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Intermittent aeration to regulate microbial activities in membrane-aerated biofilm reactors: Energy-efficient nitrogen removal and low nitrous oxide emission

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ABSTRACT

Membrane-aerated biofilm reactors (MABR) are being applied for autotrophic nitrogen removal, yet control of nitrogen turnover remains challenging in MABR counter-diffusion biofilms. In this study, we regulated microbial activities in two lab-scale MABRs by providing continuous versus intermittent aeration. Nitrogen consumption by different functional microbial groups was estimated from bulk measurements via a mass balance approach. Nitrite-oxidizing bacteria (NOB) proliferated under continuous aeration while they were significantly suppressed under intermittent aeration, and NOB suppression activated anaerobic ammonium oxidation. Nitritation performance in the MABR was studied through long-term bulk measurements and *in situ* biofilm microprofiles of dissolved oxygen (DO) and pH. During intermittent aeration pH effects rather than DO effects determined nitritation success, especially ammonia speciation, which serves as substrate and inhibitor in nitrification processes. Biofilm transition phases were monitored upon aeration switches. Canonical correspondence analysis suggested that the relative transition after anoxia and aeration intermittency were less decisive for biofilm performance than the relative aeration duration. Heterotrophic bacteria displayed minor denitrification rates with aeration control, but contributed to mitigation of nitrous oxide (N_2O) emissions. N_2O production hotspots were identified at the top of the anoxic biofilm zone under continuous aeration. Instead, under intermittent aeration an anoxic N_2O reduction zone was established. Our observations support intermittent aeration control of MABRs as a simple strategy for energy-efficient nitrogen removal with low N_2O emission.

1. Introduction

Biofilm processes are applied broadly in environmental biotechnologies, allowing for biomass accumulation and retention without the need for external devices to separate and retain biomass [1]. These processes are especially useful in retaining slow-growing microorganisms such as nitrifying bacteria [2,3]. Membrane-aerated biofilm reactors (MABRs) are a promising biofilm technology for treatment of nitrogenous (N) wastewaters relying on counter-diffusion of substrates in membrane supported biofilms [4,5]. In nitrifying MABRs, air is provided through membrane modules and redox stratification develops due to the presence or absence of oxygen within biofilms. The stratification allows to develop unique microbial communities that can achieve nitritation [6], nitrification/denitrification [7] or partial nitritation/anammox (PNA) [8].

One of the major challenges in MABRs is maintaining the process stability with an appropriate balance between microbial activities in a complex biofilm system [3,9]. For energy-efficient ammonium (NH_4^+) removal via nitrite (NO_2^-), suppression of nitrite-oxidizing bacteria (NOB) is required. While NOB suppression has been successfully tested in suspended growth systems [10,11], it is a more difficult process in counter-diffusion biofilms as both ammonia-oxidizing bacteria (AOB) and NOB thrive at the biofilm base. Besides energy savings, succesful NOB suppression in MABRs allows to exploit a more resource-efficient N removal through one-stage PNA where residual NH_4^+ and accumulated NO_2^- are utilized by anaerobic ammonium-oxidizing bacteria (AMX) [12,13]. The coexistence and stable coupling of AOB and AMX in MABRs would further lead to reduced emissions of nitrous oxide (N_2O) [13],

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which is an intermediate in N removal, but a potent greenhouse gas and ozone depleting chemical. However, manipulating the involved microbial interactions becomes really complex, because microbes in PNA systems respond differently to differences in operational conditions [9,14,15].

Aeration control can offer a practical strategy to regulate microbial activities by intermittently providing air-on and air-off conditions [8,11,16]. In suspended growth systems, intermittent aeration can favour nitritation by operating at a controlled aerobic solid retention time (SRT) that retains AOB but out-selects NOB [11], or by introducing slower responses of NOB to the transient air-off disturbances compared to AOB [17,18]. Although SRT control is less trivial in biofilms, biofilm performance still responds to aeration control [19,20]. In MABRs treating rich-N wastewater, no N removal was observed with continuous aeration but removal rates reached over 5.5 g-N/(m^2·day) with intermittent aeration, concomitant with negligible N_2O emissions [19]. A model-based study of MABRs with low-N loadings concluded that periodic pH dynamics could drive NOB suppression under intermittent aeration [21]. However, experimental characterization of the oxic/anoxic transition phase and its overall contribution to microbial activity dynamics are absent; the consequences of microbial interactions on N_2O production need to be further assessed within MABR biofilms.

In this study, lab-scale MABRs were operated under continuous versus intermittent aeration strategies to study the impact on long-term N conversions. Intermittent aeration patterns were chosen based on a previous study of Ma et al. [21]. Individual microbial activities were calculated from bulk N measurements using a mass balance-based approach. Then the regulation of microbial activities by intermittent aeration was explored. *In situ* biofilm depth profiles of pH, DO and N_2O were measured, and their transients with aeration control were analyzed. Lastly, the operational window for optimal MABR performance was discussed.

2. Material and methods

2.1. Reactor setup and operation

Two 0.8 L lab-scale MABRs were operated in parallel (Fig. S10) with aeration provided through tubular PDMS membranes chosen for their high oxygen mass transfer coefficient [22]. MABRs were inoculated with enriched nitrifying biomass, and further details of the system are available in Ma et al. [21]. Synthetic wastewater was fed continuously at an influent NH_4^+ concentration of 75 mg-N/L without external organic carbon. The influent N-loading was 9.1 g-N/(m^2·day). Reactors were operated for > 400 days with bulk DO and pH monitored (CellOX 325 and Sentix 41, WTW Germany). System temperature varied from 20 to 31 °C due to heat-loss of the recirculation pump. With the buffer capacity provided from influent, pH varied between 6.8 and 7.1 (inlet molar ratio of bicarbonate ($NaHCO_3$) to NH_4^+ = 1.8).

MABRs were operated under identical conditions, with the only exception being aeration control: $MABR_1$ was operated under either continuous or intermittent aeration, while $MABR_2$ was operated exclusively under continuous aeration (Table 1). An intermittent aeration cycle (Int_{on+off}) consisted of an air-on period (100% air) followed by an air-off period (100% N_2). Different intermittent aeration strategies were defined by the relative aeration duration (R_{on}, unitless) and aeration intermittency (f_{int}, 1/day):

$$R_{on} = t_{on}/(t_{on} + t_{off});\ f_{int} = 24/(t_{on} + t_{off})\ (1/day) \quad (1)$$

where t_{on} and t_{off} are the air-on and air-off durations of an aeration cycle (hour). Continuous aeration and air-on periods of intermittent aeration were operated at the same air flow rate and pressure (0.1 L/min, 10 kPa air).

2.2. Mass balance to estimate microbial activities

Mass balance of each N species in the system was assessed (Eqs. (2)–(4)), assuming reaction stoichiometries of nitrification, anammox, and denitrification from literature [23,24]. The following assumptions regarding the growth of heterotrophic bacteria (HB) were made: (1) HB growth was supported solely by organic carbon produced through biomass decay, as the influent did not supply any organic matter; (2) growth and decay were in balance if biofilm thickness remained constant, and assimilative N consumption for growth and N release from decay were also in balance; (3) HB grew either aerobically (using O_2 as electron acceptor) or anoxically (using NO_3^- or NO_2^- as electron acceptor); and (4) for anoxic growth, 1-step denitrification (reduction to N_2) was assumed with NO_3^- or NO_2^-. HB activity did not significantly affect estimates of the other microbial activities irrespective of the assumptions regarding respiration (discussion in Fig. S2, Table S1), essentially due to the limited amount of organic carbon. To simplify the discussion, HB growth was assumed to occur only based on anoxic NO_3^- respiration, while oxygen supplied from the membrane lumen was completely utilized by nitrifiers. N loss as gaseous nitrogen oxides were considered negligible. In this way, individual N consumption rates by AOB, NOB, AMX and HB were calculated,

$$NH_4^+ \text{ mass balance: } Q\cdot\Delta NH_4^+ = Q\cdot(NH_4^+{}_{inf} - NH_4^+{}_{eff}) = R_{NH4^+,AOB} + R_{NH4^+,AMX} \quad (2)$$

$$NO_2^- \text{ mass balance: } Q\cdot\Delta NO_2^- = Q\cdot NO_2^-{}_{eff} = R_{NH4^+,AOB} - R_{NO2^-,NOB} - 1.32\cdot R_{NH4^+,AMX} \quad (3)$$

$$NO_3^- \text{ mass balance: } Q\cdot\Delta NO_3^- = Q\cdot NO_3^-{}_{eff} = R_{NO2^-,NOB} + 0.26\cdot R_{NH4^+,AMX} - R_{NO3^-,HB} \quad (4)$$

$$\text{COD mass balance: } R_{NH4^+,AOB}\cdot Y_{AOB} + R_{NO2^-,NOB}\cdot Y_{NOB} + R_{NH4^+,AMX}\cdot Y_{AMX} - 2.86\cdot R_{NO3^-,HB}/(1 - Y_{HB}) = 0 \quad (5)$$

where $R_{NH4^+,AOB}$, $R_{NO2^-,NOB}$, $R_{NH4^+,AMX}$ and $R_{NO3^-,HB}$ are NH_4^+ consumption rate by AOB, NO_2^- consumption rate by NOB, NH_4^+ consumption rate by AMX, and NO_3^- consumption rate by HB in 1-step denitrification (mg-N/day); $NH_4^+{}_{inf}$, $NH_4^+{}_{eff}$, $NO_2^-{}_{eff}$ and $NO_3^-{}_{eff}$ are N concentrations in influent and effluent (mg-N/L); Q is the influent and effluent flow rate (L/day); Y is the observed growth yield (Y_{AOB} = 0.18 mg-COD/mg-NH_4^+_N, Y_{NOB} = 0.06 mg-COD/mg-NO_2^-_N, $Y_{HB,\ anoxic}$ = 0.54 mg-COD/mg-COD, Y_{AMX} = 0.17 mg-COD/mg-NH_4^+_N) [23,24]. Calculation of N consumption rates was implemented in Matlab R2018a (MathWorks Inc., Natick, MA, USA), and the code is provided (SI.2). Relative variations of microbial activities were evaluated,

$$\text{Degree of NOB suppression} = R_{NH4^+,AOB}/R_{NO2^-,NOB} \quad (6)$$

$$\text{Degree of AMX activation} = R_{NO2^-,AMX}/R_{NO2^-,NOB} \quad (7)$$

Table 1
Timeline of aeration control in $MABR_1$ and $MABR_2$.

Time (day)	1–67	68–94	95–143	144–196	197–255	256–301	302–368	369–430
$MABR_1$	Cont	Int_{6+6}	Cont*	Int_{6+6}*	Int_{11+1}	Int_{9+3}	Int_{6+2}	Int_{1+1}
R_{on}	1	0.5	1	0.5	0.9	0.75	0.75	0.5
f_{int}	1	2	1	2	2	2	3	12
$MABR_2$	Continuous aeration							

Cont/Cont*: continuous aeration; Int_{on+off}: intermittent aeration with a cycle comprised of air-on and air-off.

which representthe AOB-NOB and the NOB-AMX competition, respectively.

2.3. Biofilm pH, DO and N_2O: in situ microprofiles and analysis

Commercially available DO, pH and N_2O microsensors (OX-10, pH-25, N_2O-25, Unisense, Denmark) were used for *in situ* microprofile measurements within biofilms. Profiles (replicates > 3) were measured under different aeration regimes after MABR performance reached pseudo-steady state inferred from bulk N concentrations. Averaged microprofiles were used in the analysis. Microsensor measurements at the membrane-biofilm interface were further used to monitor the transient pH, DO and N_2O behavior at the biofilm base upon aeration switches. Transition time (t_{trans}) was defined as the required time for biofilm pH ($t_{trans,pH}$) or DO ($t_{trans,DO}$) to reach steady state after the air switched on.

Microprofile analyses included (1) the comparison of biofilm pH and DO between continuous and intermittent aeration, (2) the calculation of net volumetric N_2O reaction rates at different biofilm depths, and (3) the estimation of t_{trans} for pH, DO and N_2O under intermittent aeration. Oxygen penetration depth (μm) and DO concentrations at the biofilm base were included in biofilm DO comparison. Oxygen penetration depth was defined as the distance from the membrane-biofilm interface to the biofilm layer where DO concentration reached 0.01 mg/L (the detection limit). Bulk pH and pH at the biofilm base were included in biofilm pH comparison. Comparisons were performed with two-tailed student's *t*-test (95% CI). Net volumetric N_2O reaction rates under each aeration control were estimated from respective concentration profiles using Fick's second law of diffusion [25]. Statistical analyses were conducted using Microsoft office Excel 2010 with add-in solver applied for N_2O rate calculations. Values of t_{trans} were estimated with the concentration time series recorded during aeration cycles.

2.4. Total N_2O emissions and other measurements

Off-gas N_2O was measured during different aeration phases with a gas filter correlation N_2O analyzer (Teledyne AOI, San Diego, CA, USA). Calibration was performed with 200 ppm N_2O in N_2 as span gas and pure N_2 as zero gas. N_2O in the liquid phase was measured by placing a N_2O microsensor in the completely mixed bulk phase. Total N_2O emissions were compared between continuous (Cont phase) and intermittent (Int phase) aeration, including emissions in the liquid and off-gas phases. Bulk N concentrations of NH_4^+, NO_2^- and NO_3^- were measured with colorimetric test kits (Spectroquant 14776, 00683, 09713; Merck, Germany).

2.5. Statistic analysis of MABR performance

The reactor performance was described by NH_4^+ removal efficiency (ARE), Nitritation efficiency (NiE), and N removal efficiency (NRE).

$$ARE\ (\%) = (NH_4^+{}_{inf} - NH_4^+{}_{eff})/NH_4^+{}_{inf} \cdot 100\% \quad (8)$$

$$NiE = \text{Degree of NOB suppression} \quad (9, \text{Equation. 6})$$

$$NRE\ (\%) = \Delta N/NH_4^+{}_{inf} \cdot 100\% = (NH_4^+{}_{inf} - NH_4^+{}_{eff} - NO_2^-{}_{eff} - NO_3^-{}_{eff})/NO_3^-{}_{eff} \cdot 100\% \quad (10)$$

where ΔN is the soluble N loss from MABRs (mg-N/L). Canonical correspondence analysis (CCA) was used to estimate the effects of different variables on reactor performance during aeration control, using R version 3.6.3 with vegan package 2.5–6. Permutation test with Function *anova.cca* was performed to test the significance of constraints (code in SI.6). Variables with potentially high influence on microbial activities were selected in the analysis, including f_{int}, R_{on}, $R_{t,pH}$ (the ratio of $t_{trans,pH}$ to t_{on}), T (temperature, °C), pH_{bulk}, $NH_4^+{}_{bulk}$, and FA_{bulk} (bulk free ammonia), where FA_{bulk} was calculated from daily measurements of pH_{bulk}, $NH_4^+{}_{bulk}$ and temperature. Variables $R_{t,DO}$ (the ratio of $t_{trans,DO}$ to t_{on}) and $NO_2^-{}_{bulk}$ (or FNA) were not included, as $t_{trans,DO}$ was found to be excessively shorter than t_{on}, and NO_2^- concentrations remained under the detection limit (<0.1 mg-N/L) after intermittent aeration so the variations could not be distinguished among different aeration phases.

2.6. Other tests: MABR operation in batch-mode or with varying N-substrate loadings

A batch test to assess potential AMX activity was conducted at day 250 in both MABRs ($MABR_1$ and $MABR_2$), with a ~40 mg-N/L NO_2^- spike when reactors were operated in non-aeration mode (SI.8.1). A batch test to assess potential HB activity was conducted in $MABR_2$ at day 370, with a ~100 mg-N/L NO_2^- spike in non-aeration mode (SI.8.2). Besides $MABR_1$ and $MABR_2$, two additional MABRs with the same dimensions and nutrient loadings, $MABR_3$ and $MABR_4$, were operated under continuous aeration followed by intermittent aeration (Table S6). Aeration was then switched back to continuous aeration; after stable operation over 100 days, the effects of NH_4^+ and NO_2^- availability on microbial activities were studied by step-wise increasing influent NH_4^+/FA ($MABR_3$) or NO_2^- ($MABR_4$) concentrations (SI.8.3).

3. Results

3.1. Microbial activities under intermittent aeration: NOB suppression and AMX activation

$MABR_2$ was operated under continuous aeration, and developed a nitrifying biofilm mainly converting NH_4^+ to NO_3^- ($NO_2^- \leq 1$ mg-N/L, Fig. S1B). $MABR_1$ was initially operated under continuous aeration, presenting similar bulk N performance as $MABR_2$ at the end of the initial Cont aeration phase (Fig. S1A); however, the bulk concentrations changed significantly after the onset of Int_{6+6} intermittent aeration and varied with the switches between continuous and intermittent aeration at day 0 ~ 196 (Cont → Int_{6+6} → Cont*→ Int_{6+6}*, Fig. 1A). Based on the bulk measurements of $MABR_1$, microbial activities were calculated for each aeration phase (Fig. 1B). The activity variations and the concomitant bulk performance changes from continuous to intermittent aeration (Int_{6+6} and Int_{6+6}*) are presented herein below, while the influences of different intermittent aeration strategies are shown in Section 3.4.

Values of $R_{NH4^+,AOB}/R_{NO2^-,NOB}$ and $R_{NO2^-,AMX}/R_{NO2^-,NOB}$ represent the relative activities of autotrophic microorganisms. $MABR_1$ displayed low ratios at the beginning, indicating high NOB activity and low AMX activity (Cont phase, Fig. 1B). It corresponded with the accumulation of NO_3^- and the minor removal of total N at that time (18 ± 3 mg NO_3^--N/L and 10 ± 4% removal of TN). The value of $R_{NH4^+,AOB}/R_{NO2^-,NOB}$ increased dramatically during the following Int_{6+6} aeration. The ratio decreased again during Cont* phase and repeatedly increased during Int_{6+6}* phase. The repeated observation confirmed NOB prosperity under continuous aeration and suppression under intermittent aeration, and that the activity changes were controlled by aeration strategies. The value of $R_{NO2^-,AMX}/R_{NO2^-,NOB}$ also increased during Int_{6+6} aeration, suggesting AMX activation. Accordingly, bulk NO_3^- decreased and bulk NO_2^- disappeared at day 68; despite the reduced aeration supply under intermittent aeration, bulk NH_4^+ displayed no significant changes; the total N removal increased to 21 ± 7% (Fig. 1A). While AMX activity declined from Cont* to Int_{6+6}* likely due to decreasing temperatures [26], $R_{NO2^-,AMX}/R_{NO2^-,NOB}$ still increased. The variation of $R_{NO2^-,AMX}/R_{NO2^-,NOB}$ followed a similar trend with $R_{NH4^+,AOB}/R_{NO2^-,NOB}$. Hence, AMX activation under intermittent aeration was most likely related to NOB suppression as NO_2^- produced at the biofilm base by AOB could be utilized by AMX in the external anoxic layer. For instance, AMX activity increased by 200% from Cont to Int_{6+6}, meanwhile NOB activity dropped by 80%. When NH_4^+ and NO_2^- were both supplied in $MABR_4$ AMX

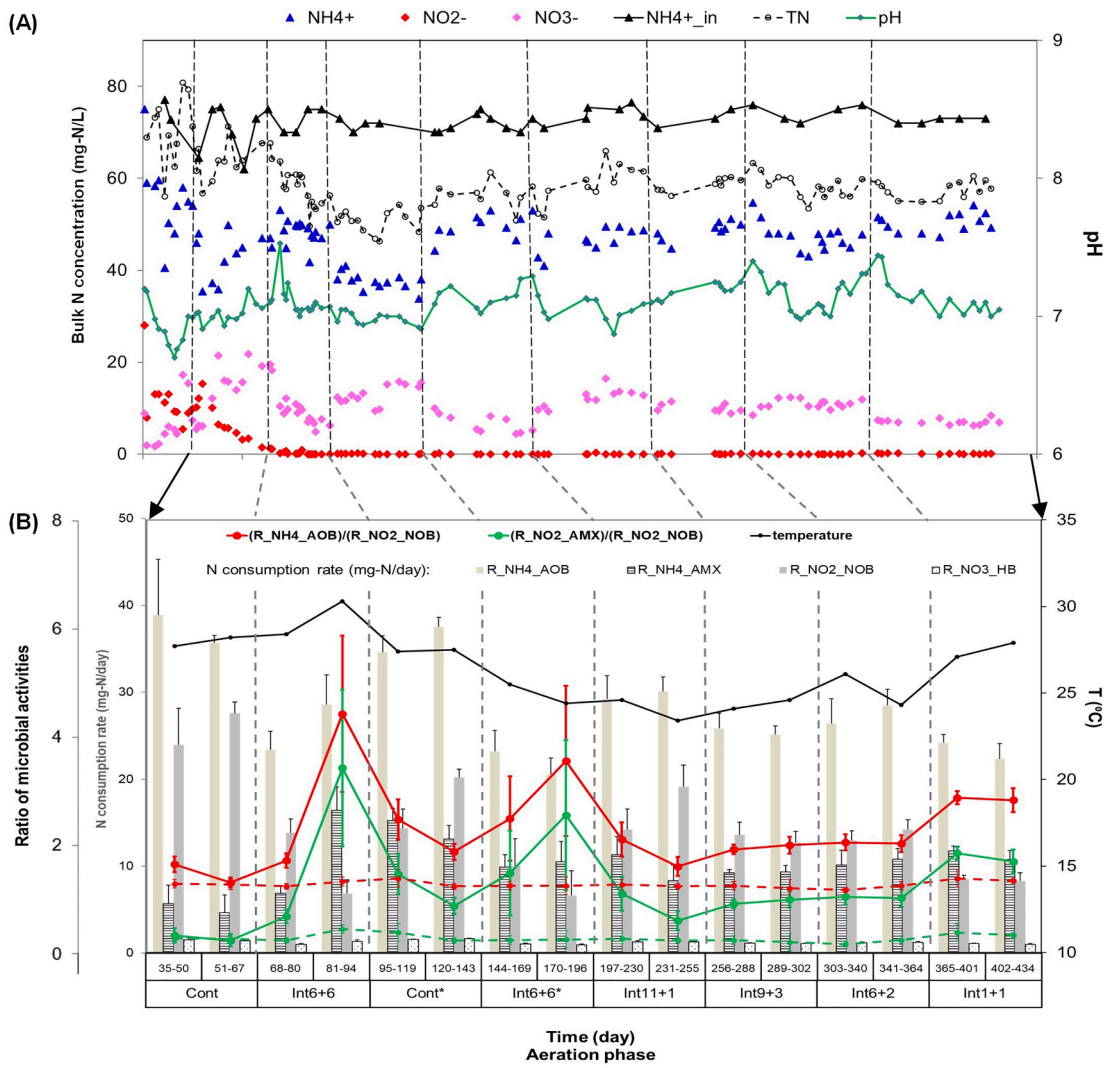

Fig. 1. $MABR_1$ performance during aeration control: (A) measurements of bulk N species and bulk pH, (B) relative variations of microbial activities represented by the activity ratios (the estimated N activities are shown in the bar chart) and the working temperature. Dash lines represent the reference ratios in $MABR_2$ which was always operated under continuous aeration.

activity was significantly enhanced (Fig. S9). AMX was enriched under intermittent aeration compared to continuous mode, which was identified in batch-mode tests as $MABR_1$ achieved a two-fold extant AMX activity over $MABR_2$ (Fig. S6).

HB activity, whether estimated as full denitrification with NO_3^- (Fig. 1) or partial denitrification with NO_2^-, was negligible in the overall N removal performance of both MABRs (Table S1). Low HB activity was further confirmed in the extant heterotrophic denitrification batch test, as bulk NO_2^- was not consumed in the bulk phase when no NH_4^+ was present (Fig. S7).

3.2. DO and pH microprofiles: comparison between continuous and intermittent aeration

In situ microprofiles of DO and pH were measured in $MABR_1$ under both continuous (Cont and Cont*) and intermittent (Int_{6+6} and Int_{6+6}*) aeration, to explore the chemical gradients within counter-diffusion biofilms (Fig. 2). Transient profiles of biofilm DO and pH upon air on–off switches were also recorded (Fig. 2C-D). Then, the local variations of DO and pH within biofilms between different aeration regimes were compared (Table 2). The main results are: (1) biofilm DO profiles during air-on periods were similar between continuous and intermittent aeration, as DO at the biofilm base ($p = 0.72$) and oxygen penetration depth ($p = 0.62$) were not significantly different; (2) upon air on–off switches, biofilm DO reached steady state rapidly ($t_{trans,DO} < 1$ min); (3) pH decreased from the bulk to the biofilm base due to nitrification when air was on, but showed an opposite trend when air was off (up to 7.52 ± 0.03 at the biofilm base), likely due to continuous CO_2 stripping from the biofilm base to the membrane lumen [21]; (4) bulk pH was significantly different between continuous and intermittent aeration ($p \ll 0.001$), while no significant difference of pH at the biofilm base was observed when air was on ($p = 0.56$); (5) upon air-on switches, biofilm pH decreased and reached steady state slowly ($t_{trans,pH} \approx 30$ min), therefore, pH stabilization in biofilm lagged behind DO stabilization under intermittent aeration.

3.3. N_2O emissions and production before and after Int_{6+6} aeration

Total N_2O emissions from nitrifying $MABR_2$ represented 0.37% of the N-load (Table S2). Contrarily, the emissions from $MABR_1$ were higher and reached the peak at 2.35% of the N-load during the initial continuous aeration, and emissions in the off-gas were comparable to those in the liquid phase (Cont phase, Table S2). Nevertheless, N_2O emissions decreased dramatically during the subsequent Int_{6+6} aeration phase and remained low even after continuous aeration was resumed (<0.35% of the N-load). Upon air on–off switches in $MABR_1$, off-gas N_2O was

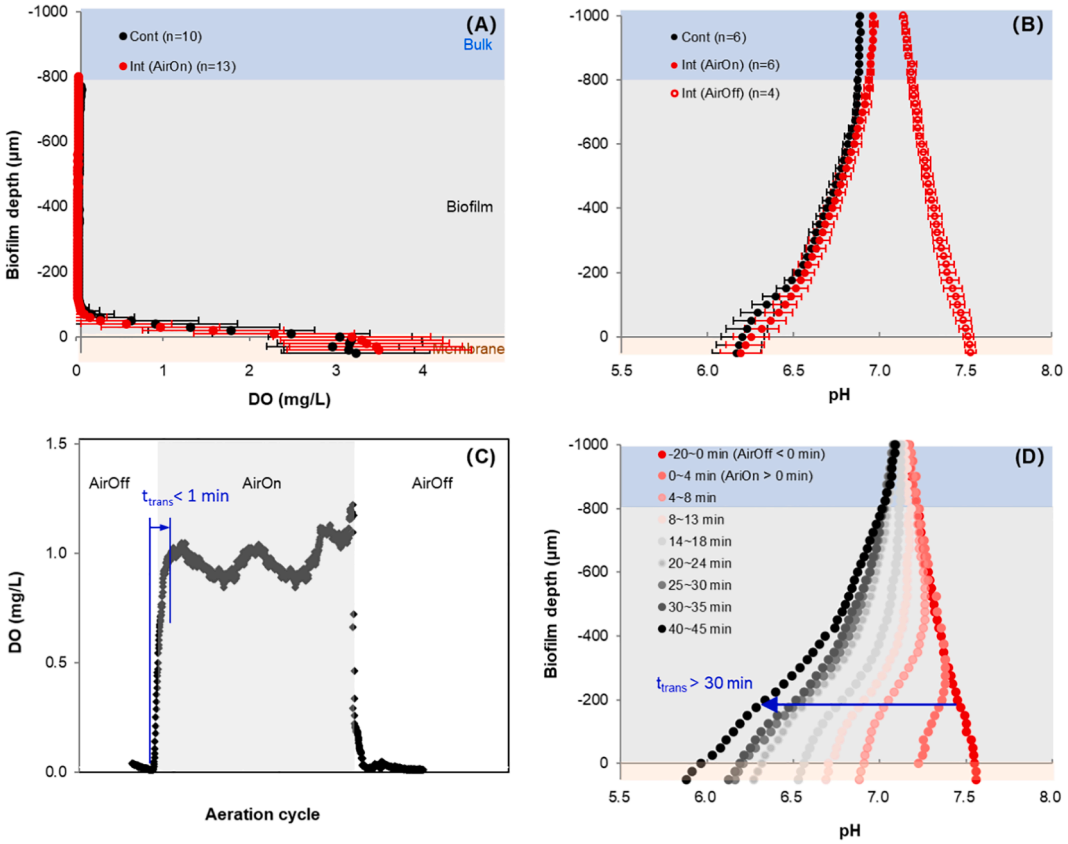

Fig. 2. Comparison of microprofiles in $MABR_1$ between continuous aeration (Cont phase) and intermittent aeration (Int_{6+6} air-on and air-off phases): (A) DO profiles, (B) pH profiles, (C) time series of DO at the biofilm base upon air off–on switches (approx. depth = −30 μm), and (D) time series of biofilm pH upon air off–on switches. Concentrations of FA and FNA within biofilms were estimated: FA = 0.032–1.17 mg-N/L, and FNA < 0.025 mg-N/L (Fig. S6.A-B). Boundary layers are not shown and assumed the same between different aeration, as the recirculation rate remained unchanged.

Table 2
Comparison of biofilm DO and pH between continuous and intermittent aeration.

	Aeration control			Intermittent aeration	
	continuous	intermittent: air-on	p-value	intermittent: air-off	t_{trans}
[a]**DO**					
oxic zone (μm)	[c]63 ± 23 (n = 10)	67 ± 28 (n = 13)	0.62	–	1 min
DO at biofilm base (mg/L)	2.95 ± 0.83 (n = 10)	3.14 ± 0.91 (n = 13)	0.72	–	
[b]**pH**					
bulk pH	6.82 ± 0.08 (n = 28)	7.03 ± 0.08 (n = 14)	<0.001	7.02 ± 0.07 (n = 10)	30 min
pH at biofilm base	6.20 ± 0.15 (n = 6)	6.25 ± 0.13 (n = 6)	0.56	7.52 ± 0.03 (n = 4)	

[a] Oxic zone was defined as the oxygen penetration depth (μm) and DO at biofilm base was measured at the membrane-biofilm interface (mg/L).
[b] Bulk pH was daily measured with pH electrodes and pH at biofilm base was measured during microprofiling.
[c] Mean of n measurements (±std).

detected as low but highly dynamic ($t_{trans,N2O} \approx 1$ h, Fig. S5), while no obvious fluctuations in the liquid phase were observed.

The N_2O concentration in $MABR_1$ was significantly higher during Cont than Int_{6+6} phase (mean value within biofilms: 0.74 and 0.08 mg-N/L, respectively; Fig. 3A). Further, it showed different trends: N_2O concentration decreased from the bulk to the biofilm base during Cont phase, while it increased within biofilm during Int_{6+6} phase (air-on periods). N_2O emissions during air-off periods were negligible. Net volumetric reaction rates were calculated to study N_2O production in counter-diffusion systems (Fig. 3B): (1) during Cont aeration phase, N_2O was produced throughout the entire biofilm, especially at high rates in the top anoxic zone, with low consumption rates in the middle part of biofilm; (2) during air-on periods of Int_{6+6}, N_2O production hotspots were located in the basal oxic zone, while consumption occurred in the top anoxic zone; (3) low N_2O reaction rates occurred during air-off periods, consistent with the observation that N_2O emissions were minimal when air was off under intermittent aeration. N_2O microprofiles in $MABR_2$ and its production were found similar as those during Int_{6+6} air-on periods of $MABR_1$.

3.4. MABR performance under different intermittent aeration

$MABR_2$ achieved surficial NH_4^+ removal rates at 4.1 ± 0.4 g-N/(m^2·day) under continuous aeration, which were in the high range of other nitrifying MABRs (1.3–3.5 g-N/(m^2·day), Table S10), despite the low ARE (45 ± 5%). NRE (12 ± 4%) and NiE (1.26 ± 0.06) were also low in $MABR_2$. Different from the stable bulk performance in $MABR_2$, $MABR_1$ displayed changing performance with aeration control as ARE, NiE and NRE varied within the ranges of 26 ~ 53%, 1.23 ~ 7.44 and 6 ~ 36%, respectively (Table S3). Overall, NiE variations in $MABR_1$were more dynamic than NRE and ARE; among all the aeration strategies, Int_{6+6} phase had the highest NiE, relatively high NRE, and comparable ARE to the initial Cont phase.

Daily reactor performance was evaluated with the operating variables (Fig. 4). CCA results showed that samples of each aeration phase

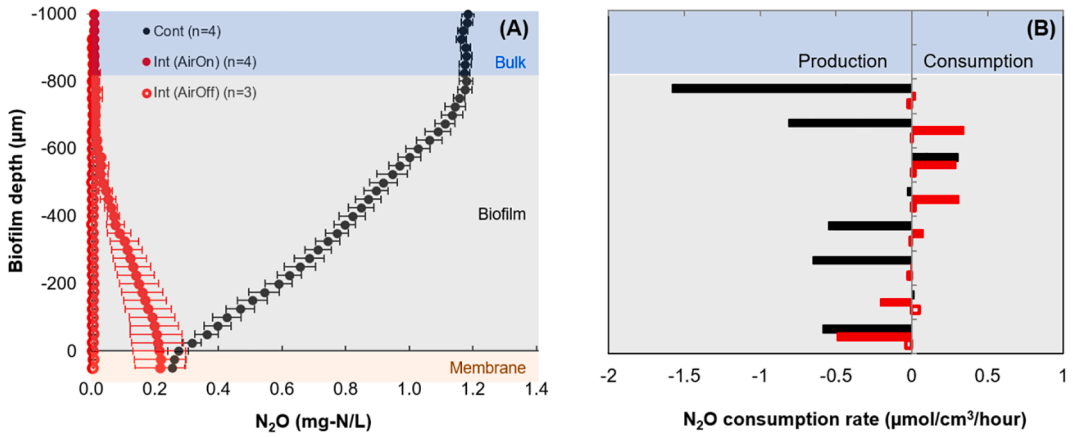

Fig. 3. Comparison of microprofiles in $MABR_1$ between continuous aeration (Cont phase) and intermittent aeration (Int_{6+6} air-on and air-off phases): (A) N_2O profiles, (B) spatial distribution of net volumetric N_2O production/consumption rates within biofilms.

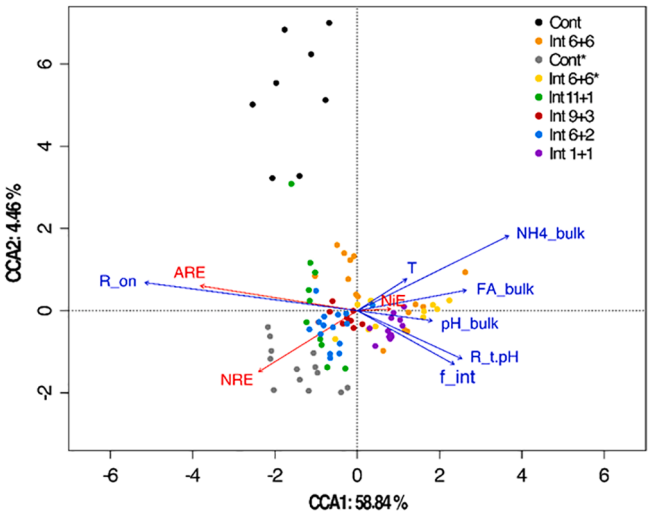

Fig. 4. Canonical correspondence analysis (scaling = II) for $MABR_1$ performance (ARE, NiE and NRE) and operating variables (f_{int}, R_{on}, $R_{t,pH}$, T, pH_{bulk}, $NH_4^+{}_{bulk}$ and FA_{bulk}) during aeration control. See data in Table S4.

assembled together. R_{on} and $NH_4^+{}_{bulk}$ had the most decisive but contrary roles in promoting N conversions, while T had minimal influence . Except R_{on}, arrows of all the other variables were adjacent with angles between two arrows <90°, implying that R_{on} had the largest effect on reactor performance. Arrows of ARE and NRE were in the opposite direction of NiE, indicating a trade-off between the suppression of NOB and the removal of NH_4^+ and total N. $R_{t,pH}$ was considered as the third control parameter of aeration, besides R_{on} and f_{int}, as $t_{trans,pH}$ was comparable to air-on duration under intermittent aeration (Table 2) and potentially exerted significant effects on microbial activities. The influence ranking of the three parameters on $MABR_1$ performance was $R_{on} > R_{t,pH} > f_{int}$. High R_{on} (with long aeration duration) led to high ARE and NRE but low NiE, e.g., in the comparison of Int_{6+6}, Int_{9+3}, and Int_{11+1}. High $R_{t,pH}$ (with long $t_{trans,pH}$ or short aeration duration) or high f_{int} (with high intermittency) generally resulted in low ARE and NRE but high NiE, e.g. the comparison of Int_{1+1} and Int_{6+2}.

4. Discussion

4.1. Nitritation under intermittent aeration and the key aeration parameter

The repeated increase of $R_{NH4^+,AOB}/R_{NO2^-,NOB}$ when going from continuous aeration to intermittent aeration indicated NOB suppression, and that the suppression was reversible and maintained through aeration control for over 200 days (Fig. 1). NOB suppression by intermittent aeration has also been observed in other studies: MABRs produced effluent with NO_3^- and not NO_2^- under continuous aeration, but the PNA process was realized with decreased NOB abundance after intermittent aeration [19]; stable nitritation was obtained under intermittent aeration, independent of the sludge age in sequencing batch reactors (SBR) [27]. In the following discussion, potential reasons why intermittent aeration resulted in MABR nitritation are discussed based on both *in situ* microprofiles and bulk observations, which are further compared with a model-based study of the same biofilm system [21].

AOB and NOB kinetics can be influenced by DO and pH via DO limitation effect, direct pH effect on enzymes, and indirect pH effects on substrate/inhibitor speciation such as FA and free nitrous acid (FNA) [21,28]. DO and pH effects on NOB activities are compared between continuous and intermittent aeration in $MABR_1$. This study reveals that DO within biofilms does not change with aeration control, so does its limitation effect which is evaluated with Monod-type kinetics. Therefore, this effect does not contribute significantly to NOB suppression in $MABR_1$. Some studies presented different observations highlighting DO limitation, as nitritation happened in continuously-aerated MABRs (3 mg-NH_4^+_N/L influent) when the membrane-biofilm interface DO was below 3.5 mg/L but it gradually deteriorated as DO increased [29]. Contrarily, Pellicer-Nàcher et al. [19] maintained nitritation in MABRs (500 mg-NH_4^+_N/L influent) with increasing DO when the lumen air increased from 2.5 to 60 kPa. It is uncertain to relate NOB suppression simply to DO limitation, consistent with our previous model study [21]. Lackner and Smets [30] further concluded that NOB suppression in counter-diffusion biofilms was less determined by DO limitation effects, compared to co-diffusion biofilms.

Biofilm pH significantly increased from Cont to Int phase due to reduced NH_4^+ oxidation at lower air (oxygen) supply. Accordingly, pH effects differed. A bell-shaped pH-dependence was proposed for nitrifying enzyme kinetics (direct pH effects), indicating a weakly alkaline optimum pH for AOB (8.2 ± 0.3) and NOB (7.9 ± 0.4) [31]. Therefore, pH upshifts during intermittent aeration might create a more suitable environment for AOB and NOB growth. While the pH_{bulk} effect on NiE was minor (Fig. 4), the effect of biofilm pH especially at the biofilm base could be higher ($pH_{biofilm}$ was not included in the analysis due to the limited measurements (n = 6, Table 2)). Indirect pH effects relate to FA and FNA speciation as both substrates and inhibitors [23]. Under intermittent aeration, FA concentration increased with increases of pH and bulk NH_4^+ (up to 1.17 mg NH_3-N/L estimated, Fig. S4A); NOB suppression likely occurred as NOB are more sensitive to FA inhibition than AOB [32,33]. $MABR_3$ operation with increasing FA loadings also

showed improved nitritation performance with an approximately two-fold activity increase in AOB compared to NOB (Fig. S8). The findings highlight FA as an inhibitor for NOB, as well as a substrate for AOB, consistent with the CCA analysis that FA_{bulk} and $NH_4^+{}_{bulk}$ were positively correlated with NiE. $NH_4^+{}_{bulk}$ has also been highlighted in supporting AOB to outcompete NOB as growth substrates, as a minimum residual NH_4^+ (~7.3 mg-N/L) was set in intermittently-aerated activated sludge to retain AOB at optimal grow rates [11], thus allowing the system to run at a critical SRT to wash out NOB. As NO_2^- concentration remained low after day 68 (<0.1 mg-N/L), FNA concentrations were likely much lower than the reported inhibition coefficient (0.04–0.1 mg-N/L in a model study [23]; 0.24–1.35 mg-N/L in a laboratory test [34]). However, variations of its inhibition effects cannot be distinguished between different aeration phases (Fig. S4B).

Aeration control has also been tested in activated sludge for successful nitritation [11,16,35,36] when, based on bulk measurements, aeration is shut down at the end of NH_4^+ oxidation and before NOB activity. This study presents a different mechanism for NOB suppression in MABRs: aeration length is manipulated (not based on real-time measurements) aiming at an appropriate NH_4^+ (FA) level to favor AOB over NOB as a growth substrate or inhibitor, most likely via intensified pH effect(s) from continuous to intermittent aeration. Temperature also affects nitritation with elevated temperature (>15 °C) usually favouring the growth of AOB over NOB [37]. But the change in temperature in $MABR_1$ (20–31 °C) had minimal influence on NiE (Fig. 4), indicating that growth in biofilms may alleviate temperature effect on AOB-NOB dynamics [26].

Assessment of nitritation with the three aeration parameters, including R_{on}, $R_{t,pH}$ and f_{int}, sheds further light on NOB suppression by aeration control. R_{on} is more determinant than $R_{t,pH}$ and f_{int} as it has a higher (negative) effect on $NH_4^+{}_{bulk}$ and FA_{bulk} acting as the growth substrate or inhibitor, supporting the pH as the determinant factor in AOB-NOB competition. Hence, a maximum R_{on} should be set to ensure sufficient NH_4^+ (or FA) for NOB wash-out. It might be the reason why NO_2^- effluent was observed with high $NH_4^+{}_{bulk}$ in a PNA reactor with 30-min air-on and 30-min air-off cycles, but NO_3^- effluent occurred with low $NH_4^+{}_{bulk}$ when R_{on} was prolonged (45-min air-on and 15-min air-off) [38]. With R_{on} fixed, higher f_{int} or $R_{t,pH}$ results in higher NiE but lower ARE (Fig. 4). This indicates that higher frequency or longer duration of pH transitions decreased the activities of NOB and AOB at the same time, due to either a common inhibitor such as FA as discussed above or their lag phases after anoxic disturbances [17,18]. However, the impact of f_{int} on nitritation is the lowest among the three parameters, although a higher aeration intermittency poses more often lag phases after anoxia. The influence of transient phases on AOB outcompeting NOB is less determinant, likely because air-on durations in $MABR_1$ (1–11 h) were much longer than the observed NOB lag phases (5–15 min [18]) or pH stabilization phases (>30 min, Fig. 2D). In agreement with the previous model evaluation [21], this experiment demonstrates that NOB suppression is promoted by low R_{on}, high f_{int} or high $R_{t,pH}$; nevertheless, aeration intermittency and transient phases are less determinant than aeration duration as the key control parameter of intermittent aeration.

4.2. Decreased N_2O emissions in $MABR_1$

While both MABRs were started with continuous aeration and nitrifying biofilms developed, total N_2O emissions in $MABR_1$ during Cont phase (2.4 ± 0.9%) were much higher than those in $MABR_2$ (0.4 ± 0.1%). $MABR_1$ emissions were comparable to emissions from partial nitritation (PN) processes either in sequencing batch operation (5.6% or 0.8% [39,40]) or continuous operation (4.0% or 1.7% [41,42]). It challenges common knowledge that conventional nitrifying processes produce less N_2O than PN processes [40], for instance, the emissions from nitrifying $MABR_2$ and in nitrifying activated sludge are as low as 0.1–0.4% of the oxidized NH_4^+-N [43]. Under continuous aeration , the high emissions from $MABR_1$ were likely due to the existence of bulk NO_2^- [44]. Although NO_2^- accumulation was as low as 1 mg-N/L at the end of Cont phase, N_2O was produced in both oxic and anoxic zones within the biofilms (Fig. 3B). Further, the production rates of HB were much higher than those of AOB in $MABR_1$ at that time. It is consistent with previous observations of N_2O production in a nitrifying biofilm under both oxic and anoxic conditions in the presence of NO_2^- [45].

After $MABR_1$ operation was changed from continuous to intermittent aeration, total N_2O emissions decreased to 0.3 ± 0.2%. This low emission level has often been reported in counter-diffusion biofilms (<0.1%) [19,46]. The significant decrease also related to bulk performance – the disappearance of NO_2^- under intermittent aeration because of the activated AMX. While NOB suppression by intermittent aeration could have resulted in NO_2^- accumulation AMX activity increased , scaenging residual NO_2^- from both the anoxic biofilm and the liquid phase. The production from heterotrophic pathways was reduced, despite bulk NO_3^- accumulation. Likely NO_2^- and not NO_3^- was the true denitrification substrate [39]. The role of AMX in competing for substrate with denitrifying HB, and thus preventing N_2O production, was also observed in a continuously-aerated MABR [13], where N_2O emissions decreased from 10% of the removed N to almost zero after AMX activation. The analysis of bulk measurements fits well with the calculated volumetric N_2O reaction rates. Different from during the Cont phase, N_2O was solely produced by AOB during the Int_{6+6} phase at the biofilm base where NH_4^+ oxidation occurred. Meanwhile, a zone of N_2O reduction by denitrifying HB established in the outer anoxic biofilm layer. As HB can compose 50% of the total bacteria in autotrophic nitrifying biofilms [47], their anoxic N_2O reduction can minimize N_2O diffusion into the liquid phase in counter-diffusion biofilms. Similarly, N_2O production in the deep part of autotrophic AMX granules could be consumed by denitrifying heterotrophs using the organic matter produced from biomass degradation [41].

Overall, the low N_2O emissions after intermittent aeration of Int_{6+6} was attributed to the low production and high consumption; AMX consuming NO_2^- contributed to the low production, and denitrifying HB contributed to the high consumption. Aeration control of the autotrophic N removal biofilms realized a desired balance between microbial activities that mitigated N_2O emissions . Aeration control has also been utilized in real wastewater for N_2O reduction, but there heterotrophic N_2O consumption with influent organic carbon was the target [48].

4.3. Ammonium and nitrogen removal as affected by intermittent aeration

For NH_4^+ and nitrogen removal in MABRs, the oxygen transfer rate (OTR, g-O_2/m^2/day) is generally the limiting factor [4]. The OTR is the oxygen gradient within the membrane times the membrane mass transfer coefficient (K_{o2}, m/day), which can be estimated based on the *in situ* DO microprofiles [25] or calculated from the microbial activities based on stoichiometry. We find that $MABR_1$ OTR during air-on periods ($OTR_{air\text{-}on}$, g-O_2/m^2/air-on hour) increased by ~20% from continuous to intermittent aeration (Table S4). As the DO concentration at the biofilm base did not significantly change with aeration control, nor did the oxygen concentration in the membrane lumen , the increased OTR was probably caused by an enhanced K_{o2}. It has been suggested that the overall mass transfer in MABR biofilms can be catalyzed by elevated biofilm activities [22]. For example in $MABR_1$ a 10–60% increase in the activity of AOB – the main oxygen consumers – was noted during air-on periods from $Cont^{(*)}$ to $Int_{6+6}{}^{(*)}$ (Table S1). In $MABR_3$ a higher AOB activity, associated with higher NH_4^+/FA loadings, also led to higher OTR under the same aeration conditions (Table S7). Therefore, $OTR_{air\text{-}on}$ (oxygen flux) in MABRs increases with increased bacterial activity , despite the reduced air supply (oxygen surface loading) under intermittent versus continuous aeration. The increased oxygen transfer into $MABR_1$ did not interfere with NOB suppression.

The enhanced OTR and the activated AMX ativity stimulated NH_4^+ removal under intermittent aeration. ARE during Int_{6+6} aeration was

therefore higher than 50% of ARE during Cont aeration (Table S3). Activated AMX also increased the total nitrogen removal. The potential AMX activity in $MABR_1$ was measured as 8.1 g-NO_2^-_N/(m^2·day) in a batch test, which revealed complete consumption of the maximal NO_2^- production of 3.1 g-NO_2^-_N/(m^2·day). NRE in $MABR_1$ was limited by NO_2^- availability, as also observed for $MABR_4$ (Table S8) and other PNA biofilms (Table S10). Provided that AMX is activated, NRE increased with the relative aeration duration under intermittent aeration (Fig. 4), as NO_2^- production increased with longer aeration phases. Competition for NO_2^- between NOB and AMX contributed little to NOB suppression, as NOB recovered once $MABR_1$ was operated again with continuous aeration, and similarly in $MABR_4$ when NO_2^- was supplied in the influent, even though AMX remained active. Our findings support that NOB can be outcompeted by AOB, but not by AMX [49]. Our study concludes that intermittent aeration has the potential to realize nitritation in MABRs, while maintaining high NH_4^+ and total nitrogen removal by activating the anammox process and enhancing oxygen flux into the biofilms.

5. Conclusions

Lab-scale MABRs were operated under continuous and intermittent aeration regimes, and the nitrogen conversions were monitored.

- A nitrifying biofilm developed in MABRs under continuous aeration, while under intermittent aeration NOB activity was suppressed and AMX activity was enhanced. NOB suppression was likely due to pH effects, as the presence of FA, a substrate for AOB and an inhibitor for NOB, was more significant under intermittent aeration. DO limitation and temperature did not seem to control NOB suppression in this study.
- This is the first experimental study that documents the dynamics of biofilm DO and pH profiles upon air on–off switches in MABRs, and reports that pH recovery lags behind DO recovery in intermittently-aeration biofilms.
- While the denitrifying activity remained low and unchanged with aeration control , heterotrophic bacteria played a critical role in N_2O dynamics as either N_2O producers or consumers in the counter-diffusion biofilms.
- Intermittent aeration regulated nitrogen conversions in MABRs with the relative aeration duration as the key determinant parameter. Aeration control is a feasible approach to realize energy-efficient nitrogen removal and mitigate N_2O emissions from counter-diffusion MABR biofilms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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