Determination of Growth Rate and Yield of Nitrifying Bacteria by Measuring Carbon Dioxide Uptake Rate

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ABSTRACT: Nitrifier growth parameters—the maximum growth rate (μ_{Amax}) and yield (Y_A)—were estimated by measuring the rate of carbon dioxide uptake and additional rates of oxygen uptake and ammonia (or nitrite) use. Batch tests in a combined titrimetric and offgas analyzer with enriched *Nitrobacter* and *Nitrosomonas* cultures and an activated sludge sample were performed. The measured μ_{Amax} values for the *Nitrobacter* and *Nitrosomonas* cultures were 0.67 ± 0.03 day^{-1} and 0.54 ± 0.09 day^{-1}, while the Y_A values were 0.072 ± 0.01 g volatile suspended solids (VSS) · g nitrogen $(N)^{-1}$ and 0.14 ± 0.02 gVSS · gN^{-1}, respectively. For the activated sludge sample, μ_{Amax} was observed to increase with pH ($\mu_{Amax} = 0.72 \cdot 3.3^{(pH-7.1)}$) over the range 6.8 to 7.1. All μ_{Amax} and Y_A values determined by this method were similar to those previously reported. Compared with other μ_{Amax} and Y_A estimation methods, this method allows for unique μ_{Amax} and Y_A estimations for given conditions from a single experiment. *Water Environ. Res.,* **79,** 2437 (2007).

KEYWORDS: growth rate, yield, nitrification, carbon dioxide uptake, activated sludge, nitrifiers, *Nitrobacter*, *Nitrosomonas*.

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Introduction

Nitrifier growth parameters, including the maximum growth rate (μ_{Amax}) and yield (Y_A), are important for the design and operation of a biological wastewater treatment plant (WWTP). Numerous methods have been reported in literature for the determination of μ_{Amax} and Y_A, either independently or simultaneously. One such method, described in Metcalf and Eddy (1991), involves operation of a nitrifying reactor at several critical sludge ages, with sufficient duration at each sludge age to attain steady-state conditions. A plot of the inverse of the sludge age against the specific nitrogen oxidation rate results in determination of the yield (Y_A, slope), the decay rate (b_A, intercept), and μ_{Amax} (Y_A multiplied by the maximum nitrogen oxidation rate, r_{Nmax}). Beccari et al. (1979) used a simplified version of this method to find only μ_{Amax}, by experimentally determining the critical sludge age that allows for nitrification. Similarly, the Water Environment Research Foundation's (WERF's) (Alexandria, Virginia) (2003) ''wash out'' method requires operation of a continuous reactor at less than the critical sludge age—not exactly at the critical sludge age, as in the Beccari et al. (1979) method. The resulting semiexponential decrease in the oxidized nitrogen concentration (nitrate-nitrogen) is fitted to a mass balance model to find μ_{Amax} at the given experimental conditions. These methods are time-consuming and, as they would be performed in the laboratory, are ex situ from the WWTP. The μ_{Amax} and Y_A determined by ex situ methods may not necessarily represent the μ_{Amax} and Y_A in a WWTP, because the ex situ bacterial culture may be somewhat altered through laboratory culturing.

The WERF (2003) method, using high food to micro-organism (F/M) ratios, and Antoniou et al. (1990), Belser and Schmidt (1980), and Painter and Loveless (1983) are among numerous reports on the determination of μ_{Amax} from the slope of the log ''activity'' versus time graph obtained from the exponential growth phase of a nitrifying culture. Activity, in these experiments, was approximated by the nitrate-nitrogen concentration or the bacterial most probable number. However, these methods do not determine a Y_A value and are again ex situ (i.e., requiring a nitrifying culture grown in a laboratory).

In situ methods for the determination of μ_{Amax} and Y_A have also been reported in literature. One method, for μ_{Amax} estimation only, involves calibrating a model, such as the IWA activated sludge model no. 1 (ASM1; Henze et al., 2000) using online WWTP data. Kabouris and Georgakakos (1996) and Larrea et al. (1992) applied this method to the estimation of μ_{Amax}, using data from laboratory experiments that were intended to mimic a nitrifying WWTP. The ASM1 was calibrated using the extended Kalman filter algorithm or the linearized maximum likelihood algorithm, respectively. However, Larrea et al. (1992) found the final estimate of μ_{Amax} to be influenced by the initial estimate chosen in the model.

Other in situ methods, such as the low F/M ratio method in WERF (2003), have made use of respirometric measures of the maximum nitrogen oxidation rate (i.e., r_{Nmax} or OUR_{max}) and determined μ_{Amax} through an assumed Y_A value (MarsiliLibelli and Giovannini, 1997; Nowak and Svardal, 1993; Nowak et al., 1994; Yuan et al., 1999). All these methods require an estimation of the nitrifier biomass concentration, X_A. In addition, Y_A is not determined in these methods.

Titrimetric measurements are quantified rates of acid or base additions required to maintain a reactor at a specified constant pH value. Titrimetric measures are particularly valuable for acid- or base-producing reactions, as the rate of the counter ion addition is a representation of the rate of the reaction. By integrating respirometric and titrimetric measurements, both μ_{Amax} and Y_A may be determined simultaneously (Petersen et al., 2001; Pratt et al., 2003). Petersen et al. (2003) concluded that, based on the structural identifiability of a nitrification model, unique estimates of both μ_{Amax} and Y_A may be attainable, provided that an experiment is

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Table 1—Rates for each component involved in nitrification.

Reaction	r_{NH3}	r_{O2}	r_{CO2}	r_X	r_{NO2-}	r_{H+}	r_{NO3-}
Ammonia oxidation	$(\frac{1}{Y_{A1}}+i_{XB})r_{A1}$	$(\frac{1.5}{Y_{A1}}-1)r_{A1}$	r_{A1}	r_{A1}	$\frac{1}{Y_{A1}}r_{A1}$	$\frac{1}{Y_{A1}}r_{A1}$	
Nitrite oxidation	$i_{XB}r_{A2}$	$(\frac{0.5}{Y_{A2}}-1)r_{A2}$	r_{A2}	r_{A2}	$\frac{1}{Y_{A2}}r_{A2}$		$\frac{1}{Y_{A2}}r_{A2}$
Overall nitrification	$(\frac{1}{Y_A}+i_{XB})r_A$	$(\frac{2.0}{Y_A}-1)r_A$	r_A	r_A		$\frac{1}{Y_A}r_A$	$\frac{1}{Y_A}r_A$

operated for a long enough duration so as to display a change in the biomass concentration (proportional to X_A) and a corresponding increase in r_{Nmax} or OUR_{max}. Using practical identifiability, which is different than structural identifiability, in that the quality of experimental data is taken into consideration, Petersen et al. (2001) found that a unique estimate of the yield of the ammonia-oxidizing bacteria (Y_{A1}) was possible. However, μ_{Amax1} could not be estimated independently using respirometric and titrimetric measurements.

Of the methods reviewed, few allow for the determination of Y_A. The parameter Y_A is important for estimation of the concentration of nitrifier biomass, X_A. The Y_A value is typically an assumed constant. However, Y_A is the ratio of the amount of biomass produced per unit substrate (in this case, nitrogen) reacted, where the energy produced from the oxidation of nitrogen is used in both maintenance and growth processes. If the fraction of energy used for maintenance was to change with a change in conditions (e.g., pH value), as is suggested in the model by Pirt (1982), then Y_A would be expected to change. Hence, there is an advantage in determining a unique Y_A for a particular set of conditions.

In this study, we propose and demonstrate an in situ method for determination of μ_{Amax} and Y_A, by measuring the maximum carbon dioxide uptake rate (r_{CO2max}), r_{Nmax}, and/or OUR_{max}, by integrated respirometric and titrimetric measurements. This is accomplished through the use of the recently developed titrimetric and offgas analyzer (TOGA sensor; Pratt et al., 2003). Nitrifiers attain carbon for growth from carbon dioxide. Hence, the ratio of r_{CO2max} to r_{Nmax} is a measure of a unique Y_A. Also, μ_{Amax} can be estimated, after determination of a constant ($1/X_A \cdot V$), as r_{CO2max} is directly proportional to it.

The proposed method was demonstrated using the following:

(1) Case 1—An enriched nitrite-oxidizing bacteria (NOB) culture grown in the laboratory.
(2) Case 2—An enriched ammonia-oxidizing bacteria (AOB) culture grown in the laboratory.
(3) Case 3—Activated sludge from a nitrifying full-scale WWTP.

Determining Y_A and μ_{Amax} from Carbon Dioxide Uptake and Nitrogen Oxidation Rates. Nitrification is a two-step process involving ammonia oxidation followed by nitrite oxidation. The first step, ammonia oxidation, is catalyzed by AOB and represented in eq 1 (note that the subscript 1 represents this reaction).

$$\left(\frac{1}{Y_{A1}}+i_{XB}\right)NH_3+\left(\frac{1.5}{Y_{A1}}-1\right)O_2+CO_2 \xrightarrow{r_{A1}} 1C_{biomass}+\frac{1}{Y_{A1}}NO_2^-+\frac{1}{Y_{A1}}H^++(\ldots)H_2O \quad (1)$$

The second step, nitrite oxidation, is catalyzed by NOB and represented in eq 2 (note that the subscript 2 represents this reaction).

$$\frac{1}{Y_{A2}}NO_2^-+\left(\frac{0.5}{Y_{A2}}-1\right)O_2+1CO_2+i_{XB}NH_3+(\ldots)H_2O \xrightarrow{r_{A2}} 1C_{biomass}+\frac{1}{Y_{A2}}NO_3^- \quad (2)$$

From eqs 1 and 2, the rates of production/consumption for each component involved in each nitrification step is summarized in Table 1.

The rate of biomass growth (r_{Xmax}) is proportional to μ_{Amax} and is equal to r_{CO2max} in both steps of nitrification, as shown in Table 1. The method for the measurement of r_{CO2max} is described in the Materials and Methods section. The parameter Y_A can be determined by dividing r_{CO2max} by r_{Nmax}, which allows for determining Y_A for given experimental conditions. Furthermore, μ_{Amax} can be determined by dividing r_{CO2max} by $X_A \cdot V$, which requires determination of the nitrifier fraction in the biomass, as discussed in the Materials and Methods section.

Materials and Methods

Enriched Nitrite-Oxidizing Bacteria Reactor (Case 1). A sequencing batch reactor (SBR) was operated to enrich NOB, the details of which are available in Vadivelu, Yuan, Fux, and Keller (2006). The reactor had a working volume of 8 L and was fed with nitrite (synthetic wastewater with 1000 mg NO_2^--N $\cdot L^{-1}$) as the sole energy source and bicarbonate as the sole carbon source (see Vadivelu, Yuan, Fux, and Keller [2006] for details). The SBR was operated with a cycle time of 6 hours and nitrogen load of 1000 mg NO_2^--N $\cdot L^{-1} \cdot d^{-1}$. Each cycle consisted of 270 minutes aerobic feeding, 20 minutes aerobic reaction, 60 minutes settling, and a 10-minute decanting period. The reactor temperature, pH, and dissolved oxygen concentration were 21 ± 2°C, 7.3 ± 0.1, and 3 ± 0.25 mg/L, respectively.

Enriched Ammonia-Oxidizing Bacteria Reactor (Case 2). A continuous reactor was operated to enrich AOB; the details of the culturing method is described subsequently, while further discussion on the derivation of this methodology is provided in Blackburne et al. (2007). The reactor was a constantly mixed continuous-flow reactor with 12 L working volume, which was inoculated with mixed liquor from a local domestic WWTP (Brisbane, Australia). The exclusion of NOBs from this culture was achieved by operating at a short sludge retention time (2.4 days) and low dissolved oxygen concentration (0.4 ± 0.05 mg/L). The feed consisted of the following (per liter): $(NH_4)_2CO_3$, 3700 mg; K_2HPO_4, 38 mg; $CaCl_2 \cdot 2H_2O$, 3.8 mg; $MgCl_2 \cdot 6H_2O$, 6.3 mg; $FeSO_4 \cdot 7H_2O$, 2.5 mg; and trace nutrients, 0.17 mg. A batch of trace nutrients was made as a stock and consisted of the following: $NaMoO_4 \cdot 2H_2O$, 0.1 g/g; $CuSO_4 \cdot 5H_2O$, 0.05 g/g; $ZnSO_4 \cdot 7H_2O$, 0.1 g/g; $MnCl_2 \cdot 4H_2O$, 0.2 g/g; $CoCl_2 \cdot 6H_2O$, 0.05 g/g; H_3BO_4, 0.1 g/g; and KCl, 0.4 g/g. Feed was provided in an on/off fashion to approximate continuous operation, as a result of very short feed and nonfeed times (approximately 1 minute feed to 3 minutes nonfeed). The temperature was maintained at 21 ± 2°C via the temperature control in the room, while the pH was maintained at 7.6, by dosing of sodium carbonate solution. Aeration was provided to the base of the reactor through an air stone.

Full-Scale Wastewater Treatment Plant (Case 3). Activated sludge from a four-stage Bardenpho process full-scale WWTP treating primarily domestic wastewater for nitrogen and chemical oxygen demand (COD) removal was used. The WWTP has primary sedimentation and on-site anaerobic sludge digesters, from which sludge dewatering liquor is produced and recycled to the bioreactors.

Data from a nitrogen balance over this plant were used to estimate X_A from this sludge (described below).

Fluorescence In Situ Hybridization and Microbial Quantification. Fluorescence in situ hybridization (FISH) (Manz et al., 1992) was used to identify the dominant NOB and AOB present in each of the reactors. The method of identification and quantification for the enriched AOB is described in detail in Vadivelu, Keller, and Yuan (2006) and Vadivelu, Yuan, Fux, and Keller (2006), and for the enriched NOB in Blackburne et al. (2007). Briefly, AOBs were identified as belonging to the *Nitrosomonas* genera using the NEU probe, as described by Wagner et al. (1995). Other AOB probes were also tested, but these did not bind to any bacteria present in this culture. Similarly, the NOBs were identified as belonging to the *Nitrobacter* genera using Nit3 (Wagner et al., 1996), with the exclusion of other NOBs that were tested for in the study.

Titrimetric and Offgas Analyzer. Oxygen and carbon dioxide uptake kinetics were determined via the Titrimetric and Off-Gas Analyzer (TOGA) (Pratt et al., 2003). The TOGA consists of a fully enclosed reactor (either 3- or 7.5-L volume), which is filled with a biomass suspension. A titrimetric pH control system maintains the reactor pH, measured with an Ionode IJ44 pH probe (TPS, Brisbane, Australia), within 0.01 unit of the desired set point (chosen for each experiment), by manipulating the hydrochloric acid and sodium hydroxide dosing pumps (ProMinent beta/4, Heidelberg, Germany). The acid/base dosage rate is recorded as the rate of proton consumption/production by the biological, physical, and chemical processes occurring in the reactor. A quadrupole mass spectrometer (Omnistar, Balzers AG, Liechtenstein) and mass flow controllers (Bronkhorst Hi-tech, El-Flow, Ruurlo, Netherlands) form the gas-phase measuring system. Specialty gases with specifically designed, known compositions are used in TOGA experiments. The measured concentrations of gaseous components of interest in the offgas, with the known concentrations of these components in the feeding gas, and the measured gas flowrates allow calculation of the transfer rates of these gaseous components through gas-phase mass balance.

In the experiments conducted in this study, gas mixtures containing both oxygen and carbon dioxide (Linde Gas, Brisbane, Australia) were bubbled through the reactor. The gas-phase mass balance allowed determination of the oxygen transfer rate (OTR) and carbon dioxide transfer rates (CTR). The measured OTR can be used directly as the biological oxygen uptake rate (OUR) when the gas/liquid transfer of oxygen (O_2) reaches equilibrium (Pratt et al., 2003).

The r_{CO2}, the carbon dioxide uptake rate by nitrifiers, is determined from the measured carbon dioxide transfer rate (CTR_{meas}) and measured hydrogen ion production rate (HPR_{meas}) signals. In the systems to be tested, the HPR_{meas} signal represents the proton production (or consumption, when HPR_{meas} is negative) rate directly caused by the biological reactions (r_H) and the actions of the ammonium/ammonia and carbonic acid/bicarbonate buffers. These dependencies are described with eq 3, the derivation of which can be found in Gapes et al. (2003) and Pratt et al. (2003).

$$HPR_{meas} = \frac{-r_{NH3}}{1 + 10^{pH-pKn}} + r_H + \frac{r_{CO2} - CTR_{meas}}{1 + 10^{(pKa-pH)}} \quad (3)$$

Equation 3 can be rearranged to give r_{CO2}, as shown in eq 4.

$$r_{CO2} = \left(HPR_{meas} - r_H + \frac{r_{NH3}}{1 + 10^{pH-pKn}}\right)(1 + 10^{(pKa-pH)}) + CTR_{meas} \quad (4)$$

Equation 4 shows that r_{CO2} can be calculated from HPR_{meas} and CTR_{meas} (measured by TOGA), r_{NH3} (determined from offline ammonium/ammonia measurement), and r_H. For case 1, nitrite oxidation, r_H is zero, because nitrite oxidation is a pH-neutral reaction (Table 1), while, for cases 2 and 3, r_H is linked with r_{NH3} through reaction stoichiometry (Table 1).

Batch Tests for Determination of Growth Parameters. The tests with enriched AOB and NOB cultures were operated at a pH of 7.8. This pH value is within the optimum pH range for both AOBs and NOBs (Grunditz and Dalhammar, 2001). To buffer the system, phosphate salts were also added. For the tests with the full-scale sludge, experiments at pH values of 6.8, 6.9, 7.0, and 7.1 were performed, as these are within the pH range found in the WWTP where the seeding sludge was collected. The AOB and NOB experiments were conducted at temperatures of 24 and 25°C, respectively, while the full-scale WWTP sludge experiments were performed at 22°C.

For all three cases tested, the experiments were operated as batch for approximately a 30- to 40-minute period, with all the substrate concentrations (dissolved oxygen, respective nitrogen substrate, and carbon dioxide) kept in excess (confirmed by regular liquid sampling for nitrogen species). Trace amounts of ammonium were also added to allow for growth in case 1. Nitrogen concentrations were determined with a flow injection analyzer (Lachat QuikChem 8000 Analytical, Milwaukee, Wisconsin).

Batch endogenous experiments were performed for each case, to estimate the activity attributable to heterotrophic organisms oxidizing decayed or lysed material from either the nitrifiers or heterotrophic organisms themselves. In all three cases, the endogenous r_{CO2} and *OUR* were subtracted from the exogenous value (as done by Pratt et al., 2003). For the full-scale sludge experiments, the mixed liquor sample used for experimentation in the TOGA was continuously aerated for 1 day before the experiments, to ensure that all the ammonium-nitrogen was oxidized and soluble biodegradable COD was removed.

The error in the signals from the TOGA used to determine r_{CO2} were attributed only to the *HPR* measurement. The error in the *HPR* measurement was estimated at 2%, because of a discrete addition of base in the TOGA, which caused spikes in the *HPR* measurement, instead of an ideal continuous addition of base. Other signals, such as *CTR* or *OUR*, had a negligible relative error, in comparison, because of the size of the sample set (measurement recorded every 8 seconds).

Methods for Biomass Concentration Estimation and Chemical Analysis. The Carlo Erba Elemental Analyzer (model 1106, Carlo Erba, Milan, Italy) was used to determine the biomass formulae in cases 1 and 2. The biomass formulae for the AOB and NOB cultures were determined as $CH_{1.82}O_{0.60}N_{0.23}$ and $CH_{1.89}O_{0.71}N_{0.22}$, respectively. For the full-scale WWTP sample, the generic biomass formula—$CH_{1.4}O_{0.4}N_{0.2}$—was used.

For all three cases, the biomass (NOB, AOB, and/or full-scale WWTP sample) concentration (volatile suspended solids [VSS]) was measured using Whatman GF/A microfiber filters (Whatman plc, Brentford, United Kingdom) at the end of each experiment. The biomass concentration was assumed to be constant over the course of the experiment (negligible increase because of growth), which is reasonable, because the time frame for the experiment is short.

For the enriched biomass samples (i.e., AOB and NOB), the concentration of active biomass, X_A, was approximated by the VSS concentration, adjusted for endogenous products, and multiplied by the fraction of enrichment of the desired bacteria. The portion of endogenous products of VSS was determined for cases 1 and 2, using the autotrophic and heterotrophic decay rates (from Henze

Table 2—Data from the full-scale wastewater treatment plant used to find the fraction of nitrifiers.

Parameter	Value	Units
SRT	13	days
Q	51 100	$kL \cdot day^{-1}$
$C_{N,in}$	48.7	$mgN \cdot L^{-1}$
$C_{N,out}$	2.58	$mgN \cdot L^{-1}$
$C_{N,cent}$	1040	$mgN \cdot L^{-1}$
Q_{cent}	508	$kL \cdot day^{-1}$
V	22 692	kL
VSS	3.1	$gVSS \cdot L^{-1}$
Y_A%	0.17	$gVSS \cdot gN^{-1}$
b_A%	0.15	$days^{-1}$
i_{XB}%	0.086	$mgN \cdot mgCOD^{-1}$

* Note: Values marked with "%" are the default values from ASM1 (Henze et al., 2000). The ASM1 default for nitrification Y_A is 0.24 $gCOD \cdot gN^{-1}$ and is adjusted to 0.17 $gVSS \cdot gN^{-1}$ using a conversion ratio of 1.41 g COD/g VSS.

et al., 2000) and the endogenous fraction, f_p (Water Research Commission, 1984). The fraction of enrichment was determined by quantitative FISH and is assumed to be equal to the mass percent. This assumption is based on the understanding that quantitative FISH is a measure of the biovolume of the bacteria, and because the density of most bacteria is approximately the same (dry density of between 1.39 and 1.62 $g \cdot cm^{-3}$ and wet density between 1.04 and 1.10 $g \cdot cm^{-3}$ [Mueller et al., 1966]), a mass percent that is equal to the biovolume percent may be assumed.

For the full-scale sludge sample, X_A was approximated by the VSS concentration multiplied by the mass fraction of autotrophic bacteria determined using data from the WWTP (eq 5).

$$f_A = \frac{Y_A \cdot \left[(C_{N,in} \cdot Q + C_{N,cent} Q_{cent} - C_{N,out} \cdot Q) - i_{XB}{}^{*} \left(\frac{VSS \cdot V}{SRT}\right)\right]}{\left(b_A + \frac{1}{SRT}\right) \cdot VSS \cdot V} \quad (5)$$

Application of eq 5 requires an assumed b_A value, which was 0.15 day^{-1} at 20°C (as suggested by Henze et al. [2000] and supported by the literature review in WERF [2003]). The $C_{N,in}$ is the average concentration of total Kjeldahl nitrogen leaving the primary sedimentation tank and entering the bioreactors. The fraction of nitrifiers in the sludge sample was determined using the data listed in Table 2.

The error associated with X_A estimation contributes to the estimation of μ_{Amax}. The standard error in the VSS measurement was approximated as 2%, which was determined by performing VSS measures in triplicate, verified consistently over numerous experiments and in agreement with the VSS measurement error quoted in *Standard Methods* (APHA et al., 1992). For case 3, the full-scale sludge sample, the standard error in X_A estimation was approximated as 8.5%. The error associated with X_A was estimated from the standard errors of the WWTP data set (approximately 2 years of data), shown in, and the errors in *HPR* and VSS measurements.

Results and Discussion

Growth Parameters of the Enriched Nitrite-Oxidizing Bacteria (*Nitrobacter*) Culture (Case 1). Three batch experiments for the determination of *Nitrobacter* growth parameters were performed. An example of the results from a TOGA batch experiment (OUR_{max}, CTR_{max} and r_{CO2max}, and nitrite concentrations), with the enriched *Nitrobacter* culture, is shown as Figure 1.

Figure 1 shows a negative r_{CO2max} value, which indicates carbon dioxide uptake for growth. The r_{CO2max} value is slightly different than the CTR_{meas} value, because of the influence of a small rate of net accumulation of carbon dioxide/bicarbonate into the solution for the attainment of the bicarbonate equilibrium. The accumulation led to production of protons in the solution, which is quantified by the HPR_{meas}. The r_{CO2max} and OUR_{max} appear to remain constant throughout the experiment, while the nitrite concentration drops linearly with time, indicating that a constant, maximum reaction rate has been attained. All rates, namely r_{Nmax}, OUR_{max}, and r_{CO2max}

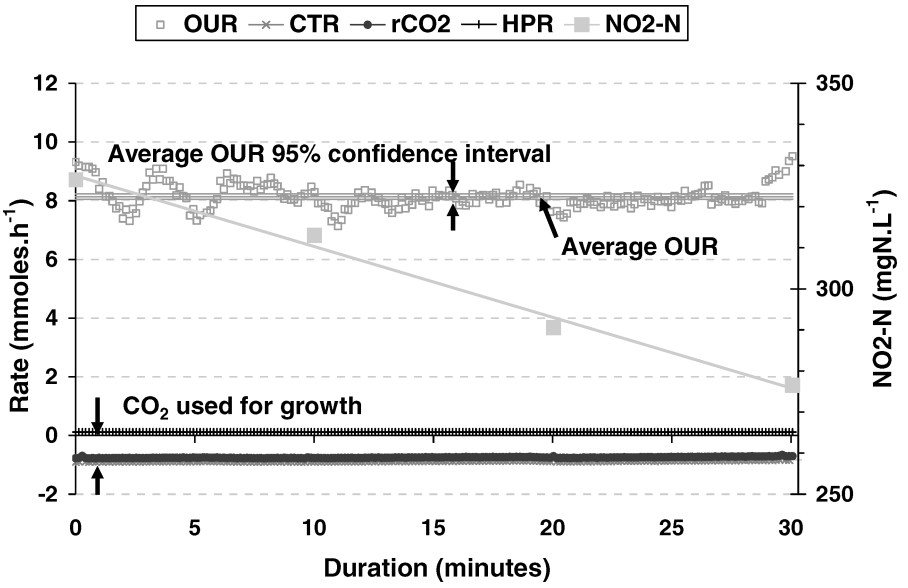

Figure 1—OUR_{max}, CTR_{max}, r_{CO2max}, and nitrite concentrations from a batch TOGA experiment. The VSS for this experiment was 360 mg/L. The OUR_{max} measurements, which were recorded every 8 seconds, were averaged over a 1-minute timeframe, with outliers discarded.

Table 3—Mass balance of TOGA rates for a *Nitrobacter* batch experiment.

	Measurement ($mmoles \cdot h^{-1}$)	Stoichiometry	Rate of reaction[a] ($mmoles \cdot h^{-1}$)
$r_{N,max}$	19.4	$\frac{1}{Y_{A2}} r_{A2}$	0.75
OUR_{max}	8.2	$(\frac{0.5}{Y_{A2}} - 1) r_{A2}$	0.68
r_{CO2max}[b]	0.75	r_{A2}	0.75

[a] Calculated with the average Y_{A2} value determined below.
[b] The endogenous r_{CO2} value was estimated at approximately 2% of the measured r_{CO2} value and was subtracted accordingly.

mass, balanced with the stoichiometric coefficients listed in Table 1, which supports that maximum reaction rates were attained and that the r_{CO2max} value matches NOB stoichiometry.

To determine the μ_{Amax2} values from the TOGA rates measured, the *Nitrobacter* mass fraction (same as volume fraction, see the Materials and Methods section) was required and determined as 73% by quantitative FISH analysis. This fraction was used, with endogenous products-adjusted VSS measurements, to estimate the active mass of *Nitrobacter*. Estimates of μ_{Amax2} and Y_{A2} resulting from the three batch tests are summarized in Table 4.

The μ_{Amax2} and Y_{A2} values, temperature-adjusted to 20°C using the coefficient $1.076^{(T-20)}$ (WERF, 2003), shown in Table 4, are in agreement with previously quoted values from literature, although it must be noted that there is a large discrepancy between these literature values, which is likely a reflection of the difficulty in performing this measurement accurately.

Vadivelu, Yuan, Fux, and Keller (2006) performed independent studies on the same *Nitrobacter* culture to determine μ_{Amax2} and Y_{A2} values with a method which was similar to, and an extension of, the low F/M ratio method from WERF (2003). They determined a Y_{A2} value of 0.071 $gVSS \cdot gN^{-1}$ and a μ_{Amax2} value of 0.48 $days^{-1}$ at pH 7.3, temperature of 22°C, and dissolved oxygen concentration of 3 mg/L. Hence, the Y_{A2} values determined in this work seem to be in agreement with the Y_{A2} values determined by an alternative method. The differences in the μ_{Amax2} values are possibly attributable to two effects—(1) the temperature difference (22°C versus 25°C), and (2) the different pH values (pH of 7.3 versus 7.8). Using the coefficient $1.076^{(T-20)}$ (WERF, 2003) to correct for temperature, the μ_{Amax2} value at 22°C would be 16% less than that at 25°C. The difference between Vadivelu, Yuan, Fux, and Keller (2006) and this work's μ_{Amax2} values is 28%. The remaining difference (12%) may be attributed to the effect of pH, which was reported by Grunditz and Dalhammar (2001) on a *Nitrobacter* pure culture, as shown in Figure 2.

Figure 2 shows that, at pH 7.8, the *Nitrobacter* activity should be close to the maximum, while, at pH 7.3, the *Nitrobacter* relative activity may be 15% less, which is close to the remaining 12% difference (as determined above) in temperature-adjusted μ_{Amax2} values. The *Nitrobacter* reaction rate versus pH value data from Srinath et al. (1976) also supports this conclusion (data not shown). Hence, both the μ_{Amax2} and Y_{A2} values attained in this work seem to be in agreement with the results obtained by Vadivelu, Yuan, Fux, and Keller (2006) and previously published data.

Growth Parameters of the Enriched Ammonia-Oxidizing Bacteria Culture (Case 2). Six batch experiments for the determination of *Nitrosomonas* growth parameters were performed. An example of the results from one of these TOGA batch experiments

Table 4—Growth parameters of the *Nitrobacter* enrichment with 95% confidence intervals. Values were determined at a temperature of 25°C and pH of 7.8. The μ_{Amax2} value in brackets corresponds to the mean value adjusted to 20°C using the relationship $\mu_{Amax2} = \mu_{Amax2} * 1.079^{(T-20)}$ from WERF (2003).

Experiment no.	μ_{Amax2} ($days^{-1}$)	Y_{A2} ($gVSS \cdot gN^{-1}$)
Experiment 1	0.68 ± 0.02	0.081
Experiment 2	0.66 ± 0.02	0.073
Experiment 3	0.67 ± 0.02	0.062
Average	0.67 ± 0.03 [0.46]	0.072 ± 0.01
Reference values	0.14 to 1.44 (Alleman, 1984; Beccari et al., 1979; Keen and Prosser, 1987).	0.02 to 0.084 (Alleman, 1984; Beccari et al., 1979).

(OUR_{max}, CTR_{max}, HPR_{max}, r_{CO2max}, and ammonium concentrations) with the enriched *Nitrosomonas* culture is shown as Figure 3.

Figure 3 shows that the *CTR* was positive, which indicates carbon dioxide stripping. After taking account the acid produced, as per eq 4, the r_{CO2max} was, in fact, negative, as would be expected. Similar to the experiment shown in Figure 1, the maximum reaction rate was attained as indicated by the constant r_{CO2max} and OUR_{max} and also the linear slope of the ammonium concentration versus time.

Again the rates, namely r_{Nmax}, OUR_{max}, and r_{CO2max}, mass balanced with the stoichiometric coefficients listed in Table 1, which supports that maximum reaction rates were attained and that the r_{CO2max} value matches the ammonium oxidation stoichiometry.

To determine the μ_{Amax1} values from the TOGA rates measured, the *Nitrobacter* mass fraction (same as volume fraction, see the Materials and Methods section) was required and determined as 82% by quantitative FISH analysis. This fraction was used, with endogenous products-adjusted VSS measurements, to estimate the active mass of *Nitrosomonas*. A summary of the growth parameters obtained with the enriched *Nitrosomonas* culture is shown in Table 6.

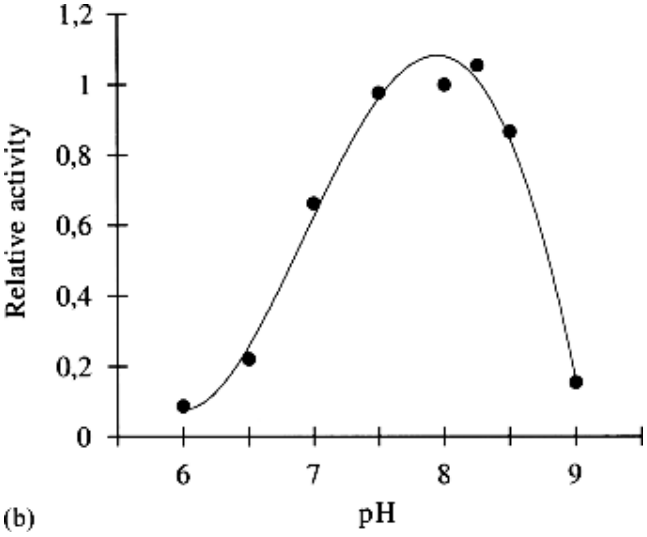

Figure 2—Effect of pH on the relative activity of a *Nitrobacter* pure culture (from Grunditz and Dalhammar, 2001).

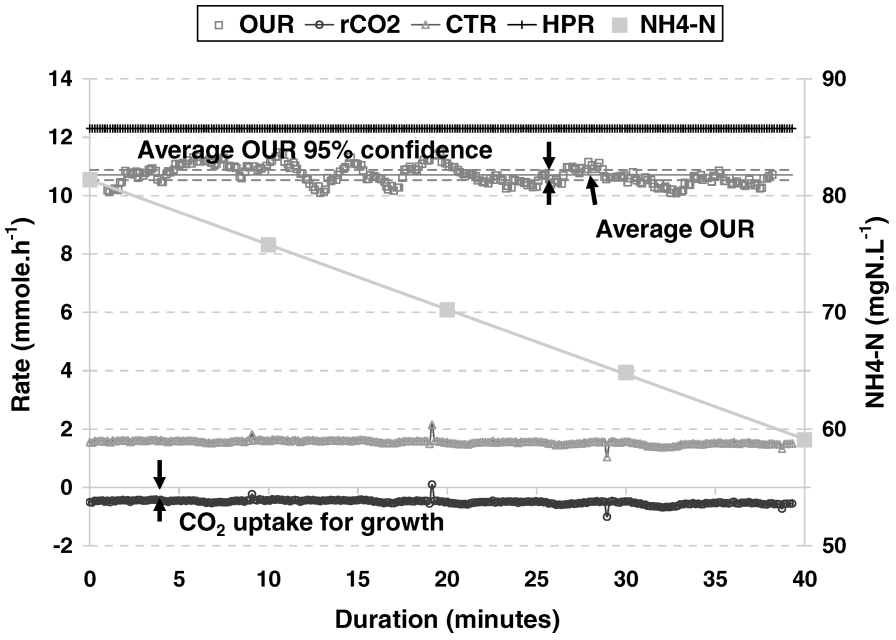

Figure 3—OUR_{max}, CTR_{max}, r_{CO2max}, and ammonium concentrations of a batch TOGA experiment. The VSS concentration for this experiment was 270 mg/L. The OUR_{max} measurements, which were recorded every 8 seconds, were averaged over a 2-minute timeframe, with outliers discarded.

Both the μ_{Amax1} and Y_{A1} values shown in Table 6 are in agreement with previously quoted values from literature, for temperature-adjusted values to 20°C using the coefficient $1.076^{(T-20)}$ (WERF, 2003). Also, for a *Nitrosomonas* culture with a similar degree of enrichment, but not the same culture as used in this work, Vadivelu, Keller, and Yuan (2006) found similar temperature- and pH-adjusted μ_{Amax1} (1.0 $days^{-1}$ at 30°C) and Y_{A1} (0.12 $gVSS \cdot gN^{-1}$) values. Therefore, both the μ_{Amax1} and Y_{A1} values attained in this work seem to be in agreement with other published results for *Nitrosomonas*.

Growth Parameters of Full-Scale Wastewater Treatment Plant Activated Sludge (Case 3). From the full-scale WWTP activated sludge sample, four different pH values were tested (6.8, 6.9, 7.0, and 7.1) in batch TOGA experiments (data not shown). Nitrite accumulation was observed in each experiment, but at a rate of nitrite-nitrogen production that was less than 6% of the rate of total oxidized nitrogen (NO_x) production; hence, nitrification was considered as a single step process. The rate of the formation of NO_x was used in the calculations of the growth parameters, instead of the ammonium consumption rate, because some ammonium is also used in bacterial synthesis (see Table 1). As per cases 1 and 2, the TOGA experiments resulted in an r_{CO2max} value determined using eq 4. However, unlike cases 1 and 2, the fraction of nitrifiers of the total VSS was determined to be 2.2 ± 0.2%, by using eq 5 and data given in Table 2. A summary of growth parameters is shown in Table 7.

The data from Table 7 can be graphically represented, as shown in Figure 4.

As shown in Figure 4, the growth rates increase with increasing pH, with a factor for pH correction ($3.3^{(pH-7.1)}$) that is similar to the Water Research Commission (1984) pH correction factor of $2.35^{(pH-7.2)}$. As indicated in Table 7, the growth rate values,

Table 5—Mass balance of TOGA rates for a *Nitrosomonas* batch experiment.

	Measurement ($mmoles \cdot h^{-1}$)	Stoichiometry	Rate of reaction[a] ($mmoles \cdot h^{-1}$)
$r_{N,max}$	7.1	$(\frac{1}{Y_{A1}} + i_{XB}) r_{A1}$	0.48
OUR_{max}	10.7	$(\frac{1.5}{Y_{A1}} - 1) r_{A1}$	0.50
r_{CO2max}[b]	0.46	r_{A1}	0.48

[a] Calculated with the average Y_{A1} value determined below.
[b] The endogenous r_{CO2} value was estimated at approximately 1.8% of the total r_{CO2} value and was subtracted accordingly.

Table 6—Growth parameters of the *Nitrosomonas* enrichment with 95% confidence intervals. Values were determined at a temperature of 24°C and pH of 7.8. The μ_{Amax1} value in brackets corresponds to the mean value adjusted to 20°C using the relationship $\mu_{Amax1} = \mu_{Amax1} * 1.076^{(T-20)}$ from WERF (2003).

Experiment number	μ_{Amax1} ($days^{-1}$)	Y_{A1} ($gVSS \cdot gN^{-1}$)
Experiment 1	0.51 ± 0.04	0.14 ± 0.006
Experiment 2	0.53 ± 0.04	0.13 ± 0.006
Experiment 3	0.60 ± 0.04	0.15 ± 0.006
Experiment 4	0.48 ± 0.04	0.18 ± 0.008
Experiment 5	0.48 ± 0.04	0.11 ± 0.004
Experiment 6	0.65 ± 0.04	0.15 ± 0.006
Average	0.54 ± 0.09 [0.40]	0.14 ± 0.02
Reference values	0.33 to 1.57 (Beccari et al., 1979; Belser and Schmidt, 1980; Keen and Prosser, 1987)	0.05 to 0.29 (Beccari et al., 1979)

adjusted to 20°C, are within the range of values quoted in literature. Therefore, the consistency of growth rate values with previous values and trends from literature suggests that growth rate values obtained via this method with full-scale activated sludge may be considered reasonable.

An interesting observation from is that the slope of the μ_{Amax} graph is different than that of the r_{Nmax} graph, which indicates that Y_A is not constant. Indeed, the slope of Y_A versus pH confirms this. General observations (as illustrated in Figure 2 for *Nitrobacter*) are that nitrification performance is improved with increasing pH, at least in the range 6.5 to 7.5. Currently, the Y_A commonly used is a ratio of the amount of nitrifier biomass produced to the total nitrogen oxidized, regardless whether the energy generated from the oxidation is used for growth or maintenance processes. For the Y_A to remain constant, maintenance energy, in particular, and the cellular mass generated must remain constant for a given unit of nitrogen oxidized. We hypothesize that this is not occurring, based on the observed increase in Y_A with pH value, as shown in Figure 4. This hypothesis seems to match with the inference from the maintenance energy model from Pirt (1982), that is, the maintenance energy is a function of activity (which is enhanced with increasing pH to approximately 7.5) and therefore may affect Y_A.

The change in Y_A with increasing pH is consistent with the other rates measured and presented in Table 7. The measured values of the slopes of each component can be verified by differentiation of eq 6, with respect to pH.

$$\mu_{A,\max} = \frac{Y_A r_{N\max}}{X_A} \tag{6}$$

Using the product rule, the differentiation of eq 6 yields eq 7.

$$X_A \frac{d\mu_{A\max}}{dpH} = Y_A \frac{dr_{N\max}}{dpH} + r_{N\max} \frac{dY_A}{dpH} \tag{7}$$

The X_A value, with a 90% confidence interval, was determined from eq 7 as 25 ± 4.2 moles, while the measured value, with a 90% confidence interval, was 20 ± 3.3 moles (using the slopes shown in Figure 4, with conversions for consistent units, Y_A of 0.1 mole · mole^{-1} and r_{Nmax} of 5.0 mole · h^{-1}). Given the confidence intervals, both the measured and calculated X_A values can be considered statistically identical to within 90% confidence. Hence, it appears that this data does support that the Y_A is not constant.

Table 7—Growth parameters with 90% confidence intervals for full-scale WWTP sludge sample (all values were determined at 22°C). The μ_{Amax} values in brackets correspond to the mean value adjusted to 20°C using the relationship $\mu_{Amax} = \mu_{Amax} * 1.076^{(T-20)}$ from WERF (2003).

Experiment number	μ_{Amax} (days^{-1})	Y_A (gVSS · gN^{-1})
Experiment pH 6.8	0.50 ± 0.15 [0.43]	0.14 ± 0.005
Experiment pH 6.9	0.57 ± 0.17 [0.49]	0.15 ± 0.005
Experiment pH 7.0	0.61 ± 0.18 [0.53]	0.15 ± 0.005
Experiment pH 7.1	0.73 ± 0.21 [0.63]	0.18 ± 0.007
Reference values	0.8 (Henze et al., 2000); 0.25 to 0.77 (Metcalf and Eddy, 1991)	0.1 to 0.15 (Metcalf and Eddy, 1991); 0.17 (Henze et al., 2000)

If the hypothesis that Y_A changes with pH is confirmed in subsequent studies, then this is an important finding, as the amount of nitrifiers, X_A, is vital for effective nitrification. Typically, nitrifier mass is determined by the sludge age, and, in some cases, the sludge age can be quite long (on the order of weeks) to ensure effective nitrification. However, an alternative may be that, by modifying the environmental conditions to enhance the nitrification reaction (e.g. operating near an optimum pH value or possibly optimum temperature), the nitrifier mass could be increased when required.

Considerations for Practical Application. A TOGA apparatus is not necessarily required to use the proposed method. A carbon

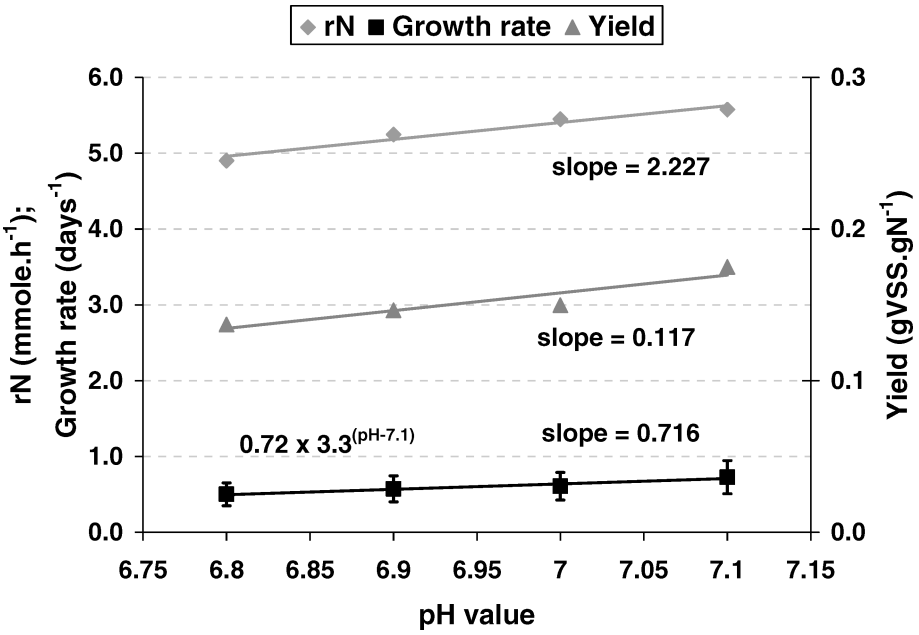

Figure 4—Effect of pH on the growth rate, yield, and rates of nitrogen oxidation. The error bars represent a 90% confidence interval. The fitted lines were determined using least sum of squares regression.

dioxide gas-measuring device, typically using infrared absorption, could be used instead of the mass spectrometer. An accurate titrimetric system would still be required, with a mass flow measurement of the inlet gas. The rate of nitrogen oxidation would need to be determined by sampling and offline analysis.

Conclusions

The key conclusions from this work are as follows:

(1) The rate of carbon dioxide uptake by nitrifiers can be measured reliably and used for estimating the maximum nitrifier growth rates and yields of ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and nitrifiers, as one function group of bacteria. The method was successfully demonstrated with three different types of nitrifying bacterial cultures, namely enriched *Nitrobacter* and *Nitrosomonas* cultures and a full-scale WWTP activated sludge sample.

(2) The maximum growth rate and yield values for the *Nitrobacter* enrichment were determined as $0.67 \pm 0.03\ \text{day}^{-1}$ and $0.072 \pm 0.01\ \text{gVSS} \cdot \text{gN}^{-1}$ at 25°C and pH 7.8, and, for *Nitrosomonas*, $0.54 \pm 0.09\ \text{day}^{-1}$ and $0.14 \pm 0.02\ \text{gVSS} \cdot \text{gN}^{-1}$ at 24°C and pH 7.8. These values for the nitrifiers in the full-scale sludge was determined to be 0.50 to $0.73\ \text{day}^{-1}$ and 0.14 to $0.18\ \text{gVSS} \cdot \text{gN}^{-1}$ at 22°C in the pH range 6.8 to 7.1. The maximum growth rate and yield values for all cases are in general agreement with previous studies, when adjusted for temperature.

(3) For the WWTP sludge sample, the growth rate was found to change with pH by a factor of $3.3^{(\text{pH-7.1})}$, which is similar to literature. The yield was also observed to change with pH value.The repeated observation of consistency between growth rate values found via this method and literature values suggests that this method is accurate, and the repeatability of experimental results suggests that this method is precise.

Nomenclature

Subscript '1'	Denotes ammonia oxidation reaction
Subscript '2'	Denotes nitrite oxidation reaction
Subscript 'max'	Denotes the maximum rate of a particular process
CTR_{meas}	Experimentally measured carbon dioxide transfer rate ($\text{mmoleCO}_2 \cdot \text{h}^{-1}$)
HPR_{meas}	Experimentally measured hydrogen ion production rate ($\text{mmoleH}^+ \cdot \text{h}^{-1}$)
OUR	Oxygen uptake rate ($\text{mmoleO}_2 \cdot \text{h}^{-1}$)
pK_a	pK_a for the carbonic acid/bicarbonate acid/base system (temperature adjusted as appropriate)
μ_A	Growth rate of nitrifying bacteria (day^{-1})
Y_A	Yield of nitrifying bacteria ($\text{mmoleC} \cdot \text{mmoleN}^{-1}$ or $\text{gVSS} \cdot \text{gN}^{-1}$)
i_{XB}	Fraction of nitrogen within bacterial cells
$C_{biomass}$	Rate of biomass formation ($\text{mmoleC} \cdot \text{h}^{-1}$)
$-r_{CO2}$	Rate of carbon dioxide production ($\text{mmoleCO}_2 \cdot \text{h}^{-1}$)
$-r_{O2}$	Rate of oxygen production ($\text{mmoleO}_2 \cdot \text{h}^{-1}$)
$-r_N$	Rate of the nitrogen production ($\text{mmoleN} \cdot \text{h}^{-1}$)
r_H	Rate of hydrogen ion production ($\text{mmoleH}^+ \cdot \text{h}^{-1}$)
r_X	Rate of biomass production ($\text{mmoleC} \cdot \text{h}^{-1}$)
$-r_{NH3}$	Rate of ammonia-nitrogen production ($\text{mmoleN} \cdot \text{h}^{-1}$)
$-r_{NO3}$	Rate of nitrate-nitrogen production ($\text{mmoleN} \cdot \text{h}^{-1}$)
r_A	Rate of reaction ($\text{mmole} \cdot \text{h}^{-1}$)
pK_n	pK_n for the ammonia/ammonium equilibrium (temperature adjusted as appropriate)
f_A	Fraction of nitrifying bacteria of all bacteria present ($\text{gVSS} \cdot \text{gVSS}^{-1}$)
$C_{N,in}$	Concentration of total Kjeldahl nitrogen (TKN) in the full-scale WWTP after primary sedimentation ($\text{mgN} \cdot \text{L}^{-1}$)
$C_{N,out}$	Concentration of TKN in the effluent of the full-scale WWTP ($\text{mgN} \cdot \text{L}^{-1}$)
Q	Flowrate into the full-scale WWTP ($\text{L} \cdot \text{d}^{-1}$)
b_A	Nitrifier decay rate (days^{-1})
SRT	Sludge retention time (days)
VSS	Volatile suspended solids concentration ($\text{mg} \cdot \text{L}^{-1}$)
V	Volume (L)
$C_{N,cent}$	Concentration of TKN in the sludge reject water (centrate) stream entering the main bioreactors of the full-scale WWTP ($\text{mgN} \cdot \text{L}^{-1}$)
Q_{cent}	Flowrate of sludge reject water (centrate) to the main bioreactors of the full-scale wastewater treatment plant ($\text{L} \cdot \text{d}^{-1}$)
X_A	Concentration of autotrophic biomass ($\text{gVSS} \cdot \text{L}^{-1}$)

Credits

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