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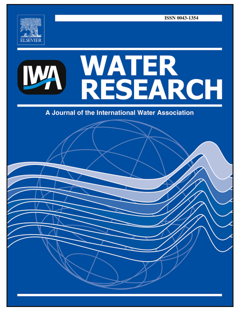


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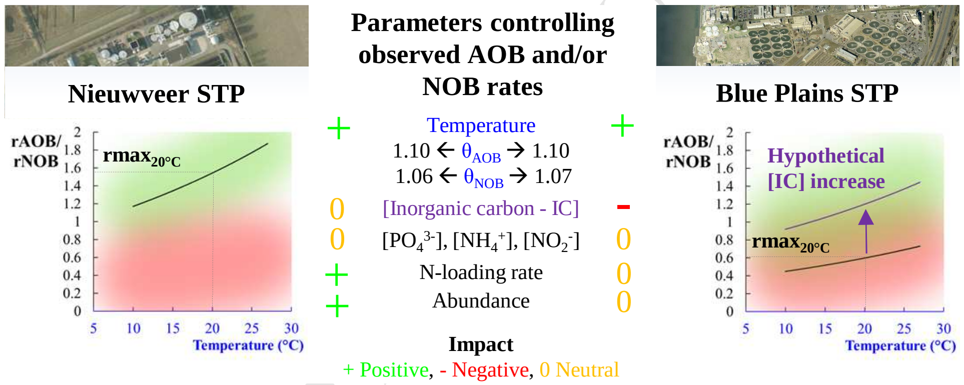
Nieuwveer STP
Parameters controlling observed AOB and/or NOB rates
Blue Plains STP
rAOB/ rNOB
rmax20°C
Temperature (°C)
Temperature
1.10 ← θAOB → 1.10
1.06 ← θNOB → 1.07
[Inorganic carbon - IC]
[PO4 3-], [NH4 +], [NO2 -]
N-loading rate
Abundance
Hypothetical [IC] increase
Impact
+ Positive, - Negative, 0 Neutral

# Pinpointing wastewater and process parameters controlling the AOB to NOB activity ratio in sewage treatment plants

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# ABSTRACT

Even though nitrification/denitrification is a robust technology to remove nitrogen from sewage, economic incentives drive its future replacement by shortcut nitrogen removal processes. The latter necessitates high potential activity ratios of ammonia oxidizing to nitrite oxidizing bacteria (rAOB/rNOB). The goal of this study was to identify which wastewater and process parameters can govern this in reality. Two sewage treatment plants (STP) were chosen based on their inverse rAOB/rNOB values (at 20°C): 0.6 for Blue Plains (BP, Washington DC, US) and 1.6 for Nieuwveer (NV, Breda, NL). Disproportional and dissimilar relationships between AOB or NOB relative abundances and respective activities pointed towards differences in community and growth/activity limiting parameters. The AOB communities showed to be particularly different. Temperature had no discriminatory effect on the nitrifiers' activities, with similar Arrhenius temperature dependences ($\Theta_{AOB}$ = 1.10, $\Theta_{NOB}$ = 1.06-1.07). To uncouple the temperature effect from potential limitations like inorganic carbon, phosphorus and nitrogen, an add-on mechanistic methodology based on kinetic modelling was developed. Results suggest that BP's AOB activity was limited by the concentration of inorganic carbon (not by residual N and P), while NOB experienced less limitation from this. For NV, the sludge-specific nitrogen loading rate seemed to be the most prevalent factor limiting AOB and NOB activities. Altogether, this study shows that bottom-up mechanistic modeling can identify parameters that influence the nitrification performance. Increasing inorganic carbon in BP could invert its rAOB/rNOB value, facilitating its transition to shortcut nitrogen removal.


# KEYWORDS

Energy-positive, inorganic carbon, Monod, partial nitritation/anammox, phosphate

# 1. INTRODUCTION

Energy-positive sewage treatment can reduce the facility's carbon footprint and nutrient emissions (N, P) to water bodies in a cost-effective manner. This can be achieved in a two-stage approach. In a first stage, organic carbon-rich constituents are redirected to a digester that produces biogas (Meerburg et al., 2015). As insufficient organic carbon is remaining to remove nitrogen via conventional nitrification/denitrification (N/DN), short-cut nitrogen removal technologies like nitritation/denitritation (Nit/DNit) or partial nitritation/anammox (PN/A) are encouraged in a second stage. In this way discharge limits are reached while the need for external carbon dosing is avoided (Verstraete & Vlaeminck, 2011). One of the key challenges to achieve a robust process operation is the suppression of nitrite oxidizing bacteria (NOB), while maximizing the activity of aerobic ammonium oxidizing bacteria (AOB). Different strategies focused on one or a combination of ON/OFF control e.g. by kinetic suppression by dissolved oxygen (DO) control, or by IN/OUT control, where selective wash-out of NOB is strived for (Cao et al., 2017). These combined strategies on real wastewater showed promising results, yet no full eradication of NOB, so more insights are necessary. As most studies focused on controllable process parameters like DO-setpoints, loading rates, residual ammonium levels or sludge retention times (SRT) to achieve NOB-suppression, they tend to overlook the additional effect of mostly location-specific wastewater characteristics like inorganic carbon, phosphorus or temperature on the activity (=ON/OFF control) of AOB and NOB.

Two strategies are commonly applied to link the activity or abundance of biomass with process parameters and wastewater characteristics in STP. The first strategy is to link these parameters with the abundance of different genera by means of easy applicable statistical exploratory data analysis tools, e.g., correlations and principle component analysis (Huang et al., 2010; Meerburg et al., 2016). These studies mostly focused on unraveling niche differentiation and did not include the link with actual microbial activities. Moreover, the uncoupling of different wastewater parameters may be challenging due to multiple correlations among wastewater parameters. The studies were however important to define which AOB and NOB are commonly selected by the long-term environmental pressures, and thus which kinetic parameters should be used in mathematical models. They showed that *Nitrosomonas* (AOB) and *Nitrospira* (NOB) were the most common nitrifying genera in STP (See Table 1) (Daims et al., 2001; Rowan et al., 2003), whereas *Nitrosospira* (AOB), *Nitrobacter* (NOB) and *Nitrotoga* (NOB) were less frequently encountered (Lücker et al., 2015; Rowan et al., 2003).

**Table 1.** Substrate affinity of *Nitrosomonas* AOB and *Nitrospira* NOB as most common nitrifiers in conventional nitrogen removal systems, according to the Monod saturation model. Numbers between brackets give the average and standard deviation, calculated from literature: $K_{NH4}$ **for ammonium oxidation (AOB) and NOB growth** (Koops et al., 2001; Henze, 2008), $K_{NO2}$ (Manser et al., 2005; Park et al., 2017; Ushiki et al., 2017), $K_{O2}$ (Summarized in Table A.1), $K_P$ (van der Aa et al., 2002; de Vet et al., 2012), $K_{TIC}$ (Guisasola et al., 2007). N.A.: not applicable, $^+$ Assumed to be non-limiting.

| | **AOB - *Nitrosomonas*** | | **NOB - *Nitrospira*** | |
|---|---|---|---|---|
| | **Literature** | **Model** | **Literature** | **Model** |
| **$K_{NH4}$ (mg N $L^{-1}$)** | 0.42-0.85 - 1 | 1.0 | <0.001$^+$ | 0.001 |
| **$K_{NO2}$ (mg N $L^{-1}$)** | N.A. | N.A. | 0.08-0.52 [0.22±0.15] | 0.23 |
| **$K_{O2}$ (mg $O_2$ $L^{-1}$)** | 0.033-1.16 [0.36±0.4] | N.A. | 0.04-0.47 [0.19±0.15] | N.A. |
| **$K_P$ (mg $PO_4^{3-}$-P $L^{-1}$)** | 0.003-0.05 | 0.045 | <0.0045$^+$ | 0.0045 |
| **$K_{TIC}$ (mM C)** | 1.78 | 1.78 | 0.1 | 0.1 |

A second strategy used process models to describe the performance in STP. These models combined transport, chemical and physical processes with earlier empirical models of bacteria's activity and growth into one complex model. Some of these software tools, both available in commercial (i.e. BioWin, Sumo, etc.) and non-commercial variants, include more advanced 2-step nitrification models. In a review on efforts on these models, most advanced models included, although not always combined, differentiation on kinetic parameters for decay, pH, growth rate, yield, anabolism (inorganic C, P), and catabolism (N, $O_2$) between AOB and NOB (Sin et al., 2008). Although these easy accessible models have been successfully applied to model shortcut nitrogen removal processes (Al-Omari et al, 2015), they still required substantial amount of time and complete sets of process data for case specific calibration and simulation (Hauduc et al., 2009). Furthermore, as they are mostly calibrated towards effluent concentrations, calibration for AOB and NOB potential activities is mostly not necessary and overlooked, yet crucial to run accurate shortcut nitrogen process models.

Since the previous statistical methods and modelling approaches not always accurately predicted AOB and NOB activity ratios, there is a need for easy adaptable methodologies that can assess limitations for the complex environment that constitutes a STP. In this study, abundances and activities were linked with commonly monitored process and wastewater parameters. We compared two STP; Blue Plains, Washington DC and Nieuwveer, NL, which had a certain degree of similarity in wastewater and process parameters; yet had a different AOB over NOB potential activity ratio (rAOB/rNOB). To acquire insight on the most important parameters that control their activity, a novel and easily implementable add-on mechanistic model was set up. The aim was to disentangle the effects of abundances and different process and wastewater parameters on rAOB/rNOB.

# 2. MATERIAL AND METHODS

## 2.1. DESCRIPTION AND SAMPLING OF THE PLANTS

Blue Plains was an advanced three-stage STP (Washington DC, USA) receiving municipal wastewater from the surrounding regions with an average flow rate of 1,140,000 $m^3$ $d^{-1}$ (about 4 million population equivalents**) (Figure B.1 for a schematic overview). The first** treatment stage is chemically enhanced primary treatment, which mainly removes particulate COD and phosphorus. The second stage is high rate activate sludge system (SRT = 2d), which removes biodegradable COD. The last stage is a plug-flow conventional nitrification/denitrification process. Here, the wastewater moves first through five aerobic (1.5 mg $O_2$ $L^{-1}$) sections of 247 000 $m^3$ total (12 parallel reactors), with the last stage being a swing zone (41,000 $m^3$; 0-0.5 mg $O_2$ $L^{-1}$). Finally, all the mixed liquor is combined and transferred to a denitrification reactor (177,000 $m^3$), where nitrogen is removed with the aid of methanol addition. Altogether, 13 grab samples of about 3L of mixed liquor were collected over the course of a year to capture the whole temperature range. They were taken from the second aerobic stage of the nitrification/denitrification process and sent to the lab within 10 minutes. For molecular analysis, mixed liquor samples were first centrifuged (10 min, 4,000 g) at 4 °C, after which the sludge pellet was stored at -80 °C for later transportation and analysis.

The Nieuwveer STP in Breda, NL functions as a two-stage STP, treating industrial and municipal wastewater from Breda and its surroundings, with over the studied period on average 72,000 $m^3$ (+- 340,000 population equivalents) of wastewater per day (Figure B.1 for a schematic overview). The first stage is a high-rate activated sludge treatment (SRT = 0.5 d), redirecting a substantial fraction of the incoming carbon stream to the anaerobic digester. $FeSO_4$ is added at the end of the A-stage to remove phosphorus. The second stage treats the effluent of the first stage, removing nitrogen by means of nitrification/denitrification. The A-stage effluent is split over four parallel reactors. Three of the four reactors are equal in configuration and built earlier, having a volume of 5400 $m^3$ with sequentially an anoxic, two facultative oxic, two oxic and again one facultative oxic section. The fourth reactor has a volume of 12,000 $m^3$ and sequentially two anoxic, four swing zones and four oxic (2.8 mg $O_2$ $L^{-1}$) sections. Both stages have the same sludge recycle ratio of 0.5. In the second stage an internal recycle ratio of 0.04-0.1 is applied. The final effluent is recirculated over the whole wastewater treatment plant, with a recirculation ratio that varied between 0.3 and 1.5 over the course of the study, since a sidestream PN/A was installed at that moment. From the first section of the second stage (4$^{th}$ reactor), over a period of 5 months, 9 samples of about 5L of activated sludge were taken. The fresh activated sludge was transported to Ghent, BE and stored overnight at room temperature to later determine the activity of AOB and NOB. It was assumed that transport and short-term storage did not affect activity of the nitrifiers. For molecular analyses, samples of 60 mL were taken at the plant and immediately centrifuged (10 min. at 4,000g). The obtained pellet was manually homogenized and subsamples of 0.5 mL were taken and flash-frozen in a cooling block of at least -20°C for transport, to be finally stored at -80°C.

### 2.2. Process and wastewater data

For Blue Plains, plant data (Table C.1) was obtained from the plant's main lab and flow measurements from the plant operation department. Physicochemical analyses ($NH_4^+$, $NO_2^-$, total soluble phosphorus, alkalinity, MLSS) were performed daily according to standard methods (USEPA Method 160.2, 1999 revision). The plant average and standard deviation data were calculated based on the measurements at the activity test days. The daily effluent samples were 24 h composite samples collected by an on-site autosampler located at the clarifier after anoxic-phase. Total inorganic carbon (TIC) present in the wastewater was calculated from the total alkalinity measurements, using the carbonate balance at a certain pH while assuming that other ions (P, volatile fatty acids) represented only 2-3% in the sewage matrix (Fairlamb et al., 2003).

$$Total\ inorganic\ carbon\ (mM\ C) = \frac{total\ alkalinity\ (mM\ H^{+})}{fraction\ HCO_3^{-} + 2 * fraction\ CO_3^{2-}} \qquad (\mathbf{eq.1})$$

For Nieuwveer, wastewater and process (SRT, HRT, R.factor) data (Table Q.1) were obtained from the Waterschap Brabantse Delta (NL), who operate the STP. Physicochemical analysis of the samples was done according to Standard Methods (Greenberg et al., 1992 ), and the measured and calculated parameters are summarized in Table 3. The wastewater parameters were not always measured on the same day that the AOB and NOB potential activity tests were run. For the continuous measured data, e.g. temperature, recirculation factor, HRT, two-day interval data before the test was used. For effluent $NH_4^{+}$-N, weekly averages before the data point were calculated from continuous measurements in reactor section 9, by an Amtax SC from Hach Lange. Some parameters were measured intermittently ($NO_2^{-}$, TP, TSS, SVI). In these cases, we always chose the data from samples collected closest in time to the batch activity tests, ranging from 2 days before to one day after.

### 2.3. AOB AND NOB POTENTIAL RATE DETERMINATION

See Appendix D.1.

### 2.4. Arrhenius model fitting

The non-linear Arrhenius temperature model fit was based on:

$$r_T = rmax_{20°C} \times \theta_T^{(T-20)} \qquad \textbf{(eq. 2)}$$

$rmax_{20°C}$ and $\theta_T$ were fitted to temperature and measured rates in the batch tests. Optimization of fit was performed by minimizing the sum of squared error (SSE) of prediction and actual measurements.

### 2.5. Statistical data analysis

A generalized linear model (glm) was fitted using the R language for statistical computing (Rstudio 0.99.903, R Development Core Team 2015). The description and mathematical details can be found in Results 3.5.1 and Supplemental Information E.1 respectively. Different limitations were added stepwise to the model, and the order was decided on 1) discovered correlations between wastewater and process parameters, and 2) the potential limitation occurring, deducted from literature saturation values.  More information can be found in Appendix D.1.

### 2.6. Molecular analyses

DNA extraction was performed by means of a DNeasy powersoil kit (Mo Bio). qPCR was performed to determine 16S rDNA abundances *Nitrospira*, *Nitrobacter* analogue to Courtens et al. (2016). In addition, 16S rDNA from AOB and all bacteria were also quantified, and specific procedure information is found in Table F.1.

For identification, the 16S rRNA gene V3-V4 hypervariable regions were amplified (De

Vrieze et al., 2016). Subsequently, absolute singleton operational taxonomic units - OTUs (i.e. OTUs with only a single read in the whole dataset) were removed and prevalence filtering was executed (McMurdie & Holmes, 2014). Subsequently, differential abundance testing was performed (based upon guidelines from the same publication) by means of the DESeq pipeline (as implemented in DESeq2, v. 1.16.0) (Love et al., 2014). In brief, size factors were estimated as well as the overdispersion parameter based upon the Negative Binomial distribution. Next, a Negative Binomial GLM was fitted with Wald statistics. Multiple comparison p-values were False discovery rate (FDR) controlled with the significance level set to 5% ($\alpha$=0.05) (Benjamini & Yekutieli, 2001).

# 3. RESULTS

## 3.1. Arrhenius model fit

To compare the temperature kinetics of both STP, an Arrhenius model was fitted in a non-linear manner to the measured potential activity data (Figure 1 and Table 2). Both STP had similar temperature theta coefficients for AOB (1.1) and NOB (1.06-1.07). Aside from this temperature effect, the fits interestingly showed the key observation that both STP had an inverse rAOB/rNOB $rmax_{20°C}$ ratio; i.e., the ratio is 0.61 for Blue Plains, and 1.68 for Nieuwveer. This indicated that some process and wastewater parameters might diversely influenced the activity or community of AOB and NOB.

## 3.2. Diversity in nitrifier communities

qPCR-based abundances revealed continuous presence of AOB, *Nitrospira* and *Nitrobacter*, with a slightly fluctuating AOB/NOB abundance ratio between 0.10-0.15 for both STP (See Figure 1). *Nitrospira* were always present in higher abundance than *Nitrobacter* - on average ~20 times higher for Nieuwveer, and ~10 times for Blue Plains.

Amplicon sequencing analysis was performed to further identify potential differences between the communities. Comparing both communities, more than half, or 1000 of 1765 OTU, differed significantly between the two STP. Different OTUs were identified as AOB and NOB on the genus level. For AOB, *Nitrosomonas* was identified as the sole genus. Out of the eight detected OTUs (see Figure 2), five had a significantly different abundance in both plants. Four OTUs, including the most abundant, were only present in either one of the STP. For NOB (see Figure G.1), only representative OTUs for *Nitrospira* were classified down to the genus level, while, in contrast to the qPCR, *Nitrobacter* was not.

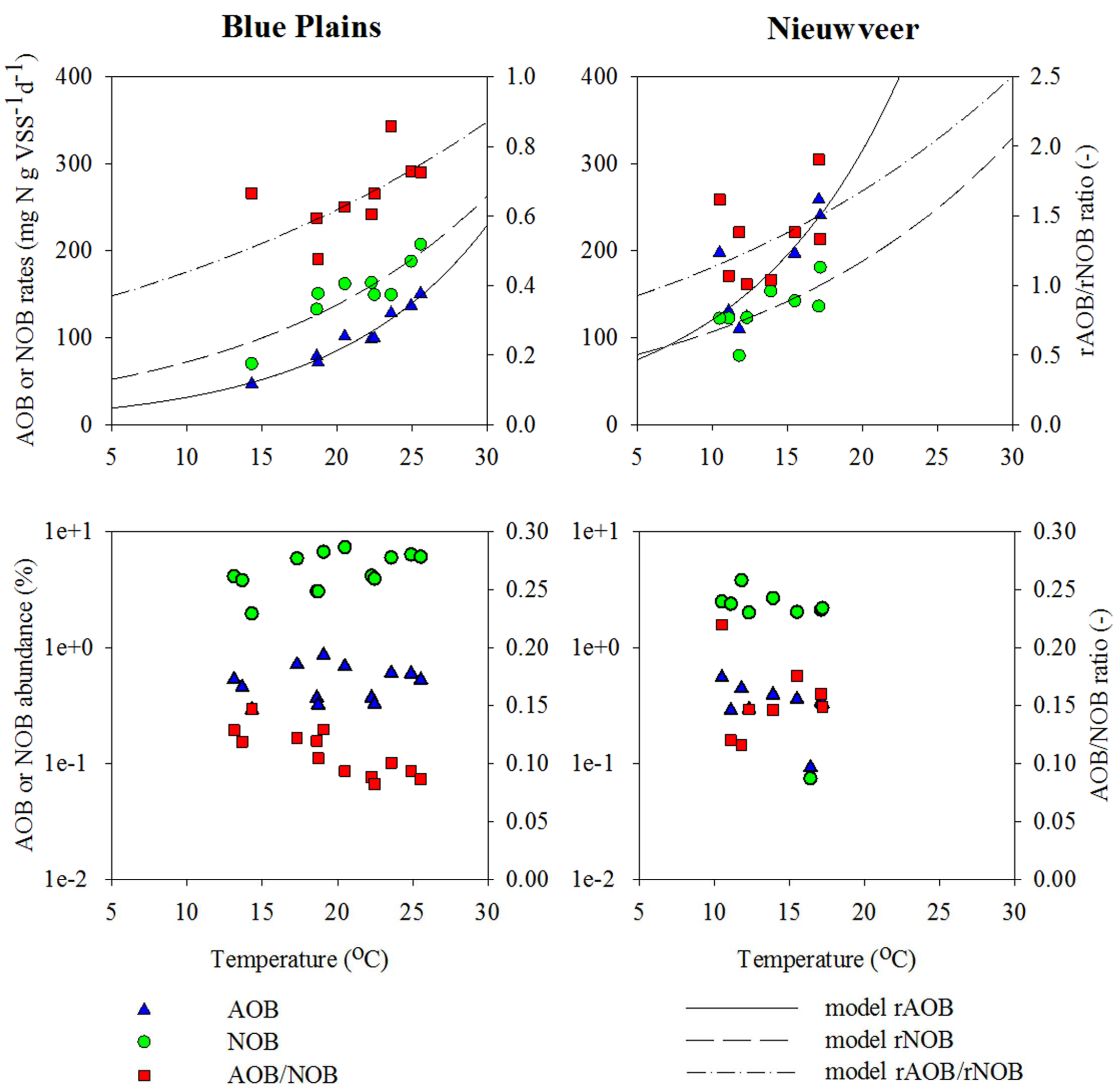



**Figure 1.** Measured potential activities (mg N g VSS$^{-1}$ d$^{-1}$) and relative abundances (16S rRNA gene count of AOB or NOB on total bacterial 16S rRNA gene count) and their respective ratios in function of temperature for Blue Plains and Nieuwveer, compared with a non-linear fitted Arrhenius temperature model.

**Table 2.** Arrhenius temperature model parameters: values obtained from data fitting to the non-linearized model (fits depicted in Figure 1), compared with literature values (Wiesmann, 1994; Wyffels et al., 2004).

| | | Blue Plains | Nieuwveer | Literature |
|---|---|---|---|---|
| **$\Theta_T$** | **AOB** | 1.1 | 1.1 | 1.10-1.12 |
| **(-)** | **NOB** | 1.07 | 1.06 | 1.06-1.07 |
| **$rmax_{20°C}$** | **AOB** | 85.4 | 315.2 | |
| **(mg N g VSS$^{-1}$ d$^{-1}$)** | **NOB** | 138 | 187.5 | |

Three of the six detected OTUs were significantly different between the two STP, of which the most abundant *Nitrospira* OTU coincided in both STP. This difference in *Nitrosomonas* community may lead to different kinetics, i.e. different $rmax_{20°C}$, while this is most likely not the case for the more similar *Nitrospira* community.

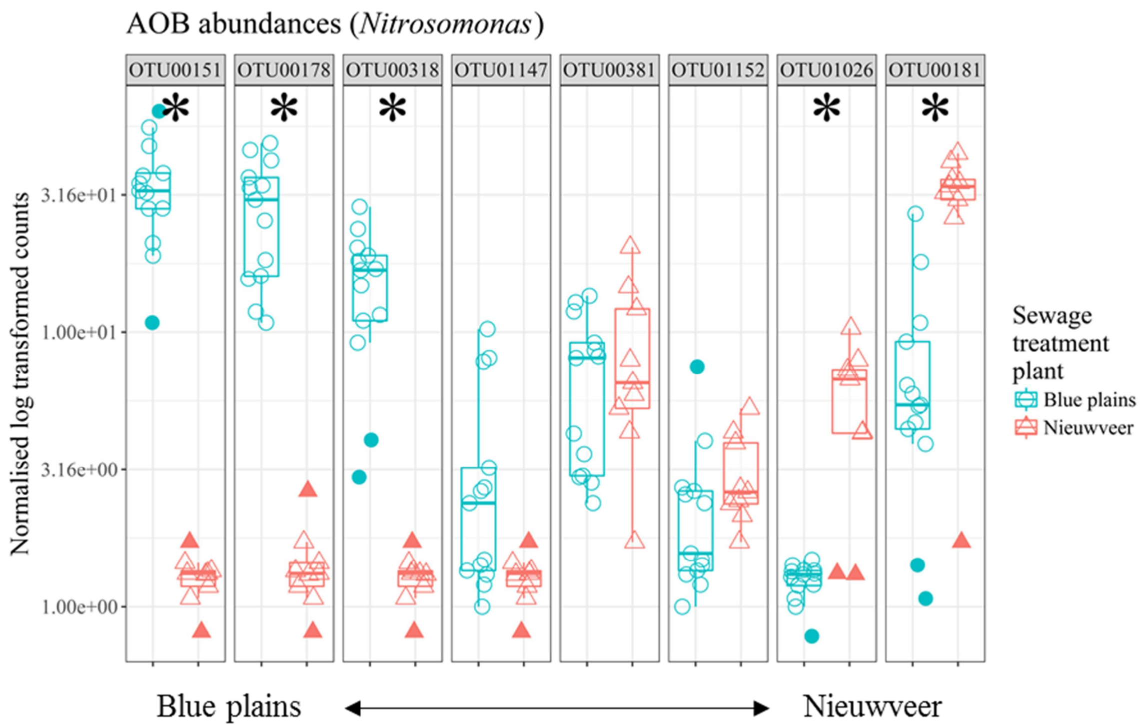



**Figure 2.** Differences in normalized log transformed counts for detected AOB OTU's for Blue Plains and Nieuwveer. Number next to the OTU is attributed when classifying the OTU's; the higher the number of reads over all samples, the lower the number. Closed symbols next to open symbols indicate outliers. OTU's with a star differed significantly (FDR=0.05).

### 3.3. DISSIMILAR RELATIONSHIPS BETWEEN NITRIFIER ABUNDANCE AND ACTIVITY

To assess the link between microbial abundance and activity, both STP were compared in Figure H.1. In this case, the difference in $rmax_{20°C}$ was not reflected in the change in average relative abundance (by qPCR). Whereas the relative NOB abundance in Blue Plains was almost twice as in Nieuwveer, the $rmax_{20°C}$ was 0.7 times lower. In contrast, AOB relative abundances were a factor 1.4 higher in Nieuwveer, but the $rmax_{20°C}$ was 3.7 times higher. The difference in measured $rmax_{20°C}$ could thus not be explained by a difference in relative abundance, yet some process or wastewater parameters or the above-mentioned community might explain the observed discrepancy.

### 3.4. (DIS)SIMILARITIES IN STP PROCESS AND WASTEWATER PARAMETERS

The Blue Plains STP, US and Nieuwveer STP, NL showed similarities and differences in their process and wastewater parameters. For Nieuwveer, no monitoring of pH and alkalinity was performed during the executed study and values depicted in Table 3 are from earlier or later measurement campaigns to acquire insight in possible limitations.

**Table 3.** Similarities and differences in process and wastewater parameters of the two plants (values represent mean ± standard deviation; difference evaluated at α=0.05). N: Nitrification; DN: Denitrification; HRT: Hydraulic retention time; SRT: Sludge retention time.

| | | **Blue Plains** | **Nieuwveer** |
|---|---|---|---|
| **Similar** | Reactor configuration | Plug-flow N/DN | Plug-flow DN/N/DN + internal recycle |
| | Carbon dosage | Methanol | None |
| | Year-round temperature (°C) | 14.3 - 25.7 | 9.5 - 22.6 |
| | Sludge concentration (g VSS $L^{-1}$) | 2.42 ± 0.60 | 2.49 ± 0.55 |
| | Reactor loading rate (g N $m^{-3}$ $d^{-1}$) | 88 ± 17 | 117 ± 14 |
| | Reactor N removal efficiency (%) | 94 ± 5 | 77 ± 10 |
| | Aerobic HRT (d) | 0.22 ± 0.03 | 0.10 ± 0.05 |
| | Anoxic HRT (d) | 0.15 ± 0.02 | 0.07 ± 0.02 |
| | Anoxic SRT (d) | 11.5 ± 7.6 | 11.7 ± 2.7 |
| | Aerobic SRT(d) | 16.0 ± 10.6 | 21.7 ± 4.9 |
| **Different** | Effluent alkalinity (mg $CaCO_3$ $L^{-1}$) | 75 ± 9 | 182 ± 31* |
| | Effluent inorganic carbon (mM C $L^{-1}$)° | 1.09 ± 0.13° | 3.48 ± 0.59° |
| | Effluent $PO_4^{3-}$-P (mg P $L^{-1}$) | 0.04 ± 0.04 | 1.39 ± 0.99 |
| | Effluent ammonium (mg N $L^{-1}$) | 0.22 ± 0.54 | 1.10 ± 0.83 |
| | Effluent nitrite (mg N $L^{-1}$) | 0.00 ± 0.01 | 0.52 ± 0.2 0 |
| | Reactor pH | 6.57 ± 0.10 | 7.65 ± 0.10+ |

* Measured values during a measurement campaign in June-August 2016 (Average of 3 dry weather values)
° Calculations based on alkalinity measurements and pH bicarbonate balance
+ Measured values are an average of the pH evolution between 1997-2009

Over the measurement campaign, both plants had similar nitrogen loading rates (~100 g N

$m^{-3}$ $d^{-1}$) and anoxic sludge retention times (±12d), although the aerobic sludge retention time is shorter in Blue Plains (16 vs. 22 d). Yearly temperatures are higher in Blue Plains (14.3-25.7 °C) than in Nieuwveer (9.5-22.6 °C), with a similar seasonal temperature DT of 11-13°C. The influent raw sewage and different pretreatment steps prior to N/DN resulted in different wastewater parameters in the reactor. These differed significantly ($p<0.05$) between the two STP: effluent inorganic carbon (as alkalinity), ammonium ($NH_4^+$-N), nitrite ($NO_2^-$-N), and phosphorus (P) were much more limited in Blue Plains than Nieuwveer, creating potential activity and growth limitations (see Table 1). For Nieuwveer, only nitrogen levels ($NH_4^+$-N /$NO_2^-$-N) could limit their potential activity.

## 3.5. SPECIFIC PROCESS AND WASTEWATER LIMITATIONS CONTROL ACTIVITIES

To further investigate what process and wastewater parameters affected AOB and NOB activities in the two STP, a Spearman non-linear correlation analysis (Table I.1 and J.1) was executed. In Table 4, all significant correlations ($p<0.05$) for a selection of wastewater parameters are depicted. For Blue Plains, positive correlations were found between the measured potential activities and their ratios with temperature, phosphorus (P), inorganic carbon (IC) and pH. For the latter three, temperature can act as a possible confounder since it also had positive correlations with these variables. For Nieuwveer, aside from temperature, these wastewater characteristics didn’t show positive correlations. In contrast, the sludge-specific N-loading rate and sludge retention time (SRT) showed positive correlations with the measured rates and their ratios. Temperature could also act as a confounder here, so elucidation required further assessment.

**Table 4**. Spearman’s rank correlation coefficients between biomass properties (activities and relative qPCR abundances) and process and wastewater parameters in the effluent/mixed liquor (‘-’ indicates no significant

correlation; $p<0.05$). Cells with a grey background were not included in the analyses: for Blue Plains, nitrite was below the detection limit, and for Nieuwveer, inorganic carbon (as alkalinity) and pH were not monitored. TIC and $HCO_3^-$-C were calculated from alkalinity measurements and the pH balance. NOB: sum in abundances of *Nitrospira* and *Nitrobacter.* SRT: total sludge retention time.

| | | **Temperature** | **$NH_4^+$** | **$NO_2^-$** | **$PO_4^{3-}$** | **Inorganic carbon (TIC)** | **Inorganic carbon ($HCO_3^-$-C)** | **pH** | **Sludge-specific loading rate** | **SRT** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Blue Plains** | **Temperature** | 1.00 | - | | 0.78 | 0.78 | 0.77 | 0.63 | - | - |
| | **$r_{AOB}$** | 0.91 | - | | 0.71 | 0.83 | 0.79 | 0.57 | - | - |
| | **$r_{NOB}$** | 0.83 | - | | 0.66 | 0.79 | 0.73 | 0.59 | - | - |
| | **$r_{AOB}/r_{NOB}$** | 0.71 | - | | 0.61 | 0.86 | 0.84 | 0.62 | - | - |
| | ***Nitrosomonas*** | - | - | | - | | | - | - | - |
| | ***Nitrospira*** | - | - | | - | 0.69 | 0.65 | - | - | - |
| | ***Nitrobacter*** | - | 0.71 | | - | | | - | - | - |
| | **NOB** | 0.55 | - | | - | 0.75 | 0.70 | - | - | - |
| | **AOB/NOB** | 0.79 | - | | - | | - | - | - | - |
| **Nieuwveer** | **Temperature** | 1.00 | - | 0.83 | 0.88 | | | | 0.81 | - |
| | **$r_{AOB}$** | - | - | - | - | | | | 0.90 | 0.71 |
| | **$r_{NOB}$** | 0.88 | - | - | - | | | | 0.76 | - |
| | **$r_{AOB}/r_{NOB}$** | - | - | - | - | | | | - | 0.77 |
| | ***Nitrosomonas*** | - | - | - | - | | | | - | - |
| | ***Nitrospira*** | - | - | - | - | | | | - | - |
| | ***Nitrobacter*** | 0.79 | - | 0.71 | - | | | | 0.71 | - |
| | **NOB** | - | - | - | - | | | | - | - |
| | **AOB/NOB** | - | 0.76 | - | - | | | | - | - |

### 3.5.1. **Uncoupling temperature from other limitations**

To separately evaluate the effects of temperature (T) and other wastewater parameters, the

Arrhenius model was sequentially complemented with specific terms for P, $NH_4^+$-N, $NO_2^-$-N, inorganic carbon, and pH, called add-on mechanistic model (Supplemental Information E.1. For the impact of the N-loading rate, a different approach was applied (Section 3.5.4)

The modelled approach (see Supplemental Information E.1 for a detailed mathematical description) was based on the linearization of a combined Arrhenius temperature model and (separate) addition of different substrate activity models: i.e. a Monod saturation model for $NH_4^+$-N, P, inorganic carbon or a sigmoidal inorganic carbon activity model. An example equation of this linearized model, including Monod saturation is as follows:

$$\ln(r_T) = \mathrm{T}\ln(\theta_T) + \mathrm{a}\ln\left(\frac{S_1}{S_1+K_1}\right) + \mathrm{b}\ln\left(\frac{S_2}{S_2+K_2}\right) + \cdots + \mathrm{n}\ln\left(\frac{S_n}{S_n+K_n}\right) + C \qquad \text{(eq. 3)}$$

With $\mathrm{C} = -20ln(\theta_T) + \ln(rmax_{20°C})$; S = wastewater parameter; K = Saturation constants, and a, b, …, n fitted coefficients.

The model was fitted to the measured potential rates in the external batch tests, with literature values of the model constants (i.e., saturation constants) (see Table 1). This means that the model is not fitted on the actual limited AOB/NOB activities in the STP, since they cannot be measured unless the system is overloaded, but on the measured potential activities, given possible limitations that were present in the batch test (e.g. P, inorganic carbon). This potential activity differs from the model estimated maximum achievable activity, $rmax_{20°C}$, which will be apparent under unlimited growth-conditions over a longer period for the same community.

The model estimated Θ coefficients for temperature and pH, maximum achievable activities at 20°C, $rmax_{20°C}$, and the presence or absence of a certain substrate limitation by estimation of the limitation coefficients a, b, …, n. The affinity constants were fixed, and the weight of each substrate limitation, e.g. Monod term, on each model fit was evaluated by the fitting of the limitation coefficients a, b, …, n. The coefficient for each limitation was interpreted as follows: a) close to 1: substrate limitation is occurring, b) close to 0: no substrate limitation is occurring, possibly because the real affinity constant was smaller than the used literature value c) all other values (>> or << [0,1]): unrealistic fit of the model. To evaluate the goodness of the model fit, the following four things, in order of importance, were analyzed: 1) The estimated parameters were realistic (limitation coefficients; either 0 or close to 1, $rmax_{20°C}$ and $\Theta_{T;}$ in line with expected literature values), 2) The increase in model complexity (i.e. extra limitation added) led to a minimum of residuals, 3) The estimated parameters were contributing significantly ($p < 0.1$) to the model fit and 4) The model fit was statistically different ($p<0.05$), by means of an F-test, from a baseline Arrhenius temperature model.

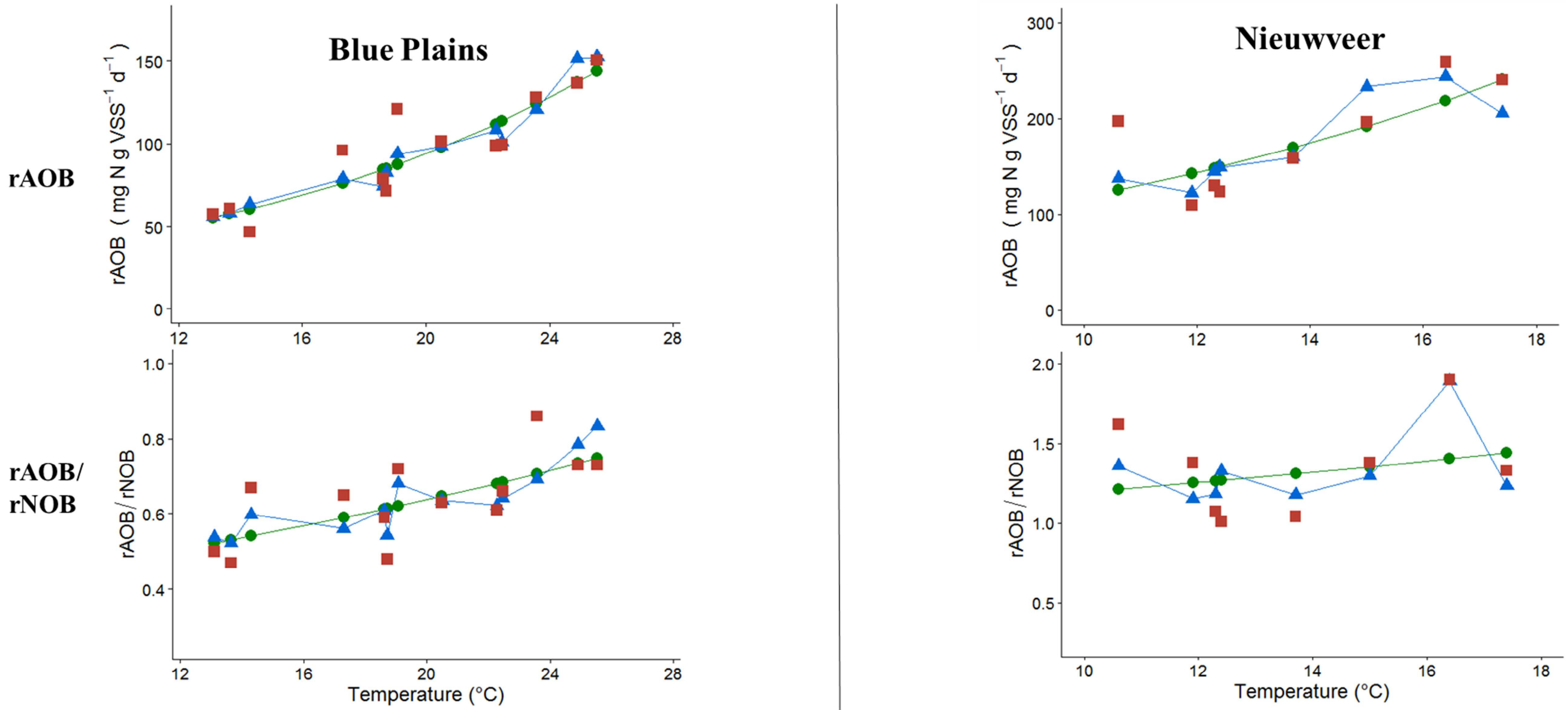
Finally, to assess the impact of the abundance of AOB and NOB, the model fits of bulk activity in mg N g $VSS^{-1}$ $d^{-1}$ were compared to model fits of genera-specific activities in mg N g $VSS_{AOB}^{-1}$ $d^{-1}$ or mg N g $VSS_{NOB}^{-1}$ $d^{-1}$, by correcting the VSS mass for the relative AOB or NOB abundance obtained from qPCR data (16S rRNA gene count of AOB or NOB on total eubacterial 16S rRNA gene count). When both model types had the same realistic model fit, the bulk sludge activity reflected the abundance of AOB and NOB in the sludge, meaning that the sludge was limited by lacking substrate. If only the bulk activity was showing a realistic model fit, the sludge was substrate-limited and abundance does not reflect immediate activity.

#### 3.5.2. **Overall model fit**

In Figure 3, for both STP the best model fits for rAOB and rAOB/rNOB, chosen from Table K.1 and L.1 according to the goodness-of-fit criteria listed above, are depicted together with their estimated model parameters. These model fits (blue line) were compared to a simple Arrhenius temperature model (green line) and the actual data (red squares). For Blue Plains, the best model fit included all limitations; T, P, $NH_4^+$-N, inorganic C, whereas for Nieuwveer, the model that included phosphate did not yield realistic fits (= unrealistic P-coefficient, Table K.1), and thus only temperature and nitrogen levels ($NH_4^+$-N/$NO_2^-$-N) were considered.

For Blue Plains, the variation in the data could be better described by this more complex model compared to a simple Arrhenius temperature model. Inorganic carbon limitations were playing a major role in the measured activities of AOB and thus rAOB/rNOB ratio, while nitrogen levels and phosphorus were not. In the case of Nieuwveer, the variation in the data could not be explained by the limitation in ammonium or nitrite alone, adverting to other unknown factors (e.g. during transport or storage) or limitations that played an important role in the measured activities and model fits.

When we compared the modeled limitations of genera-specific activity rates with those of the bulk specific activity (Table K.1 and L.1), the model types were not in line with each other for Blue Plains, indicating that there were activity limitations that are independent from the abundance of AOB and NOB. For Nieuwveer, the fits are in line with each other, and it




| | Model fit/ Range → | $\Theta_{temp}$ | $rmax_{20°C}$ | P-coefficient | IC-coefficient | $NH_4^+$-coefficient |
|---|---|---|---|---|---|---|
| | | 13.1 - 25.5 °C | | 0.01 - 0.08 mg P L$^{-1}$ | 0.88-1.03 mM TIC L$^{-1}$ | 0.01 - 2.35 mg N L$^{-1}$ |
| rAOB | T | 1.08*** | 93.9*** | | | |
| | T +$P_{sat}$ +$IC_{sigm1.TIC}$ +$NH_{4sat}$ | 1.05** | 165.5. | 0.0309 | 0.7558 | -0.0154 |
| rAOB/ rNOB | T | 1.03*** | 0.6* | | | |
| | T +$P_{sat}$ +$IC_{sat.TIC}$ +$NH_{4.sat}$ | 1.02 | 1.8 | -0.0161 | 1.0878 | 0.0292 |

| | Model fit/ Range → | $\Theta_{temp}$ | $rmax_{20°C}$ | $NH_4^+$-coefficient | $NO_2^-$-coefficient |
|---|---|---|---|---|---|
| | | 10.6 - 17.4 °C | | 0.7 - 2 mg N L$^{-1}$ | 0.22- 0.81 mg N L$^{-1}$ |
| rAOB | T | 1.1*** | 307.7. | | |
| | T +$NH_{4sat}$ | 1.09* | 495.1. | 0.77 | |
| rAOB/ rNOB | T | 1.03 | 1.54 | | |
| | T +$NH_{4sat}$ +$NO_{2sat}$ | 0.94 | 0.77 | 0.66 | 1.4 |

**Figure 3.** Ammonia oxidizing bacteria rate (rAOB; top panels) and nitrifier rate ratio (rAOB/rNOB; bottom panels) in the two sewage treatment plants (STP) as a function of temperature: measured data (red squares), Arrhenius temperature model (green circles), and best fitting add-on mechanistic model (blue triangles). The embedded tables display the measurement range for each parameter and the fitted variables and coefficients with their respective significance of fit (p < *** 0.001, ** 0.005, *0.05, . 0.1). IC: Inorganic carbon. TIC: Total inorganic carbon.

is thus suggested that the observed activities were at least partially, attributable to differences in abundance, rather than sole activity limitations.

### 3.5.3. **Inorganic carbon**

Inorganic carbon (IC), calculated from total alkalinity measurements, was only monitored in Blue Plains during the experiments, whereas in Nieuwveer a later measurement campaign raised insight in possible limitations.

For Blue Plains, to investigate the effect of IC, different IC-models were added to a T + $P_{sat}$ + $NH_{4sat}$ model: 1) two Monod saturation models: One with total inorganic carbon, $IC_{sat,TIC}$, and one with bicarbonate as source of inorganic carbon, $IC_{sat,HCO3^-}$, 2) three sigmoidal activity models, with two sets of widely varying literature kinetic constants for AOB or nitrification; $IC_{sigm1,TIC}$ or $IC_{,sigm1,HCO3^-}$ ($K_{ICAOB}$ = 1.11 mM C, $K_{ICNOB}$ = 0.1 mM C, a = 0.57 mM C) (Guisasola et al., 2007) and $IC_{sigm2,TIC}$ ($K_{ICAOB}$ = 4.17 mM C, a = 0.83 mM C)(Wett & Rauch, 2003) , and 3) an exponential pH model for nitrification, $IC_{pH}$, that describes both carbon limitation and loss of activity due to changes in pH (Henze, 2008) . Only for the Monod saturation models, differentiating kinetic constants for AOB and NOB were found in literature (Table 1). For the sigmoidal model, a $K_{ICNOB}$ was assumed based on the parameters of Guisasola et al., (2007).

Blue Plains rAOB showed to be sensitive to inorganic carbon limitation, with a significant parameter contribution of $rmax_{20°C}$ and $\Theta_T$ to all inorganic carbon models, except for the pH model. The best rAOB model fits (Table K.1) were achieved by using the $IC_{sigm1,tic}$ or $IC_{sat,tic}$ model, with an IC-coefficient close to 1 (=0.75-1.18). For rNOB, results showed that the activity was less sensitive to changes in inorganic carbon, with only a minor increase in the estimation of $rmax_{20°C}$. For rAOB/rNOB, the best fit (IC-coefficient of 1.08) was achieved with a Monod saturation model based on TIC. The $\Theta_T$ and $rmax_{20°C}$ changed from [1.03, 0.6] to [1.02, 1.8], compared to a simple T model, although only for the latter, a significant estimation of both parameters was achieved. The sigmoidal model did not yield better results.

#### 3.5.4. **Sludge-specific nitrogen loading rate**

For Nieuwveer, correlation analysis (Table 4) indicated a significant positive correlation of the loading rate with temperature, rAOB and rNOB. For Blue Plains, no significant correlations were found. To better understand the Nieuwveer case, one would expect, a higher correlation coefficient with rAOB and rNOB when the loading rate is within the timeframe of 1 SRT (~30d). This is because historical loading rates (past operation, values in the negative side of the x-axis) influence the abundance of AOB and NOB and thus increase or decrease their observed activities.

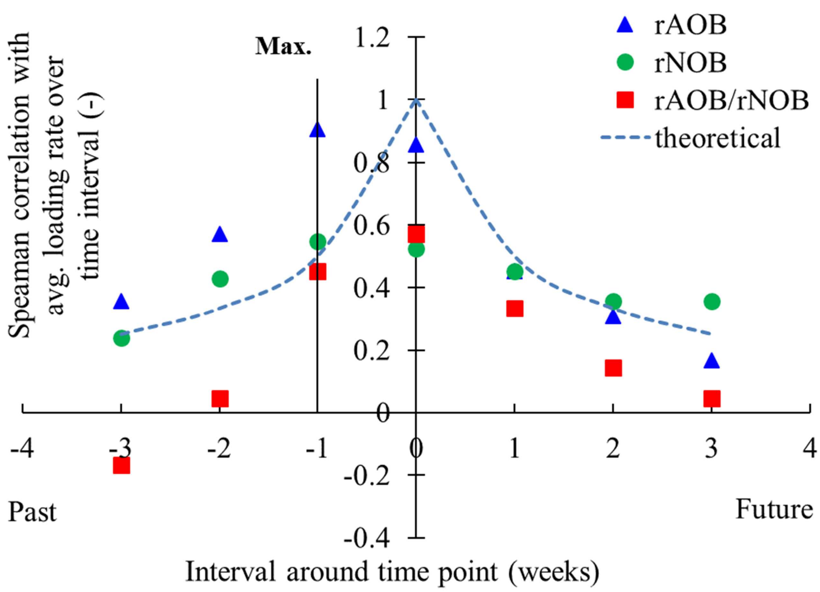


**Figure 4.** Spearman rank correlations between rAOB, rNOB and rAOB/rNOB at any time point, and the average sludge-specific $NH_4^+$ loading rate over the given interval around that time point. A correlation frequency of 1-week was used due to limitations in data-availability. The blue dashed line represents the theoretical curve if there would be a perfect correlation only at time t=0; i.e. the correlation strength would decrease as the interval widens e.g. $(0+1)/(1+2d) = 0.33$ at t±2d.

The calculated correlation coefficient, depicted in Figure 4, showed a peak for rAOB and rNOB at week -1, indicating that for both bacterial genera, the activity was largely affected by loading rate that occurred in the plant one week earlier; more so than by the instantaneous loading rate. This was not reflected in the rAOB/rNOB ratio, suggesting that the activity ratio was not dependent on the historical loading rate.

# 4. DISCUSSION

A more detailed discussion on some parts, including goodness-of-fit, can be found in Supplemental Information M.1.

## 4.1. Limitations in nitrifiers' growth and activity

### 4.1.1. Temperature

The results confirmed that AOB were more temperature-sensitive than NOB, with indifferent temperature kinetics for the two STP, although AOB communities were different (Wiesmann, 1994). Furthermore, *Nitrospira*, which was the most abundant NOB in both STP, showed temperature kinetics similar to the reported literature values for *Nitrobacter*, confirming previous research (Blackburne et al., 2007). For modelling purposes, these were important results, because no differentiation must be made between different AOB or NOB communities.

Interestingly, at temperatures lower than 13°C, the rAOB/rNOB ratio increased for Nieuwveer, which could not be explained by other limitations (Figure 1, data points at lower temperatures). For Blue Plains, no data at these temperatures was acquired. These results are in line with previous research of Gilbert et al. (2015), who recorded similar rAOB/rNOB temperature behaviors in 3 different PN/A sludges, and reported nitrite accumulation in STP at lower temperatures. This would implicate that Arrhenius modelling would only work in a range from 13 to 35 °C, and that below 13°C, either more specialist AOB might take over, or that AOB were more resistant to colder temperatures. Since no shifts in AOB or NOB community were revealed at low temperatures by 16S amplicon sequencing, the data supports the latter possibility.

#### 4.1.2. **Phosphate**

Results from both treatment facilities suggested that phosphate limitation was no important parameter. The scarce amount of literature reported $Kp_{AOB}$ in the range of 0.003-0.05 mg P $L^{-1}$ in groundwater filters (van der Aa et al., 2002; de Vet et al., 2012), and a $KP_{NOB}$ = 0.02 mg P $L^{-1}$ in highly N-loaded waters (Nowak et al., 1996). With a P-range of 0.01-0.08 mg P $L^{-1}$, limitations could have occurred in Blue Plains, yet sensitivity analysis (Figure N.1 and O.1) indicated no impact when increasing $KP_{AOB}$ or $KP_{NOB}$. Thus, although both STP differed in pretreatment prior to N/DN and resulting P-concentrations, this impacted most likely not the activity of AOB and NOB.

#### 4.1.3. **Inorganic carbon**

Model fits of Blue Plains showed that inorganic carbon potentially limited AOB activity, suggesting that [$\Theta_T$, $rmax_{20°C}$] of rAOB/rNOB would increase to [1.021-1.027, 1.2-1.8] when no IC limitations were present. This kinetics would coincide with the ones of Nieuwveer, where inorganic carbon and other limitations were almost not present, with an estimated $\Theta_T$ and $rmax_{20°C}$ of [1.03, 1.54]. This showed that inorganic carbon was most likely the only limiting factor for AOB in Blue Plains. Further discussion (see Supplemental Information M.1) suggested that modelling with bicarbonate and a sigmoidal model most likely resulted in a better description of bicarbonate limitation, which is in line with literature (Guisasola et al., 2007; Jiang et al., 2015; Mellbye et al., 2016).

Previous studies suggested that IC limitation for nitrification mainly prevailed in highly loaded N-removal systems, such as side stream PN/A with a low influent bicarbonate-over-ammonium ratio. STP in regions with low influent alkalinity and BOD, or with deep aeration tanks with a high oxygen uptake rate also risked limitations (Sin et al., 2008; Wett

& Rauch, 2003). This was also reflected in modelling efforts, where usually AOB and NOB kinetics were not differentiated by using very low saturation constants (0.008-0.1 mM C). This study suggests that inorganic carbon is an important parameter to consider under the conditions of a conventional STP. Literature supported that IC limitation affects the rate of AOB in a range of 0-3.5 mM TIC, which lie well within the range of process parameters of most STP, as well as for Nieuwveer STP (3-3.5 mM TIC, pH 7.1-7.6) (Guisasola et al., 2007; Mellbye et al., 2016). Furthermore, reduced inorganic carbon levels to ~40 % of the required growth demand, resulted in overgrowth of NOB in PN/A, mainly due to lower AOB and AnAOB activity (Ma et al., 2015). To further optimize the rAOB/rNOB ratio for purpose of shortcut nitrogen removal, it can be helpful to increase inorganic carbon effluent levels to 3-3.5 mM C.

#### 4.1.4. Nitrogen levels ($NH_4^+$ & $NO_2^-$)

Nitrogen levels played an important role in Nieuwveer, and not in Blue Plains, although more limiting. For Nieuwveer, the indication of nitrogen limitation by the model fits were in line with the dependency of the activities on the sludge-specific loading rate, and with the similar response of the abundance-corrected model compared to the bulk activity model. This showed that both AOB and NOB were capable to process a higher nitrogen load, and no obvious activity/growth limitations by other parameters were experienced. For further applications, AOB rates can thus be boosted by increasing levels of residual ammonium, as was already previously done (Poot et al., 2016).

### 4.2. ADD-ON MECHANISTIC MODELLING METHODOLOGY

Overall, the add-on mechanistic modelling enabled to disentangle and pinpoint effects of different wastewater parameters on the activities of AOB and NOB. Since not always sufficient data was measured in STP to run short-cut nitrogen removal process models, this approach can act as a quick-and-easy methodology to identify limitations. This can be done by using commonly measured data in STP combined with non-calibrated, kinetic literature data. Furthermore, by highlighting potential limitations, it can assist to prioritize calibration of parameters for process models, where multiple parameters need to be calibrated simultaneously. In this way, time can be saved for calibration and detailed measured campaigns, which now consume significant amount of time, i.e. several weeks (Hauduc et al., 2009).

In general, the fitted $rmax_{20°C}$ and $\Theta_T$ showed, although not always, significantly contributions to the fits. The fitted limitation coefficients (for P, N, IC) were most of the time realistic (close to 0…1), however not significantly contributing. Also, compared to the baseline T-model, the different limitation model fits were never statistically different ($p<0.05$, anova F-test). One reason for this lack of significance or realistic fitting could be due to the limited amount of data; 13 sample points for Blue Plains and 8 sample points for Nieuwveer. Larger data sets will be able to achieve better fits and estimate coefficients more reliably. Another reason was that other unrecorded parameters could have influenced the activity of the nitrifiers. Results from the current datasets thus should be interpreted with sufficient care regarding the possible limitations in both STP, and always be accompanied with a sensitivity analysis (See Figure N.1-R.1) for the applied kinetic constant to understand the model fit.

In the case of Nieuwveer, model fits pointed out that nitrogen levels could have influenced rAOB and rNOB. Furthermore, IC limitations appeared to be limiting for rAOB in Blue

Plains. In both cases, the concentrations of both substrates were present in higher concentrations in the batch test as they were in the STP, indicating that the impact of historical growth conditions on rAOB and rNOB could be resolved with this add-on mechanistic model. For inorganic carbon limitation, Guisasola et al. (2007) showed that, when inorganic carbon was spiked at high concentrations to an enriched AOB community (after a 2-3-day IC limitation), high-activity was regained within minutes, yet ~20% lower than initial maximum rate. Due to the short timeframe of the test (2h), IC-limitations thus could have impacted the measured rates. For N-levels, previous studies on ammonium limitation showed that AOB have a base level of ammonium oxygenase (AMO) transcription to rapidly respond to sudden availability of ammonium, while having a higher expression of AMO after longer exposure to increased substrate concentrations (Geets et al., 2006; Stein & Arp, 1998). In contrast, NOB suffer much more from anoxic disturbances, and their activity is likely more susceptible to variations in nitrogen levels (Kornaros et al., 2010). Since low substrate concentrations (<2 mg N $L^{-1}$) were present in STP and a short exposure (2-6h) to higher concentrations (4-10 mg N $L^{-1}$) prevailed in the batch test, nitrogen levels might have slightly influenced the measured potential activities. To conclude, to study whether certain limitations are influencing activity, batch tests should be set up with STP-identical limitations on the anabolic side (e.g., T, pH, P, IC), and sufficient data points should be used to fit the model. In contrast, to study whether historical limitations influenced the potential activity, no limitations should be present in the batch test.

# 5. CONCLUSIONS

Blue Plains and Nieuwveer are two similarly operated nitrifying STP in terms of nitrogen loading rate, total SRT and temperature profile. Nonetheless, Nieuwveer displayed a much higher rAOB/rNOB ratio (1.6 at 20°C, versus 0.6 in Blue Plains), likely facilitating the transition to a shortcut nitrogen removal process. A bottom-up, step-by-step approach enabled to unveil determining factors for this:

1. Disproportional and dissimilar relationships between AOB or NOB relative abundances and respective activities pointed towards differences in community and growth/activity limiting parameters.
2. Nitrifying communities differed mainly in AOB: the five most abundant AOB *Nitrosomonas* OTU were present in either one of the STP, whereas both STP shared the most abundant NOB *Nitrospira* OTU.
3. Arrhenius temperature model fits showed different $rmax_{20°C}$ yet similar $\Theta_T$ ($\Theta_{AOB}$ = 1.1, $\Theta_{AOB}$ = 1.06-1.07), not discriminating the rAOB/rNOB activity ratio in the two STP.
4. The developed add-on mechanistic model could disentangle the effect of temperature from different wastewater parameters ($NH_4^+$, $NO_2^-$, P, inorganic carbon) on AOB and NOB activities.
5. Nieuwveer AOB and NOB activities were limited by lack of nitrogen substrate due to the loading rate of the STP.
6. Blue Plains AOB activity was limited by inorganic carbon (not for N and P), while no limitations for NOB were found. It is hypothesized that addition of inorganic carbon (e.g. as $CaCO_3$) to the process would increase the rAOB/rNOB ratio from 0.6 to >1, facilitating the transition to more energy efficient sewage treatment.

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# HIGHLIGHTS

- STP Nieuwveer has a higher AOB/NOB potential activity ratio than STP Blue Plains.
- The AOB (not NOB) communities differed greatly.
- Add-on mechanistic modelling disentangled temperature from wastewater parameters.
- Blue Plains AOB (not NOB) were activity limited by inorganic carbon.
- Nieuwveer AOB and NOB were nitrogen limited, by the loading rate of the STP.