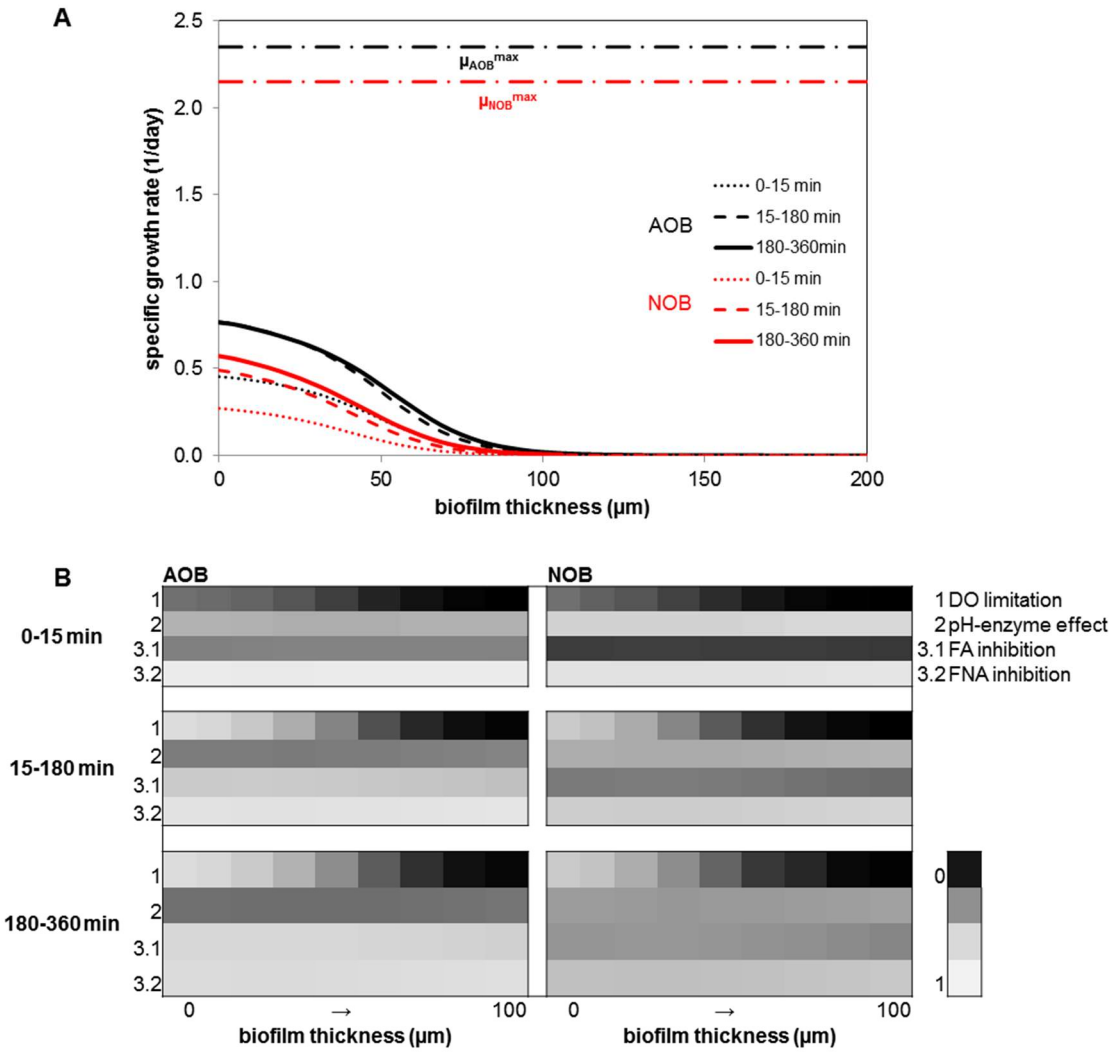
[1]Aeration strategy 6+6 meant a 12-hour intermittent aeration cycle consisting of a 6-hour aeration phase and a 6-hour non-aeration phase. [2]Buffer capacity in the influent was recorded as the molar ratio of bicarbonate (HCO_3^-) to ammonium (NH_4^+_N). [3]FA was calculated with the averaged NH_4^+ concentrations and bulk pH during a full aeration cycle (equation 6). [4]For a clear comparison, NE was normalized to the Nitritation efficiency in the default simulation case A (NE = 48.5%). MSNBM was run in continuous aeration (200 days) to achieve a mature nitrifying biofilm, followed by various intermittent aeration strategies: (A-D) different intermittent aeration but the same influent; (A,E-G) the same aeration intermittency but different influent concentrations. NEs in the NOB suppression process in intermittent aeration were recorded (e.g. at day 215) (Table S6). In simulations E-G, oxygen loadings proportionally varied with NH_4^+ influent concentrations (more simulations in Table S8).



562

563 **Figure 1.** Experimental (discrete symbols) and predicted (line) concentrations in MABR at steady state (A)
564 microprofiles in the first aeration hour, (B) microprofiles in the last aeration hour, and (C) bulk profiles in a
565 12-hour intermittent aeration cycle. For each micro profile, replicates (n>3) were made and the average was
566 shown.

567

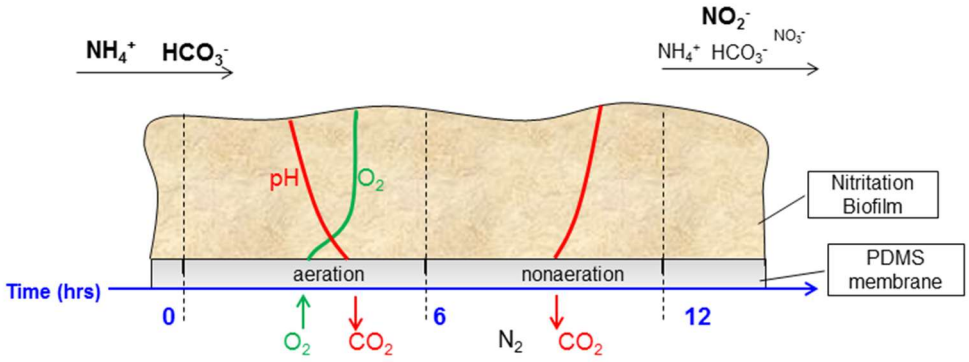


568

569 **Figure 2.** A- Specific growth rates of AOB and NOB within the biofilm in a 6-hour aeration period at day 15
570 (AOB- black, NOB- red). B- Individual effect on AOB and NOB within the 100μm-aerated biofilm base in a
571 6-hour aeration period at day 15. (0- strong limitation/inhibition effect, 1- no limitation/inhibition effect)

572

573



574

575 **TOC-** Graphical abstract